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Evidence of clonal propagation in *Cryptomeria japonica* D. Don distributed on Pacific Ocean side in Japan

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Abstract

Cryptomeria japonica is distributed on the Pacific Ocean side and reproduces only by seedling; however, *C. japonica* var. *radicans* is distributed on the Japan Sea side and reproduces by both seedling and layering. Until now, there has been no report that *C. japonica* on the Pacific Ocean side regenerates by layering. In this study, the regeneration system of *C. japonica* in a national forest of Miyagi Prefecture Ishinomaki City was investigated using DNA analysis. *C. japonica* growing in the study plot was divided into seven genets and 40 single ramets, clearly suggesting that *C. japonica* at this site regenerates not only by seedling, but also by layering. The strong winds and low temperature appear to be key factors in layering reproduction at this site.

Keywords: *sugi, C. japonica var. radicans, SSR, microsatellite, clone reproduction*

Introduction

Cryptomeria japonica D. Don (Sugi) is an allogamous and wind-pollinated coniferous species endemic to Japan (Tsumura, 2011). Natural forests are discontinuous and restricted to small areas ranging from Aomori Prefecture (40º42′ N) in the north to Yaku Island (30º15′ N) in the south. The altitudinal distribution ranges from 0 m in Wakayama Prefecture to 2,070 m in Toyama Prefecture (Maeda, 1983; Taira, 1985). This species has adapted to a wide range of environmental conditions and is the most important forestry species in Japan. Variations among the natural forests of *C. japonica* throughout Japan were investigated using diterpene components (Yasue et al., 1987), which suggested two varieties, known locally as ura-sugi (*C. japonica* var. *radicans*, growing on the Japan Sea side) and omote-sugi (*C. japonica*, growing on the Pacific Ocean side) (Yamazaki et al., 1995). Regarding the morphological features of leaves, there is no clear difference between *C. japonica* and *C. japonica* var. *radicans* (Tohyama, 1960). In contrast, there is a clear difference in their reproduction systems, i.e., *C. japonica* on the Pacific Ocean side reproduces only by seedling, but *C. japonica* var. *radicans* on the Japan sea side reproduces both by seedling and layering (Yamazaki et al., 1995, Ohashi et al., 2015). Layering generally occurs when a sprout touch to the ground by snow pressure. In the mountainous, high-altitude regions on the Japan Sea side, *C. japonica* var. *radicans* reproduces only by layering because it is not possible to regenerate through seedlings (Taira et al., 1997; Moriguchi et al., 2001). Kimura et al. (2013) reported the clonal propagation of 13 natural populations (3 populations on the Pacific Ocean side and 10

DOI:10.1515/sg-2017-0007
edited by the Thünen Institute of Forest Genetics

populations on the Japan Sea side), and confirmed that layering is a peculiar characteristic of populations on the Japan Sea side (i.e., *C. japonica* var. *radicans*). For a significant period, this was the adopted theory.

Taira (2011) recently described the possibility of clonal propagation of *C. japonica* in the Ishinomaki population based on its morphological features (curved stem and/or sprouts from the stem). This population is located on the Pacific Ocean side, being classified into the group of the Pacific Ocean side by Yasue et al. (1987). In this study, we analyzed the clonal structure in the Ishinomaki *C. japonica* population by using simple sequence repeat (SSR) markers, and investigated whether *C. japonica* can reproduce by layering.

Materials and Methods

Study plot

A study plot (36 x 36 m) was established at Miyagi Prefecture, Ishinomaki City at a latitude-longitude of N38 19.026, E141 27.953. *C. japonica* was dominant in the canopy. *Pinus densiflora, Abies firma, Chamaecyparis pisifera, Aesculus turbinate,* and *Acer palmatum* were also present in this plot. All *C. japonica* ramets over 5 cm in diameter at breast height (DBH) were mapped, and the measured DBH, tree height, and needle tissues were collected for DNA analysis. Root connections and large curved stems (*Figure* 1) were carefully checked. The basal area (BA) was calculated based on the DBH.

Figure 1
A *Cryptomeria japonica* tree in the study plot with a large curved stem.

Environmental factors

We assessed the wind speed, snow depth, and slope angle at the study plot. The snow depth was calculated from the 1-km mesh snowfall data over a period of 30 years (from 1971 to 2000), which were obtained using the program provided with the Mesh Climatic Data of Japan, published by the Japan Meteorological Agency (2000), to compare the data to those reported by Kimura et al. (2013). The wind data of Ishinomaki, Hamamatsu (Shizuoka Prefecture), Kaiyo (Tokushima Prefecture), and Kito (Tokushima Prefecture) (the three areas other than Ishinomaki used as study sites of *C. japonica* described by Kimura et al., 2013) were calculated from the data over a period of approximately 8 years (from 2009 to 2016), which were collected from the Japan Meteorological Agency. The slope angle was measured using a laser telemeter (TruPulse 200, Laser Technology, Inc., Centennial, CO, USA).

DNA analysis

Total DNA was extracted from the young needle tissue of each ramet using a modified cetyltrimethyl ammonium bromide method (Tsumura et al., 1995). We determined the genotypes of all ramets using the eight highly polymorphic genomic SSR markers described by Kimura et al. (2013) and used for clone identification (CS1525, CS1579, CS1219, CJS0520, CS1364, CS2169, CJG0077, and CJS0333; Moriguchi et al., 2003; Tani et al., 2004). PCR amplifications were performed in 6-µL reaction volumes containing 5 ng genomic DNA, 3 µL of 2× QIAGEN Multiplex Master Mix (QIAGEN, Hilden, Germany), and 2 µM of each primer (forward primer labelled with dye in each primer pair). Amplifications were performed on a Takara PCR Thermal Cycler (Takara, Tokyo, Japan) under the following conditions: initial denaturation for 15 min at 95°C, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 90 s at 57°C, extension for 60 s at 72°C, and a final extension for 60 min at 60°C. PCR products and the DNA size marker (LIZ600; Life Technologies, Foster City, CA, USA) were separated by capillary electrophoresis on an ABI 3130 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). DNA fragments were detected using GeneMarker software (ver. 2.4.0; SoftGenetics, State College, PA, USA).

Statistical analysis

Differences in wind strength among the four populations were examined by the Tukey-Kramer test. Statistical analysis was performed using R software (ver. 3.0.3; R Development Core Team, Vienna, Austria).

Results and Discussion

The BA of *C. japonica* had a biased distribution due to its smaller size; however, there were some large ramets with a BA of over 10,000 cm^2 (Figure 2). The wind strength at Ishinomaki was significantly stronger than that at the other three sites on the Pacific Ocean side (*Figure* 3). The strong wind increased the possibility of fallen trees. In fact, there were some fallen trees in the study plot, which improved the light conditions, thus exerting a positive effect on the establishment of *C. japonica* seedlings.

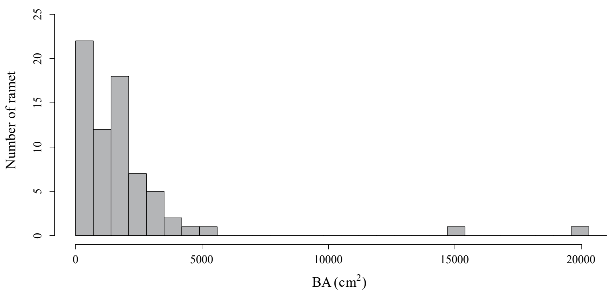

Figure 2
Basal area (BA) of *C. japonica* distributed in the study plot.

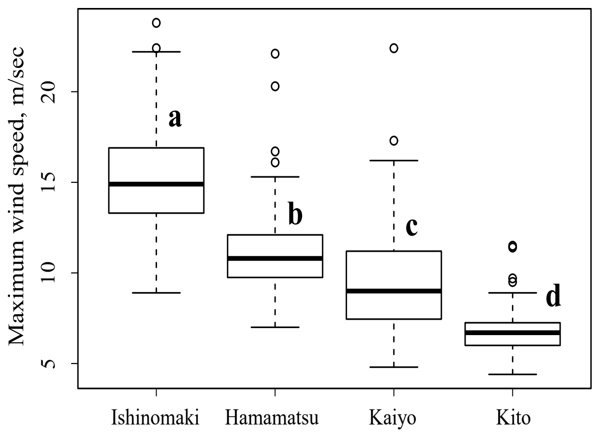

Figure 3
Comparison of wind data for four areas on the Pacific Ocean side of Japan. Three of the areas, excluding Ishinomaki, were used as study sites by Kimura et al. (2013).

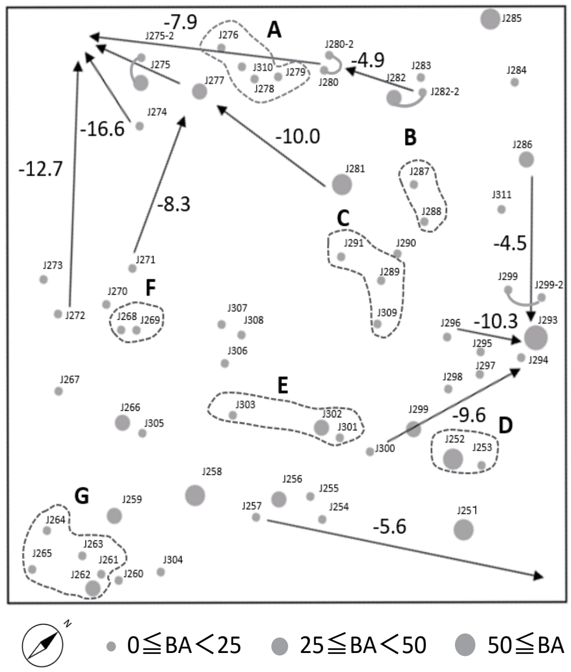

Figure 4
Location of *C. japonica* in the study plot. Circles indicate the BA. The sets of trees enclosed by dashed lines show genets A to G. Large curved stems are shown by solid lines. The arrows and values indicate the direction and degree of the slope, respectively.

There were 61 ramets in the study plot, four ramets of which had a large curved stem (*Figure* 4). According to SSR analysis, *C. japonica* growing in the study plot was divided into seven genets and 40 single ramets. Most of genets (B, C, E, F and G) were spread flat area. The largest genet consisted of five ramets, and the smallest genet consisted of two ramets. These results clearly show that *C. japonica* in the Ishinomaki population, which is located on the Pacific Ocean side, regenerates not only by seedling, but also by layering. Taira (1994) classified the layering of *C. japonica* into two major types: sprout-layering and seedling-layering. Sprout-layering is derived from sprouts with the stem touching the ground, and seedling-layering is derived from seedlings creeping on the ground. In the Ishinomaki population, both layering types are possible.

The average snow depth over the 30-year period in Ishinomaki was only 7 cm, which is typical for the Pacific Ocean side (average snow depth of Hamamatsu, Kaiyo, and Kito = 1, 3, and 6 cm, respectively (Kimura et al., 2013)). Kimura et al. (2013) detected layering in various populations on the Japan Sea side (associated with *C. japonica* var. *radicans*; average snow depth = 26–132 cm). Although snow pressure is one of the most important triggers for layering propagation, heavy snow may not always be necessary. In Ishinomaki (the northernmost population on the Pacific Ocean side), snow does not disappear readily due to the low temperature, which may lead to successful seedling-layering and/or sprout-layering even given the low snow depth.

Kimura et al. (2013) suggested that genetic factors have a more significant effect on clonality than environmental factors, such as snow depth. Kimura et al. (2014) investigated the genetic characteristics of 37 natural populations and suggested that the Ishinomaki *C. japonica* population has its main gene pool on the Pacific Ocean side. However, the sprouts and large curved stem of *C. japonica* observed in Ishinomaki are typical morphological features of *C. japonica* var. *radicans.* Because all populations have gene pools both on the Pacific Ocean side and the Japan Sea side (Kimura et al., 2014), layering propagation may occur not only in *C. japonica*, but also in *C. japonica* var. *radicans* if environmental factors are stable.

Acknowledgement

The authors are grateful to Dr. M. Kimura for valuable discussions. We thank R. Katayama and Dr. K. Uchiyama for their assistance in collecting samples and weather data, respectively.

This study was in part supported by a GLocal AGE 2020 Japanese Scholarship program.

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