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Species composition and distribution of *Coleosporium* species on the needles of *Pinus densiflora* at a semi-natural vegetation succession site in central Japan☆

Hiroyuki Suzuki [a], Dai Hirose [b], Yuichi Yamaoka [a, *]

[a] Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki, 305-5872, Japan
[b] College of Pharmacy, Nihon University, Funabashi, Chiba, 274-8555, Japan

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Coleosporium species cause pine needle rust. Most species have heteromacrocyclic life cycles, and 12 species use *Pinus densiflora* as aecial hosts. To understand the biology of rust fungi and develop better methods for controlling rust diseases, it is necessary to clarify that which *Coleosporium* species affect pine trees. However, *Coleosporium* on pine trees have rarely been identified at the species level because of their morphological similarities. We used polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) to clarify the species composition, abundance, and distribution of *Coleosporium* in a *P. densiflora* forest. We surveyed a site where several *Coleosporium* species might complete their life cycles. PCR-RFLP revealed four species on the pines: *C. asterum*, *C. clematidis-apiifoliae*, *C. lycopodis*, and *C. phellodendri*. *Coleosporium phellodendri* was distributed throughout the forest and was the most abundant. Aecia of *C. phellodendri* formed mainly on 2-y-old needles. The abundance and distribution of *C. phellodendri* appeared to be affected by the longer effective dispersal range of basidiospores and the existence of abundant inoculum sources. The age of leaves where *C. phellodendri* form aecia mainly was thought to be influenced by the characteristic life cycle, with aecial formation requiring 2 y after basidiospore infection.

1. Introdnuction

Coleosporium species, in the order Pucciniales, cause pine leaf rust. In Japan, 28 species have been recorded (Harada, 1994; Hiratsuka et al., 1992). Most species have heteromacrocyclic life cycles, with five spore states, and require two unrelated groups of host plants. Their spermogonial and aecial states are found on the needles of pines. The uredinial and telial states occur on various woody and herbaceous angiosperms, which are referred to as alternate hosts (Hiratsuka, Sato, & Kakishima, 1984; Kaneko, 1981). Teliospores disperse basidiospores without dormancy under suitable conditions. Basidiospores usually infect current-year needles of pines. Spermogonia and aecia are usually formed on needles the following spring. The host alternation of *Coleosporium* spp. has been elucidated by inoculation experiments. However, little is known which species are present on pine trees in the field because the morphological characters of the aecial state are similar among the species.

The accurate identification of a large number of aecia obtained in the field is necessary for analyzing the abundance of *Coleosporium* in the habitat being surveyed. In the past, *Coleosporium* on pine needles were identified by experimental inoculations together with close examination of the sorus and spore morphology of the uredinial and telial states (Hama, 1972; Saho, 1963a; Zinno & Chiba, 1967; Zinno & Endo, 1964). This method requires a longer period of time to obtain results and is difficult to utilize for the large number of aecia that are often required in ecological studies. Therefore, a method of identifying large samples of aecia on pine needles collected in the field within a short period of time is needed.

In recent years, molecular techniques have frequently been used to identify fungal species with similar morphological characteristics. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) is a technique for detecting variations in

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* Corresponding author. Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki, 305-5872, Japan.
E-mail address: yamaoka.yuichi.gp@u.tsukuba.ac.jp (Y. Yamaoka).

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homologous DNA sequences. Because it uses various restriction enzymes working at different sites, PCR-RFLP is highly adaptable for targeting various DNA regions. This method has often been used to identify or distinguish species with minor morphological differences or races within a species (Imazu et al., 2000; Moricca & Ragazzi, 1998; Nakamura, Kaneko, Yamaoka, & Kakishima, 1998; Santos, Souza, Junior, Thalhari, & Souza, 2010). In addition, PCR-RFLP was used to evaluate the diversity of soil fungi and clarify the species composition of fungi isolated from fruit, as this method can identify fungi more easily, rapidly, and inexpensively than sequencing (Diguta, Vincent, Guilloux-Benatier, Alexandre, & Rousseaux, 2011; Muriel, Aymeric, & Yves, 2000). Therefore, PCR-RFLP seemed to be useful for identifying aecia of many *Coleosporium* species collected from the field.

Pinus densiflora Siebold & Zucc. (Japanese red pine) is an aecial host species of *Coleosporium* species, and 12 species of *Coleosporium* spend their aecial state on the needles of Japanese red pine (Hiratsuka, 1933; Kaneko, 1981; Saho, 1966; Sakuyama, 1973). Japanese red pine is widely distributed in Japan throughout Kyushu, Shikoku, Honshu, and the southern region of Hokkaido. Although the distributions of the aecial state of the 12 *Coleosporium* species are unknown, they might have different distribution areas because their uredinial and telial states have different distribution areas. Therefore, species in different distribution areas are likely adapted to different climate conditions, and species that have the same distribution areas should have evolved ecological strategies such as the segregation of niches in a pine and viability of spores for competition. To understand the biology of rust fungi and develop better methods of controlling rust diseases, researchers need to clarify the differences in their ecological strategies and functions.

The Sugadaira Research Station, Mountain Science Center, University of Tsukuba is located at an elevation of 1300 m on the Sugadaira plateau, in Ueda, Nagano Prefecture, central Honshu, Japan. At Sugadaira Research Station, different types of vegetation are arranged semi-naturally along a typical successional gradient in central Japan (i.e., *Miscanthus* grassland, Japanese red pine forest, and deciduous forest; Fig. 1). A survey of rust fungi from 2006 to 2008 found that four species that potentially infect *P. densiflora* existed on their alternate hosts. The four species were *C. asterum*

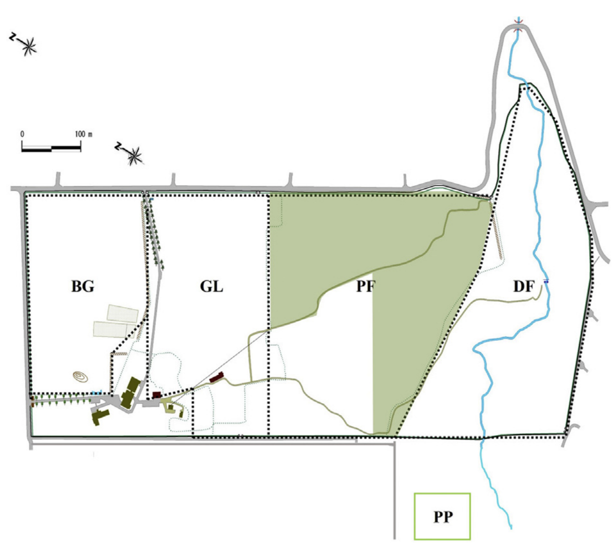

Fig. 1. –Map of the Sugadaira Research Station. GL: grassland of *Miscanthus sinensis*, PF: forest of *Pinus densiflora*, DF: deciduous forest, BG: botanical garden, PP: plantation of *Phellodendron amurense*. The green area in the grassland represent the area whose forest floor is covered by *Sasa senanensis*.

(Dietel) Syd. & P. Syd., *C. clematidis-apiifoliae* Dietel, *C. lycopodis* Syd. & P. Syd, and *C. phellodendri* Kom. These four species might complete their life cycles using *P. densiflora* at the research station. Sugadaira Research Station is a suitable site for studying the ecology of *Coleosporium* on pines, which requires large numbers of samples.

Before performing biogeographical studies, it is necessary to establish a reliable method for identifying numerous aecial samples on pine needles and a method to evaluate the abundance of the aecial state of *Coleosporium*. Here, we clarify the species composition, distribution, and abundance of *Coleosporium* on the needles of *P. densiflora* at the research station using a newly established PCR-RFLP method.

2. Materials and methods

2.1. Study site

The study site at the Sugadaira Research Station, Mountain Science Center, University of Tsukuba (36° 31′ N, 138° 21′ E) is located on the Sugadaira plateau, Ueda in Nagano Prefecture, central Honshu, Japan, at about 1300 m above sea level. The annual mean temperature (from 1981 to 2010) at the site is 6.6 °C, and the monthly mean temperature ranges from 19.4 °C in August to −5.5 °C in February. The mean annual precipitation is 1342 mm (http://www.sugadaira.tsukuba.ac.jp/nature/weather.html). The region belongs to the humid continental climate (Dfb) in Koppen's classification of climates. At Sugadaira Research Station, three vegetation zones are established, in addition to the botanical garden: *Miscanthus* grassland, Japanese red pine forest, and deciduous forest (Fig. 1). The typical pattern of secondary vegetation succession in central Honshu progresses from grassland dominated by *Miscanthus sinensis* Andersson to deciduous forest through Japanese red pine forest (Kashiwagi, 1991).

Miscanthus sinensis grassland is a typical mountain grassland found in central Honshu, Japan. In addition to *M. sinensis*, various herbs and shrub such as *Pteridium aquilinum* (L.) Kuhn, *Lespedeza bicolor* Turcz., and *Artemisia indica* Willd. var. *maximowiczii* (Nakai) H. Hara grow in the grassland. The grassland progresses to Japanese red pine forest under natural conditions. To prevent succession, plants in the grassland are artificially cut in the middle of October every year. Several alternate hosts of *Coleosporium* spp. grow in the grassland: *Adenophora triphylla* (Thunb.) A.DC. var. *japonica* (Regel.) Hara for *C. lycopodis*, *Aster iinumae* Kitam. and *A. microcephalus* (Miq.) Franch. & Sav. var. *ovatus* (Franch. & Sav.) Sejima & Mot.Ito for *C. asterum*, and *Clematis apiifolia* DC. for *C. clematidis-apiifoliae*. *Adenophora triphylla* var. *japonica* is distributed throughout the grassland, and the coverage of *A. triphylla* var. *japonica* is the highest among all *Coleosporium* alternate hosts in the grassland (Hayashi, 2003).

The *P. densiflora* forest is located southeast of the grassland and is approximately 8.5 ha in area. The age of the Japanese red pine trees is approximately 50 y at the northwestern edge of the forest and gradually increases up to approximately 70 y toward the southwest. The forest density is 137 trees in an area of 400 m^2 (Kashiwagi, 1991). Most trees, except for those at the edges and in gaps in the forest, have dropped their lower branches because of a lack of sunlight. Although *C. apiifolia* is distributed on the forest floor, most of the forest floor is covered by *Sasa senanensis* (Franch. & Sav.) Rehder (Fig. 1).

The deciduous forest adjacent to the Japanese red pine forest is mainly composed of *Quercus crispula* Blume. In addition, *Betula platyphylla* Sukaczev var. *japonica* (Miq.) H.Hara, *Acer* spp., and *Prunus* spp. grow in the forest. A few *Phellodendron amurense* Rhpr., which is the alternate host species of *C. phellodendri*, and *C. apiifolia*

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