The Genus *Cronartium* Revisited

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Abstract

Cronartium is a prominent rust pathogen (Pucciniales, Basidiomycota). The type species of the genus was *Cronartium asclepiadeum*, which is currently synonymized under *C. pini*. *Cronartium* species infect branches, cones and stems of *Pinus* species, which are economically and ecologically valued crops in many countries in North and Central America, Asia and Europe. *Cronartium* species show host specificity alternating between two unrelated hosts such as *Pinus* and *Ribes* species. In this macrocyclic, heteroecious lifecycle, the five different spore stages are produced. They are the causal agent of numerous blister rust diseases such as cone rust disease, gall rust disease and limb rust disease on pine forests worldwide. Mainly, *Cronartium comptoniae* causes sweet fern blister rust, *C. ribicola* causes white pine blister rust, *C. comandrae* causes comandra blister rust, *C. pini* (= *Peridermium pini*) causes resin-top disease, *C. quercuum* causes gall rust disease in pine-oaks, *C. arizonicum* causes limb rust disease and *C. strobilinum* and *C. conigenum* cause cone rust disease. All species of *Endocronartium* have been synonymized and transferred to *Cronartium*. The updated phylogenetic analysis for the genus *Cronartium* is performed based on ITS sequence data from GenBank. This study is focused on morphology, phylogeny, pathogenicity and mitigation methods as well as future directions for research on the genus *Cronartium*.

Key words – Five spore stages – pathogens – rust diseases

Introduction

Rusts are obligate parasites of plants, including many economically valuable plants such as bean, coffee, peanut, wheat and others (Littlefield & Heath 1979, Zuluaga et al. 2011). They were identified as autoecious species in carboniferous period in two original hosts, ferns and mosses (Millar & Kinloch 1989). After heteroecism evolved, they became specialized on their primary hosts, but required a secondary, usually unrelated host plant on which to complete their life cycle. In the past, ancient angiosperms and old pine hosts were shared by rusts including *Cronartium comptoniae*, *C. flaccidum* (= *C. pini*), *C. occidentale*, *C. quercuum* and *C. ribicola* (Millar & Kinloch 1989). Each *Cronartium* species has high pathogenic variability on both pine and the alternate host (Powers 1982).

Cronartium species have a heteroecious life cycle. Most *Cronartium* spp. alternate between aecial (primary) host and telial (secondary) hosts. Mainly, *Cronartium* spp. affect *Pinus* spp.

(*Pinaceae*) as aecial hosts. Spermatia and aeciospores are formed on the aecial host. Main telial hosts of *Cronartium* spp. are *Ribes* spp. and different dicotyledonous hosts (*Fagaceae*, *Grossulariaceae*, *Myricaceae*, *Orobanchaceae*, *Santalaceae* and *Scrophulariaceae*). For example, *Cronartium conigenum*, and *C. strobilinum* infect *Quercus* spp. while *C. orientale* and *C. quercuum* affect *Castanea* spp., *Castanopsis* spp. and *Quercus* spp. (EFSA Panel on Plant Health et al. 2018). Both urediniospores and teliospores are formed on the telial hosts. The aeciospore stage, in particular, is the economically damaging stage. The infection and production of aecia leads to different symptoms according to the *Cronartium* species on host tissues such as, branch and stem dieback, cankers, deformity, galls and cone death (EFSA Panel on Plant Health et al. 2018), leading to lower quantity and poorer quality timber yields.

Classification

Phylum Basidiomycota contains rusts under class Pucciniomycetes, order Pucciniales (previously known as Urediniomycetes and Uredinales). There are 13 families within Urediniomycetes separated according to telial morphology, spermogonial and aecial type, basidiospores and uredinial characteristics (Cummins & Hiratsuka 2003, Kolmer et al. 2009). The genus *Cronartium* was described by Fries (1815) with *Cronartium asclepiadeum* (Willd.) Fr. as the type species of the genus. The type species was later synonymized under *C. flaccidum* (Hiratsuka (1995) and currently synonymized as *Cronartium pini* (Species Fungorum 2019). *Cronartium* is the only genus of family *Cronartiaceae* (Maier et al. 2003). Sydow & Sydow (1915) accepted 22 *Cronartium* species while Peterson (1973) documented 36 species according to the telial morphology. Regarding the spermogonia types and other morphological features, 19 species have been transferred to *Cianothrix*, *Crossopsora*, *Didymopsora* and *Endophylloides* (Hiratsuka 1995). Hiratsuka (1995) recognized 16 *Cronartium* species. With complexity of species level, known closely related species of *Cronartium* are divided into five species complexes viz. *Cronartium coleosporioides*, *C. comandrae*, *C. flaccidum* (= *C. pini*), *C. ribicola* and *C. quercuum* (Hiratsuka 1995).

Ninety-two species epithets are listed under *Cronartium* (Index Fungorum 2019). Species which show a genetic similarity to *Cronartium*, but exclude autoecious and endocyclic characters, were included in *Endocronartium* (Aime et al. 2018). *Endocronartium* was established by Hiratsuka (1969) using spore and germ tube characteristics (Hiratsuka 1995, 1969, Aime et al. 2018). *Endocronartium harknessii*, the type species for *Endocronartium* is the main causal agent of western gall rust (Aime et al. 2018). Including *Endocronartium harknessii*, *E. sahoanum* var. *hokkaidoense*, *E. sahoanum* var. *sahoanum* (synonymized as *Cronartium harknessii*, *C. kurilense*, *C. sahoanum*, respectively) all species of *Endocronartium* have been synonymized and moved to *Cronartium* (Aime et al. 2018, EFSA Panel on Plant Health et al. 2018). *Endocronartium pini* and *E. yamabense* are currently known as *C. pini* and *C. yamabense*, respectively (Hantula et al. 2002, Aime et al. 2018, Index Fungorum 2019).

Geographical distribution

In past, *Cronartium* species are naturally limited to the Northern hemisphere in Asia, Europe and North America. Until 1900, few *Cronartium* rust diseases are highlighted in United States, North America. They are eastern gall rust disease (*C. cerebrum*), southern fusiform rust disease (*C. fusiforme*) and western gall rust disease (*C. coleosporioides*) (Smith 1970). *Cronartium ribicola* is a well-studied rust, related to white pines, currants and gooseberries trees which are distributed in Asia, Europe and North America (Geils et al. 2010). The first record of *C. ribicola* was from Western Russia (Transcontinental country in Eastern Europe and North Asia) in 1856 (Stewart 1906, Hummer 2000). According to Hummer (2000) *C. ribicola* probably originated in central Eurasia (all of Eastern Europe and North Asia) (Leppik 1967, Spaulding 1929). Further, he mentioned, it may spread from east of the Ural Mountains (from north to south through Western Russia) through central Siberia (Russia) reaching to the Pacific coast of Asia in the east and southwards to the Himalayas (Asia). Also, Stewart (1906) has mentioned that *C. ribicola* may have been inadvertently imported from

Russia to Eastern Europe. In the same time, in 1906, *C. ribicola* was observed in Germany (Western Europe) and in 1921 it was found in British Columbia on a shipment of white pines from France. In 20th century, *C. ribicola* spread from its native Siberia (Leppik 1970) into Europe and became widespread in Northern and Western Europe. Some species have been introduced to new areas in North America from Europe. For example, *C. ribicola* was native to Asia and Southern Europe affecting white pines (Zambino et al. 2005, Kolmer et al. 2009, Kaitera & Hiltunen 2012, Brar et al. 2015). It was introduced to Eastern and Western North America with imported pine seedlings from Europe in 1890s (Smith 1970, Powers 1982, McDonald & Hoff 2001, Brar et al. 2015). *Cronartium ribicola* was spread to Northern Europe also (Kaitera & Hiltunen 2011, 2012).

Cronartium pini (as *C. flaccidum* and *Peridermium pini*) is the common native pine stem rust pathogen in Asia and Europe (Hiratsuka et al. 1992, Kaitera et al. 2011) and Mediterranean countries (Moriondo 1975, Kasanen 2001, Kaitera et al. 2018). Hiratsuka (1995) mentioned that the European and Asian forms of *C. pini* can be found in China, Japan and Russia also. In North America *C. comandrae*, *C. coleosporioides* and *C. comptoniae* were identified (Hiratsuka et al. 1992). *Cronartium harknessii* (western pine gall rust) is prominent in North America while *C. kurilense* and *C. sahoanum* are prominent in Russia and Japan (EFSA Panel on Plant Health et al. 2018).

Importance as pathogens

Cronartium species have a deleterious effect in forest pine trees which have great ecological value. *Cronartium ribicola* is an invasive alien fungal species, which causes white pine blister rust disease (Powers 1982, Ma et al. 2019). Five-needle pines (*Pinus* spp.) are main primary hosts and currant and gooseberries are the secondary hosts (Kolmer et al. 2009, Geils et al. 2010). It mostly infects *Pinus cembra* (Pawsey 1963, Kaitera & Hiltunen 2012) also other most important hard pine species *Pinus lambertiana* (sugar pine), *P. monticola* (western white pine) and *P. strobus* (eastern white pine) and other five-needle pines (Gäumann 1959) in North America where it was introduced from Europe in 1906 (Bergeron et al. 2019, Schoettle et al. 2019). By the 1950s it had spread to most of the commercial white pine regions in North America (Maloy 2003).

In Finland (Europe) 1800s, *C. ribicola* was first reported on exotic five-needle pines and in Britain on *Pinus strobus*, currants and gooseberries in 1892 (Liro 1908, O'Hara et al. 2010). Also, they have affected other less commercially important five-needle white pines i.e. *Pinus albicaulis* (white bark pine), *Pinus flexilis* (limber pine) (Maloy 2003). In Europe, they damage *Pinus palustris* subsp. *palustris* and trees in *Apocynaceae* and *Tropaeolaceae* families (Kaitera & Hiltunen 2012, Singleton & Oblinger 2017). This pathogen also infects *Ribes* sp. (*Grossulariaceae*) and *Pedicularis* (*Orobanchaceae*) in Asia, and *Loasaceae* in North America (Kaitera & Hiltunen 2012). *Ribes nigrum* is most susceptible to *C. ribicola* in Finland (Kaitera & Hiltunen 2012). *Cronartium* species naturally sporulate on *Euphrasia stricta*, *Melampyrum arvense*, *Odontites vulgaris*, *Ribes nigrum*, *R. rubrum*, *R. uva-crispa* and *Vincetoxicum hirundinaria*. Other than natural habitats they can be sporulated on several garden plants such as *Asclepias*, *Paeonia*, *Tropaeolum*, *Vincetoxicum* and *Papaver* in Germany of central Europe (Kaitera et al. 2018).

Cronartium pini infects on two-needle pines with significant economic loss throughout Europe and Asia (Kaitera & Hiltunen 2012, Kaitera et al. 2018). This is listed as a harmful organism (as *C. flaccidum*) in different countries such as Colombia, New Zealand, and Taiwan (Sullivan 2010). It is a host-alternating fungus which has reported for resin top disease (Scots pine blister rust) in young Scots pine (*Pinus sylvestris*) with dead tops of green crowns (Kasanen 2001, Kaitera et al. 2018). *Cronartium pini* affects needles of pine by basidiospores and the aecia formation is prolonged for several years. The alternate hosts of *Cronartium pini* are in many angiosperm genera: *Asclepias*, *Castilleja*, *Euphrasia*, *Gentiana*, *Grammatocarpus*, *Impatiens*, *Loasa*, *Melampyrum*, *Nemesia*, *Paeonia*, *Papaver*, *Pedicularis*, *Schizanthus*, *Tropaeolum*, *Verbena*, *Veronica*, and *Vincetoxicum* (Kaitera et al. 2015, 2018). *Melampyrum* spp. are the main alternate hosts for *C. pini* and it spreads in natural pine forests via *Vincetoxicum hirundinaria* in Southern Europe and *Melampyrum sylvaticum* in Northern Europe (Kaitera & Hiltunen 2011, 2012, Kaitera et al. 2015, 2018). *Cronartium pini* does not infect five-needle pines or *Ribes* spp. (Kaitera & Nuorteva 2006). The

autoecious *C. pini* is also causes resin top disease especially on *Pinus mugo* (Kasanen 2001, Kaitera & Nuorteva 2008).

Cronartium comandrae causes comandra blister rust in *Pinus banksiana* in Canada, *P. contorta* (lodgepole pine), *P. ponderosa* (ponderosa pine) and *P. sylvestris* (European Scots pine) plantations in North America (Jacobi et al. 1993, 2002, Reich & John 2018). Also, European maritime pine (*P. pinaster*), Austrian pine (*P. nigra*) and mountain pine (*P. mugo*) have been reported to be vulnerable in North America. This pathogen is widely distributed in Western USA and Canada (Jacobi et al. 2002). Severe impacts of comandra blister rust occur where aecial and telial hosts grow near to each other (Jacobi et al. 1993). The aecial hosts of *C. comandrae* are two- and three-needled *Pinus* spp. in North America. The common telial host is *Comandra umbellata* (pale comandra) in Northern Montana and Canada, and *Geocaulon lividum* (northern comandra) in Canada (Jacobi et al. 2002). Comandra rust cankers can girdle the pine stem and kill branches, crown and the entire tree with a growth loss, deformation and lower seed production (Jacobi et al. 2002).

Sweet fern blister rust (*C. comptoniae*) is a serious canker disease that girdles and kills native two and three needle *Pinus* spp. in North America (Smeltzer & French 1981, Horst 2008). The cankers can appear on small plants with elongated swellings. *Pinus banksiana*, *P. contorta*, *P. muricata*, *P. ponderosa*, *P. resinosa*, *P. rodinata* and *P. taeda* are main native pine hosts. Sweet fern (*Comptonia peregrina*) and sweet gale (*Myrica gale*) are main alternate hosts in North America (Pawsey 1974, Smeltzer & French 1981). European pine species planted in North America such as *P. sylvestris* and *P. nigra* are more susceptible to sweet fern rusts (Pawsey 1974).

Stalactiform blister rust, *C. coleosporioides* also induces perennial cankers on two and three needle pine trees such as *Pinus contorta*, *P. banksiana* and *P. ponderosa* in North America (Mordue & Gibson 1978, Nevill et al. 1990). Van der Kamp (1994) also reported *C. coleosporioides* infections on *P. contorta* trees mostly occurring on branches around 2 m above the ground. Indian paintbrush (*Castilleja* spp.) and members of figwort family (*Scrophulariaceae*) are main alternate hosts for stalactiform rust. This pathogen affects lumber content, and growth rate of hosts. Hiratsuka et al. (1988) revealed *C. coleosporioides* is less aggressive than *C. comandrae* and *C. comptoniae* in girdling pine trees.

Pine-oak rusts infect oak hosts and also stems, branches and cones of pines and are widely distributed in North America. The severity of parasitism by pine-oak rusts depends on their alternate hosts and it is difficult to identify on oak hosts (Vogler 2008). *Cronartium quercuum* f.sp. *banksianae*, *C. quercuum* f.sp. *echinatae* and *C. quercuum* f.sp. *virginianae* are pine-oak rusts which cause eastern gall rust disease. Jack pine, shortleaf pine and Virginia pine are the primary hosts for these rusts, respectively (Vogler 2008). *Cronartium quercuum* f.sp. *fusiforme* is a pine-oak rust which causes fusiform rust on shortleaf pine in Southeastern US and Southern North America (Vogler 2008, Kaneko 2000). *Cronartium fusiforme* is recognized as a significant disease of two needle pines in Asia, North America (Kaitera & Nuorteva 2008, Snieszko et al. 2014). *Cronartium harknessii* (*Peridermium harknessii*) causes western gall rust disease of hard pines in North America and is not present in southern hemisphere (Allen et al. 1998). The aeciospores of *C. harknessii* produce globose galls on branches and stems of host trees 1–2 years after infection (Allen et al. 1998). *Cronartium orientale* causes Asian pine-oak rust disease (Kaneko 2000). The spermogonia and aecia of *C. orientale* are produced on hard pine trees while uredinial and telial forms are on oak trees (Kaneko 2000). *Cronartium strobilinum* and *C. conigenum* are other pine-oak rusts reported in some *Pinus* spp. such as longleaf, slash pine and ponderosa pines (Vogler 2008). *Cronartium appalachianum*, Virginia pine blister rust infects *P. virginiana* as primary host by girdling the pine bark lesions (Vogler & Bruns 1998, Horst 2008, Ratliff et al. 2015) with *Buckleya distichophylla* as the alternate host (Horst 2008, Ratliff et al. 2015). *Cronartium arizonicum* causes limb rust disease mainly on ponderosa pines in Arizona (Fairweather et al. 2006). Initially, *C. arizonicum* attacks needle-bearing twigs to enter the main stem of pine trees, and damage the branches. *Castilleja* spp. are main alternate hosts for *C. arizonicum* (Fairweather et al. 2006).

Cronartium ribicola creates a huge ecological devastation by killing economically important hosts in many countries, thereby affecting plant economy. Singleton & Oblinger (2017) reported that

white pine trees have great economic value compared to other crops of Warm Springs Reservation (WSR) in Western North America. The annual economic loss caused by white pine blister rust in Western North America has been estimated at US\$ 450 million (Hamelian 2012). Fusiform rusts (*Cronartium quercuum* f.sp. *fusiforme*) which infect economically valuable loblolly pine (*P. taeda*) and slash pine (*P. elliotii*) and *P. palustris* in Southern and Southeastern US (Snieszko et al. 2014) cause over \$140 million annual estimated economic losses (Hamelian 2012, Snieszko et al. 2014).

Morphology

Species of *Cronartium* may have up to five different spore stages, often referred to by the symbols 0, I, II, III and IV: 0 = spermatia, I = aeciospores, II = urediniospores, III = teliospores and IV = basidiospores (Kolmer et al. 2009, Voegelé et al. 2009, Bergeron et al. 2019). These develop, respectively, in spermatogonia, aecia, uredinia, telia, and on basidia (probasidia). They alternate between dikaryotic and haploid nuclear status in both primary (*Pinus* sp.) and secondary (mostly *Ribes* sp.) hosts (Kolmer et al. 2009). The typical development of different spore stages are basidiospores (n) and spermatia (n), aeciospores (n+n), urediniospores (n+n) and teliospores (n+n) (Littlefield & Heath 1979). The different stages of *C. orientale* spores and germinating basidiospore of *C. fusiforme* are indicated in Fig. 1 (Littlefield & Heath 1979, Kaneko 2000).

Spermatia, which can be considered as haploid gametes, are produced in spermatogonia on pine hosts (Hiratsuka & Sato 1982). Spermatogonia develop between periderm, as flat, yellowish at the beginning, producing spermatia in orange droplets, darkening later, gradually becoming disrupted because of aecial enlargement (Sullivan 2010). Aecia are peridermioid, dehiscent circumscissile or irregular. Aecial peridium has several thick rhomboid ellipsoid cells that are strongly verrucose (wart-like), with hair like peridial filaments (Sullivan 2010). *Cronartium* aeciospores are single-celled and thick-walled, the surface of which is covered in small knob-like structures, globose to ovoid-ellipsoid, hyaline (Hansen & Lewis 1997, Sullivan 2010). Uredinia bear urediniospores on *Ribes* sp. hosts. Urediniospores are single-celled and darkly pigmented, thick-walled, with germ pores and spine like structures (echinulate). Telia are hypophyllous, often developing in uredinia, and erumpent. Teliospores are produced in chains, adhering in columns to 2 mm or longer, pale orange to cinnamon brown. Teliospores are ellipsoid to cylindrical, not separating, and smooth, with yellowish wall. They can germinate without dormancy. Usually, upper telial columns have whitish powdery form because of basidia and basidiospores formation (Sullivan 2010). In germination process, teliospores form four-celled promycelium that give rise to basidia and basidiospores (Hansen & Lewis 1997, Maloy 2003, Liu et al. 2015). Basidia bear basidiospores which produce initial infections on pine species. Mature basidia are septate, four cells, each with a conical protuberance (sterigma).

Basidiospores form at the apex of every sterigma and there are four basidiospores for each basidium. Basidiospores are rounded, smooth, hyaline, and 3–4 µm in diameter (Ragazzi et al. 1987, Sullivan 2010). Basidiospores germinate to produce branched germ tubes, 200 µm or more in length, 2–5 µm diameter (Ragazzi et al. 1987, Sullivan 2010). When morphological distinctions are lacking, the host association can be taken as the best identification method for rusts (Hansen & Lewis 1997).

Life cycle

Rusts are highly specific organisms showing host specificity (Kolmer et al. 2009). In the heteroecious life cycle, two taxonomically dissimilar hosts (e.g. *Pinus* and *Ribes*) are infected as part of the life cycle. The alternation of meiosporic and mitosporic reproduction of the rust pathogens can be seen in two hosts (Taylor et al. 1999). Mostly angiosperms are the primary hosts for *Cronartium* rusts (Hansen & Lewis 1997). Scots pine blister rust, *C. pini* completes its life cycle by alternating between pines and seed-plants of different angiosperm families such as *Acanthaceae*, *Apocynaceae*, *Balsaminaceae*, *Gentianaceae*, *Loasaceae*, *Orobanchaceae*, *Paeoniaceae*, *Papaveraceae*, *Tropaeolaceae* and *Verbenaceae* (Geils et al. 2009). The life cycle of *C. ribicola* is shown in (Fig. 2). *Cronartium ribicola* disease cycle needs many years to complete (Kolmer et al. 2009) and it begins with infection of pine needles by basidiospores. Teliospores, produced in telia in late summer on underside of *Ribes* leaves, germinate to form basidia that produce basidiospores after meiosis.

Fig. 1 – Different spore stages of *Cronartium orientale* (a-e) and germinating basidiospore of *C. fusiforme* (f) redrawn from Littlefield & Heath (1979) and Kaneko (2000). a Aeciospores on *Pinus densiflora*. b Urediniospores on *Quercus crispula*. c SEM view of urediniospore. d Apical part of telial column on *Q. crispula*. e Basidiospores on *Q. crispula* (from fresh teliospores). f Germination of basidiospore with appressorium of *Cronartium fusiforme* Scale bars: a-b, e= 20 μ m, c=5 μ m, d=100 μ m, f= $\times 2800$.

There are four haploid basidiospores produced from each basidium (Kolmer et al. 2009, Liu et al. 2015). The basidiospores disperse by wind and germinate on pine needles producing a germ tube that penetrates through the stomata (Smith 1970, Maloy 2001, 2003, Kolmer et al. 2009, Liu et al. 2015). For a successful attack viable pathogenic spores and living pine needle with moisture are required (O'Hara et al. 2010). Mycelium that originates from basidiospores grows throughout the pine needles, branches and stems (Lachmund 1933, O'Hara et al. 2010, Liu et al. 2015). Infected needles show disease spots after 4–10 weeks. Base of infected needle bundles are discoloured and become swollen. These swellings on pine base needles, branches and stems develop into cankers, which can exude a large amount of resins (Lachmund 1933, Smith 1970, Maloy 2003). Rapid death of trees may occur after a canker encircles the bole of young five-needle pine tree (Hummer 2000). *Cronartium* rusts produce spermatia (pycnia) at canker margins (Maloy 2003, EFSA Panel on Plant Health et al. 2018). *Cronartium pini* and *C. ribicola* produce spermatia in late summer 1-2 years after infection and aecia in the next early summer. These haploid spermatia contain spermatia (pycniospores) in hypertrophy on host branches, cones and stems (Maier et al. 2003, Kolmer et al. 2009).

Fig. 2 – Life cycle of *Cronartium ribicola* redrawn from (Miller et al. 1959).

After plasmogamy, *Cronartium* rusts produce aeciospores in an aecium which develops at canker regions (Maloy 2003, Kolmer et al. 2009, Ratliff et al. 2015). In spring (or summer at high elevation), aeciospores can emerge through bark of pine trees from the area previously produced spermagonia onto *Ribes* plants and also *Castilleja* and *Pedicularis* species (Kolmer et al. 2009, Liu et al. 2015). Other than wild species aeciospores can disperse artificially on to *Bartsia* (*Orobanchaceae*), *Euphrasia* and *Mentzelia* (*Loasaceae*) species. Aeciospores can be dispersed for long distance by wind, as far as 480 km (Maloy 2003) and after contact with a susceptible host species they germinate, penetrate the stomata and grow rapidly to infect the leaf (Smith 1970, Kolmer et al. 2009). Aeciospores of *C. comandrae* show optimum germination around 15°C when free water and dark environmental conditions are available (Powell 1974). Aecial infection produces cluster type aecial colonies by spreading disease up to death of host (Kolmer et al. 2009). Aecia of *C. fusiforme* induce swelling or galls and *C. ribicola* aecia induce necrotic cankers (Littlefield & Heath 1979). Peridermioid aecium is typical of *Cronartium* spp. The peridium is blister-shaped and has thick-walled cells with several layers covering the whole aecium surface (Littlefield & Heath 1979, Maier et al. 2003). The mycelium grows through leaves of *Ribes* sp. and sporulates to produce urediniospores from uredinia over summer (Liu et al. 2015). This stage can repeatedly re-infect the same host throughout summer (Maloy 2003, Kolmer et al. 2009). Urediniospores are transported over short distance (about 1.6 km) by wind (Maloy 2003, Richardson et al. 2005). Telial columns, which often develop from the uredinia (Maloy 2003), contain the teliospores. In late summer telia start to produce teliospores in rows (Liu et al. 2015). They germinate *in situ* to produce basidiospores that are ejected into the air by force but are sensitive to dry conditions (Kolmer et al. 2009). Basidiospores infect the pine host, starting to result in swollen cankers in following spring or summer to complete the life cycle (Kolmer et al. 2009, Liu et al. 2015). The different pathogenic stages, characteristics

and disease symptoms in life cycle of *C. ribicola* related aecial host and telial host are summarized in Table 1.

Isolation

Rusts are obligate biotrophs, which obtain nutrients and survive only on living hosts. Rusts are not lethal to the host until they form and release their spores (Vogler 2008). To study vegetative phases of rust fungi, laboratory practices related to culture preparations are used (Moricca & Ragazzi 2001). Isolates and growing techniques were reported for single genotype axenic cultures of *Cronartium* species by single hyphal fragments (Hu et al. 1994, Moricca & Ragazzi 2001).

Table 1 Different pathogenic stages of *Cronartium ribicola*.

Pathogenic state	Symptoms and characters	Reference
Spermatia from spermatogonia on white pine host	Chlorotic spots on pine needles Yellow-orange color spindle-shaped swollen cankers on pine branches and stem Orange color bark at canker margins	Lachmund (1933), Maloy (2003), Liu et al. (2015)
Aeciospores from aecia on white pine host	Aecial blisters on pine host Yellow-orange aeciospores release from cankers Dead pine branches and stem (within several years) Killing all plant parts above the stem Red colored dead needles from affected branch	Maloy (2003), O'Hara et al. (2010), Liu et al. (2015)
Uredinia on <i>Ribes</i> leaves	Chlorotic (yellow) spots on the upper leaf surface Orange color uredinia on underside of leaves Dark brown, hair-like telial column emerge from uredinia	Maloy (2003), Liu et al. (2015)
Telia on <i>Ribes</i> leaves	Teliospores on underside of leaves Producing basidia No infection	Maloy (2003)
Basidia on <i>Ribes</i> leaves	Producing basidiospores	Maloy (2001, 2003), Liu et al. (2015)

Cronartium stem rusts from pine hosts such as *C. fusiforme*, *C. pini*, *C. quercuum* and *C. ribicola* have been studied for *in-vitro* growth (Moricca & Ragazzi 2001). According to Hu et al. (1994) the axenic culture production from *C. quercuum* f.sp. *fusiforme* from basidiospores was first reported by Hollis et al. (1972). Successful saprophytic isolates from *C. pini* using individual basidiospores was first reported by Moricca & Ragazzi (2001). Previously, the Modified Harvey and Grasham (HG1) and modified Schenk and Hildebrandt (SH1) culture media (Schenk & Hildebrandt 1972, Harvey & Grasham 1974) were used for axenic growth of *C. pini*. Modified HG1 medium (HG1Y) with bovine serum albumin (BSA) has also shown excellent colony growth for single-genotype axenic cultures (Moricca & Ragazzi 2001, Moricca & Ginetti 2015). This culture media has been used to confirm the growth of *C. pini* from aeciospores and basidiospores. However, the capacity for axenic growth of rust fungi from their urediniospores has never been tested (Moricca & Ginetti 2015).

In isolation process, basidiospores are released onto agar media, showing considerable germination rate. The spores collected in the field can be contaminated with other microorganisms. The single-genotype rust cultures formation in pure culture techniques must overcome this barrier. The low contamination rate should be maintained (<2%) by removing the contaminant moulds (Moricca & Ragazzi 2001). The fungal colony shows fast growth on HG1 and HG1Y media amended with BSA. BSA positively affects fungal growth in media which form comfortable environment to fast hyphal growth (Moricca & Ragazzi 2001). The pure fungal mycelium can be used for molecular-based investigations for taxonomic development. It is also useful in analysing pathogenic variation.

Single genotype isolation has high genetic purity than multi-genotype isolation of rust fungi (Moricca & Ragazzi 2001).

Morpho-molecular studies

There is taxonomic confusion with white pine blister rusts because of insufficient information about host range and phylogeny (Geils et al. 2010). Spaulding (1922) resolved this and now *C. ribicola* is considered as a single, sexually reproducing rust that alternates between white pines and *Ribes* (Geils et al. 2010, Schoettle et al. 2019). Previously, *Ribes* spp. (*Grossulariaceae*) was considered as the natural alternate hosts of *Cronartium ribicola* in North America (Zambino et al. 2005). Presently, *C. ribicola* damage was revealed on wild *Castilleja miniata* and *Pedicularis racemosa* (*Orobanchaceae*) was proved by using ITS rDNA sequence analyses for teliospores while *Ribes* alternate hosts do not grow in Northern Idaho, USA (Zambino et al. 2005). The first reported molecular analyses of rust fungi were carried out using 5.8S rRNA (Gottschalk & Blanz 1984, Maier et al. 2003). ITS gene region was selected to confirm closely related species of many genera including *Cronartium* (Vogler & Bruns 1998, Maier et al. 2003). Maier et al. (2003) reported the phylogenetic relationships of selected genera in order Uredinales by using nuclear large subunit rDNA data also. Analyzing isozyme patterns and protein arrangements of aeciospores, differences among *C. appalachianum*, *C. comandrae*, *C. harknessii*, *C. ribicola* and *C. quercuum* can be distinguished (Powers et al. 1989, EFSA Panel on Plant Health et al. 2018).

The relationship between anamorphic *Peridermium* and *Cronartium* species was debatable (Vogler & Bruns 1993, 1998). *Peridermium harknessii* (anamorphic) species has higher similarities with (teleomorph) *C. quercuum* complex and *C. coleosporioides* complex (Vogler & Bruns 1993, 1998). They were difficult to distinguish using morphology and symptoms on the host. Analysis of ITS sequence results with *P. harknessii* has shown a closer relationship to *C. quercuum* complex than to *C. coleosporioides* complex and identical to *C. quercuum* f.sp. *banksianae* (Vogler & Bruns 1993). *P. harknessii* was synonymized as *Endocronartium harknessii* following the cytological evidence (Hiratsuka 1969, Vogler & Bruns 1993). Vogler & Bruns (1993) described the close phylogenetic relationships between *P. harknessii* with *C. quercuum* f.sp. *banksianae*, and *P. bethelii* with *C. comandra*.

The problematic relationship between *Cronartium flaccidum* and *Peridermium pini* (currently both known as *C. pini*), has arisen because of different host specificity and some morphological characters related to aeciospores between the two species (Kasanen 2001). Hiratsuka (1968) mentioned that aeciospores of *P. pini* has different aeciospore germ tube morphology in meiosis than *C. flaccidum*. Kaitera et al. (1999b) measured the morphological variation of aecial germ tubes within populations between above two species. Similar observations in variations of germ tube morphology between *C. flaccidum* and *Peridermium pini* have been reported by Klebahn (1938), Gibbs et al. (1988) and Kaitera et al. (1999). However, the studies of the hyphal ultrastructure of *P. pini* in pine cortex by Walles (1974), was compared with ultrastructure of *C. flaccidum* by Longo (1982) and no differences could be found in septa or haustoria. According to the suggestion from Moricca & Ragazzi (1998) *P. pini* and *C. flaccidum* were trying to distinguish by the ITS genes of rDNA (Kasanen 2001). Hantula et al (1998) successfully amplified the random amplified microsatellite (RAMS) markers from both fungi (Kasanen 2001). After phylogenetic analysis some RAMS markers were shown to be co-dominant. These markers are heterozygous within all *C. flaccidum* isolates, while *P. pini* is homozygous. Hantula et al. (1998) mentioned heterozygosity of marker loci of Italian *C. flaccidum* samples also went against to the homozygosity of *P. pini* samples (Kasanen 2001).

Following the molecular analyses, mistakenly identified *C. coleosporioides* were resolved as *C. ribicola* on *Orobanchaceae* (Zambino et al. 2005). Another study explored hybrid aecial states between *C. comandrae* and *C. ribicola* on limber pine species in British Columbia. By using ITS sequence data from *Cronartium* teliospores, it was revealed that no hybridization occurs between species on *Orobanchaceae* hosts (Zambino et al. 2005). *Cronartium asclepiadium* var. *quercuum* was the original reported name for pine gall rust disease and Shirai (1899) then synonymized it as *C.*

quercuum (Kaneko 2000). Morphological studies were done between *C. fusiforme* and *C. quercuum* and genetic plasticity has identified to understand the breeding resistivity of *Pinus* species.

Materials & Methods

Molecular data analyzing

Sequence data for the ITS gene region of *Cronartium* were obtained (Table 2) from GenBank (National Center for Biotechnology Information) and the literature (Vogler & Bruns 1993, 1998, Vogler 2008). Single gene alignment was automatically generated from MAFFT v. 7 (<https://mafft.cbrc.jp/alignment/server/>, Katoh & Standley 2013). The alignment was improved manually when necessary using BioEdit v. 7.0.9.0 (Hall 1999). Phylogenetic analyses were performed using maximum likelihood (ML) and Bayesian inference (BI). Fifty-nine strains are included in ITS gene region with 702 total characters in the final alignment. Maximum likelihood tree was generated using the RAxML-HPC2 on XSEDE (8.2.8) (Stamatakis 2014) in the CIPRES Science Gateway platform (Miller et al. 2010) using the GTR+I+G model of evolution.

Bayesian analysis was conducted with MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001) to evaluate Bayesian posterior probabilities (BYPP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) by Markov Chain Monte Carlo sampling (BMCMC). GTR+I+G was used in the command. Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generation. The distribution of log-likelihood scores was examined to determine stationary phase for each search and to decide if extra runs were required to achieve convergence, using the program Tracer 1.5 (Rambaut & Drummond 2007). Phylograms were visualized with FigTree v1.4.0 program (Rambaut 2012) and reorganized in Microsoft power point (2010).

Results

We have constructed a phylogenetic analysis of *Cronartium* species using ITS locus to show their intra specific relationships (Fig. 3). No topological conflicts occurred between trees generated from ML and BI analyses; the ML tree is shown in Fig. 3 with a final ML optimization likelihood value of -2922.652206. The matrix had 308 distinct alignment patterns, with 12.97% undetermined characters or gaps. In BI analysis, first 10 % of generated trees were discarded and remaining 90 % of trees were used to calculate posterior probabilities of the majority rule consensus tree. To discuss tree output of the phylogenetic analysis (Fig. 3), we divided the taxa in the phylogram into eight clades (A–H). In this attempt to illustrate phylogenetic relationships, we observed that there are close phylogenetic affinities from the species those are responsible for specific rust diseases. The species that are reported as causing blister rust diseases, nested in sub clades A, B, D and E. *Cronartium appalachianum*, *C. bethelii*, *C. coleosporioides*, *C. comandrae*, *C. comptoniae*, *C. pini*, *C. ribicola* and *C. occidentale* were grouped in above sub clades (Fig. 3). There is only one known species responsible for limb rust disease (*Cronartium arizonicum*) and it nested in sub clade C with close phylogenetic affinity to *C. coleosporioides* and *C. pini*. The species responsible for Scots pine blister rust diseases (*Cronartium pini*) nested in sub clade D with high bootstrap support (100% ML, 1.00 PP) as a monophyletic group. The species causing gall rust diseases viz. *Cronartium harknessii* and *C. quercuum* nested in sub clade F with high bootstrap support (96% ML, 1.00 PP) (Fig. 3) as a monophyletic group. The sub clade F is proven in the literature that anamorphic *C. harknessii* is the teleomorph state of *C. quercuum* reported by Vogler & Bruns (1993). There are two species reported for causing cone rust disease (*C. conigenum* and *C. strobilinum* and) and the strains of these species grouped in clade H as the most basal lineage in our phylogenetic analysis. There are three strains grouped in clade G that are still not recognized causing any damage to agriculture.

Our choice of ITS as the phylogenetic marker used herein has been based on the availability of ample sequence data. Other gene sequence data are simply not available for almost all of the species. It is recommended to obtain more gene markers such as LSU, RPB2, TEF and tub2 to perform analyses on different combinations of datasets to come up with reliable phylogenies to ensure that species definitions and justifications for establishing new species are scientifically valid in this genus.

Control of *Cronartium* diseases

In North America, chemical control of *Cronartium* using fungicides is practiced in a large scale, even in nursery stock (Pawsey 1963). High contribution of large-scale planting programs with nursery site selection and disease-free planting stocks should be established to explore the silvicultural potential and pathology of species (Pawsey 1963). Geils et al. (2009) gave general methods to minimize the effect of rust diseases such as fungicides for pine hosts, separation of alternate hosts from pine trees, biological agent for decreasing rust reproduction and silvicultural practices (Geils et al. 2009, Li et al. 2014). *Tuberculina maxima* the purple mould, has been used as a biological control agent in Western North American white pine blister rust disease (Kimmey 1969, Powell 1971). *Tuberculina maxima* can restrict aeciospore production of *C. comandrae* and all active cankers on lodge pole pine (Powell 1971). In Europe and Pacific Northwest, *T. maxima* has been observed and documented with the ability to reduce the inoculum of target rust organisms through inhibition of rust cankers (Maloy 2003). Destroying aeciospores is a promising way to control Armandii pine blister rust in Yunnan, China because of aeciospore generation is important stage which spreads blister rust (Li et al. 2014). Li et al. (2014) showed the destructive effect on aeciospores of Armandii pine blister rust by using mycoparasite SS003. Mycoparasite SS003 originally extracted from *C. ribicola* aeciospore piles and it was identified as *Trichoderma atroviride* by its morphology and ITS sequence analysis (Zhou et al. 2008, Li et al. 2014). Kaitera (2002) reported that thinning increased growth, and removal of infected Scots pine seeds avoid susceptible seed dispersal and removal of trees reduces disease spread. Consideration of genetic manipulation on selection, breeding, and spreading of hosts with high resistance is needed to mitigate the diseases (Geils et al. 2009). White pine blister rust can be prevented and decreased on young white pine trees by pruning of lower branches (Singleton & Oblinger 2017). Also, eradication of infected *Ribes* species is most controllable method for *C. ribicola*. In China, *C. ribicola* is a quarantine pest and the control measures are mainly involved with pruning and thinning of infected plants, chemical treatment for blister rust cankers and herbicide removal of telial hosts (Zhang et al. 2010, Li et al. 2014). Continuous investment of pruning treatments can increase survival probability of young white pine (McDonald & Hoff 2001). Geils et al. (2009) suggested that in North America, high safety policies and diagnostic techniques should be applied to confirm the rust infections for Scots pine blister rust. At the social level, awareness about the diseases and disadvantages to forestry, nursery and horticultural activities and in scientifically, genetic analyses of Scots pine blister rust to predict potential invasive risk should be developed (Geils et al. 2009).

The awareness of density and diversity of rusts on host trees, host populations, communities, and ecosystems are very useful to mitigate their pathogenic impacts (Geils et al. 2009). Regeneration for Resilience (R4R) framework is a powerful management method to control white pine blister rust disease caused by *C. ribicola* in North America (Schoettle et al. 2019). This is an integrated approach of forest health, genetic resistance, risk and hazard of pathogen and host population (Schoettle et al. 2019). Bulk seeding with qualitative genetic resistant genes is effective in low risk areas. By this, the acceleration of *C. ribicola* virulent strains can be reduced (Schoettle et al. 2019). When the number of healthy trees become too low the population of trees can be increased through regeneration. Also, nutrient-rich sites with susceptible alternate hosts should be avoided in regeneration of pines and deciduous trees can be used as mixture.

Genetic maps are also reported as a successful method to understand disease resistance (Liu et al. 2019). The high-density genetic map was constructed for *Pinus flexilis* (Limber pine) by exome-seq to understand the evolution of resistance (Liu et al. 2019). The rust resistance orthologous loci and evolutionary pressure on them were revealed by the genetic map (Liu et al. 2019). Genetic mapping has provided a practical tool for breeding and genetic conservation of limber pine and another *Pinus* sp. (Liu et al. 2019). It is very effective to provide a long-term breeding program on rust resistance against blister rust (Snieszko et al. 2011, Singleton & Oblinger 2017). Keane et al. (2017) mentioned that the active restoration design which fits the local community is needed for conserving ecosystems of white bark pine. It should be implemented with strategies to effects of climate change as well as considerably reducing the anthropogenic threats (Keane et al. 2017).

Fig. 3 – RAxML tree based on a dataset of partial ITS sequence analyses (*Cronartium*). Bootstrap support values for ML equal to or greater than 70 %, Bayesian posterior probabilities (PP) equal to or greater than 0.95 are shown as ML/PP above the nodes. The tree is rooted to *Coleosporium tussilaginis* and *Chrysomyxa rhododendri* (*Coleosporiaceae*). The scale bar represents the expected number of nucleotide substitutions per site. Species groups, where known for specific rust diseases are indicated with coloured blocks.

Table 2 Taxa used for molecular study and their corresponding GenBank numbers.

Species	Strain/Voucher	GenBank accessions
		ITS
<i>Coleosporium tussilaginis</i>	LB08615/ZT_Myc_57992	KY810485
<i>Chrysomyxa rhododendri</i>	1457CHR-RHF-FR/QFB25059	JF491434
<i>Cronartium appalachianum</i>	Ca-1	L76484
<i>Cronartium arizonicum</i>	FSprP-1	L76504
<i>Cronartium bethelii</i>	BYoC-1	U75987
<i>Cronartium bethelii</i>	BLvC-8	L76478
<i>Cronartium bethelii</i>	BRfC-10	L76479
<i>Cronartium bethelii</i>	BCpC-15	L76477
<i>Cronartium coleosporioides</i>	Pbract2-2006	EF185858
<i>Cronartium coleosporioides</i>	RNCast2	AY955828
<i>Cronartium coleosporioides</i>	RNPB2	AY955835
<i>Cronartium coleosporioides</i>	SPC-21	L76513
<i>Cronartium coleosporioides</i>	CsSr-1	L76500
<i>Cronartium comandrae</i>	1195CRM-GEL-BC	JN943253
<i>Cronartium comandrae</i>	393CRM-PNB-X12	JN943251
<i>Cronartium comandrae</i>	42CR-PNB-RN6	JN943242
<i>Cronartium comandrae</i>	44CRT-PNB-BJ4	JN943235
<i>Cronartium comandrae</i>	CcND-1	U75985
<i>Cronartium comandrae</i>	Cc-1	L76485
<i>Cronartium comandrae</i>	CPeEl-1	L76481
<i>Cronartium comptoniae</i>	1148CRT-PNB-LJ	JN943254
<i>Cronartium comptoniae</i>	BLD-B6-FP	JN943209
<i>Cronartium comptoniae</i>	INC-1-FP	JN943208
<i>Cronartium comptoniae</i>	CcomMn-1	L76487
<i>Cronartium conigenum</i>	CcL-1	L76486
<i>Cronartium harknessii</i>	40CR-PNB-LP1	JN943249
<i>Cronartium harknessii</i>	424PEH-PN-MW1	JN943248
<i>Cronartium harknessii</i>	425PEH-PN-X15	JN943247
<i>Cronartium harknessii</i>	428PEH-PN-QC13	JN943244
<i>Cronartium occidentale</i>	OMpM-1	L76507
<i>Cronartium pini</i>	GREEK1	X83908
<i>Cronartium pini</i>	GREEK2	X83909
<i>Cronartium pini</i>	GREEK3	X83910
<i>Cronartium pini</i>	THET1	X83911
<i>Cronartium pini</i>	THET2	X83912
<i>Cronartium pini</i>	WS4	KX147063
<i>Cronartium pini</i>	ALS3	KX147062
<i>Cronartium pini</i>	11001	JN802139
<i>Cronartium pini</i>	38A	AY566270
<i>Cronartium quercuum</i>	CqvGa-1	L76495
<i>Cronartium quercuum</i>	CqbMn-7	L76492
<i>Cronartium quercuum</i>	CqeGa-1	L76493

Table 2 Continued.

Species	Strain/Voucher	GenBank accessions
		ITS
<i>Cronartium quercuum</i>	CqfGa-1	L76494
<i>Cronartium quercuum</i> f. sp.	CqE3WM-FP	JN943199
<i>Cronartium quercuum</i> f. sp.	CqE7WM-FP	JN943198
<i>Cronartium quercuum</i> f. sp.	CqE9WM-FP	JN943197
<i>Cronartium ribicola</i>	RM-77	GU727731
<i>Cronartium ribicola</i>	MCA5066	KF387533
<i>Cronartium ribicola</i>	Geils 2005	KX574672
<i>Cronartium ribicola</i>	DAOM 234424	HQ317509
<i>C. ribicola</i> x <i>C. comandrae</i>	HYBRID_ITS_consensus	MG806113
<i>C. ribicola</i> x <i>C. comandrae</i>	COD	MG806112
<i>C. ribicola</i> x <i>C. comandrae</i>	ComA	MG806106
<i>Cronartium</i> sp.	PUR N11655	KY587788
<i>Cronartium</i> sp.	CqL-1	L76489
<i>Cronartium</i> sp.	CqQ-1	L76490
<i>Cronartium strobilinum</i>	G-317-HGS1-FP	JN943192
<i>Cronartium strobilinum</i>	807-ISFSL-FP	JN943191
<i>Cronartium strobilinum</i>	CSt-2	L76482

Discussion

Cronartium species are rust pathogens causing severe damage to conifer forests and to several *Ribes* species by decreasing plant growth, causing stem deformation, and reducing quality of wood. Applications of natural regeneration using silvicultural practices and artificial regeneration with disease-free plant materials can mitigate this catastrophe. Genetic bottlenecks on pine species should be mitigated by exploring new genetic resistivity probabilities to *Cronartium* rust diseases for ecosystem balancing with high diversity.

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Accessibility of data

All the ITS sequence data were extracted from GenBank (Table 2).

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