Recent clonal reproduction of *Cryptomeria japonica* in a snowy region revealed by a survey of small-sized ramets

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Abstract

Clonal reproduction may contribute to population maintenance in areas where disturbance caused by snow limits tree recruitment. To understand the importance of clonal reproduction in the population dynamics of canopy tree species, it is necessary to determine the frequency of clonal reproduction in the early stages of seedling establishment. We found 106 ramets, including "small-sized" ramets of less than 5 cm in diameter at breast height, aggregated within 4 patches in a 70 × 50 m plot and also identified 20 genets among these ramets with the use of nuclear microsatellite markers. The size structure of the ramets revealed an inverse J-shaped distribution, suggesting that continuous recruitment of new ramets occurs. However, the number of intermediate-sized ramets (around 10 cm DBH) at the present study site was small, suggesting that most new ramets die while they are still small by pressure from heavy snow. Of the 20 genets, 12 included one or more small-sized ramets, which indicated recent recruitment. Of the 12 genets, 3 included only a single small-sized ramet, which suggested seedling recruitment, whereas the other 9 included multiple ramets (39 small-sized ramets in total), which indicated clonal recruitment. The frequency (9/12) and number (39/9) of recent clonal recruits suggest that clonal reproduction effectively maintains the population of *Cryptomeria japonica* in snowy regions.

Keywords: *Clonal diversity, microsatellite marker, layering, seedling recruitment, conifer*

Introduction

Many forest tree species have the capacity to grow clonally *via* root suckers, rhizomes, and stolons and by sprouting and layering. The purposes of clonal reproduction are multifold, which include restoration following disturbances and population expansion (Silvertown and Charlesworth, 2001). Sprouting and layering are particularly common phenomena in tree species and are efficient means of recovering biomass and reducing mortality following a disturbance (Bond and Midgley, 2001; Nagamitsu et al., 2004; Nagashima et al., 2015). It is necessary to determine the frequency of clonal recruitment at a given time in order to elucidate the role of clonal reproduction in canopy tree species.

Investigations focusing on ramets of a small-sized class (e.g., those smaller than 5 cm in diameter at breast height [DBH]) are required to determine clonal recruitment at a given time point. However, clonal reproduction in small-sized ramets has not been well studied, especially in canopy tree species. Also, distinguishing seedlings from clonal shoots is difficult

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when conducting field observations. The rate of seedling recruitment is still unknown as there is no clear understanding of when genets are recruited. Highly polymorphic co-dominant markers, such as microsatellite markers (i.e., simple sequence repeats), are useful not only for the identification of genets but also for the estimation of genetic relationships among genets (Kimura et al., 2013a; Kimura et al., 2013b; Nagashima et al., 2015; Doğan et al., 2017).

Cryptomeria japonica D. Don (Cupressaceae *sensu lato*) is a coniferous species endemic to Japan. Natural forests of *C. japonica* are distributed in areas of diverse environmental conditions in the Japanese archipelago, from Aomori Prefecture (40°42′N) to Yakushima Island (30°15′N) (Hayashi, 1960). This species can regenerate either from seedlings or by layering. Several studies have revealed a higher frequency of layering in regions where snowfall is heavy (Moriguchi et al., 2001; Hirayama and Sakimoto, 2008; Kimura et al. 2013a); in such areas, environmental and genetic factors affect the frequency of clonal reproduction of *C. japonica* (Kimura et al., 2013a). In the present study, we assessed the frequency of recent clonal reproduction in *C. japonica* by investigating the clonal structures of all ramets, including small-sized ramets with DBH less than 5 cm.

Materials and Methods

Study site

The study was conducted in Niigata University Forest (NUF), Niigata University, Sado Island, Niigata, Central Japan (38°12.2′N, 138°27.1E, 880–895 m above sea level). The environmental data in the study area was estimated from the meteorological data in a 1-km grid for a 30-year period from 1971 to 2000 (Japan Meteorological Agency, 2002), and the mean annual temperature, precipitation and maximum snow depth were 9.7 °C, 2,700 mm and about 2 m, respectively. The area encompassed a well-developed cool-temperate mixed forest (ca. 500 ha) dominated by *C. japonica*, *Fagus crenata*, *Acer* spp., and *Quercus* spp. No record of either catastrophic or artificial disturbances in this area has been found for centuries. In May 2012, during the thawing season, a plot (about 70 m × 50 m) was established on flat ground within the study area, and the locations of all *C. japonica* ramets in the plot were measured. The canopy layer in the plot consisted of only *C. japonica*, and the height of the trees was about 18 m. The canopy was partially damaged by the wind from the sea. The canopy was partially damaged by the wind from the sea. The forest floor was covered by about 70 % of shrubs (e. g., *Lindera umbellata* var. *membranacea*, *Ilex leucoclada*, *Ilex crenata* var. *radicans*, *Pachysandra axillaris*), herbs (e. g., *Schizocodon soldanelloides* var. *magnus*, *Carex multifolia* var. *multifolia*) and ferns (e. g., *Matteuccia struthiopteris*). We identified 4 *C. japonica* patches with 106 ramets in total. All ramets with a DBH greater than 5 cm were treated as different ramets (even if connected at the base) and DBH was measured as ramet size. It is difficult to distinguish between sprouts and seedlings that have germinated at the mossy butt swell of *C. japonica* trees. The ramets with DBH less than 5 cm were categorized as "small-sized ramets", and the diameter of the base (D_0) was measured as ramet size.

DNA analysis

Total DNA was extracted from the needle tissue of each ramet applying a modified cetyltrimethylammonium bromide (CTAB) method (Tsumura et al., 1995). We determined the genotypes of all ramets using eight nuclear microsatellite loci (*Cjg0077*, *Cjs0333*, *CS1219*, *CS1525*, *CS1364*, *CS1579*, *CS2169*, *Cjs0520*) (Moriguchi et al., 2003; Tani et al., 2004). The average non-exclusion probability for the identity of two unrelated individuals (Waits et al., 2001) was very low (7.13×10^{-12}) using these eight microsatellite loci (Kimura et al., 2013a). Therefore, ramets that shared the same multilocus genotype were regarded as ramets of the same genet. If only one of the 16 alleles was different, it was considered a somatic mutation and determined to be the same genet (three of the 106 ramets). At least four of the eight loci differed between the different genets in this study. The PCR conditions and genotyping method were as described by Kimura et al. (2013a).

Clonal diversity

Multilocus genotypes were detected using eight microsatellite markers developed by GenAlEx 6.41 (Peakall and Smouse, 2006). The clonal reproductive frequency, which was expressed as the *G*/*N* ratio, was calculated for each sampling patch; *G* is the number of observed distinct genotypes (genets), and *N* is the total number of individual trees (ramets). The genotypic evenness (*E*; Fager, 1972) was also calculated for the sampling patch as follows: $E = (D - D_{min})/(D_{max} - D_{min})$, where $D = 1 - [\Sigma n_i (n_i - 1)]/[N(N - 1)]$, $D_{min} = [(G - 1)(2N - G)]/[N(N - 1)]$, and $D_{max} = [N(G - 1)]/[G (N - 1)]$; *N* denotes the total number of ramets in each sampling patch, and n_i denotes the number of individual ramets of genet *i*. To elucidate the contribution of seedlings and clones to recruitment, these values were calculated for small-sized ramets (DBH < 5 cm).

The sibling relationships among the sampled genets were inferred by the likelihood method based on the genotype data of 20 sampled genets and allele frequency data from other natural populations using the COLONY 2.0.3.3 software (Wang, 2004; Wang and Santure, 2009; Jones and Wang, 2010). We calculated the allele frequencies of eight microsatellite markers in 1,239 individuals from 38 natural *C. japonica* populations and 20 sampled genets. Subsequently, we calculated the likelihood of a given categorization of genets into half- and full-sib families, and in each case, the family structure with the maximum likelihood was identified applying the Markov chain Monte Carlo method with a simulated annealing technique (Wang, 2004). Five independent runs with the same dataset were conducted to confirm that all runs converged to the same family structure with the same maximum likelihood. Each run had a 0.5 paternity score or a 0.5 maternity score, an allelic dropout rate of 0, a genetic error rate of 0.0001, and no selfing.

Results

The frequency distribution of ramets was inverse-J-shaped (Figure 1). In total, 46 ramets (43.4 %) were "small-sized ramets (DBH < 5 cm)", and 20 multilocus genotypes (genets) were identified for all of the ramets (n = 106) in the 4 sampling patches analyzed using the eight microsatellite loci. The number of ramets per genet ranged from 1 to 23, with a mean of 5.3. Of the 20 genets, 6 had only one ramet (defined as solitary genets), whereas the rest had more than two ramets (defined as multiple genets). The total *G*/*N* ratio was 0.19, and the *E* value was 0.52.

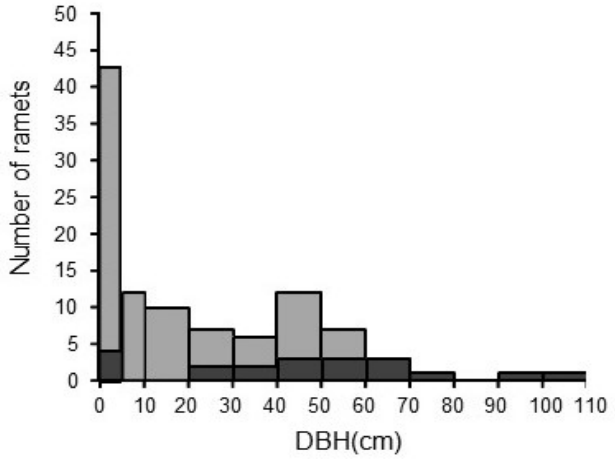


Figure 1
Frequency distribution of *Cryptomeria japonica* ramets of different sizes (diameter at breast height). Dark bars indicate the largest ramet of each genets, and shaded bars indicate the other ramets of various genets.

The number of small-sized ramets per genet ranged from 1 to 16, with a mean of 3.8. The total *G*/*N* ratio and *E* value were 0.26 and 0.53, respectively; these values are similar to those for large ramets (0.27 and 0.59, respectively). The size distributions of ramets varied among genets (Figure 2). Of the 14 multiple genets, 9 had several small-sized ramets produced by recent clonal reproduction.

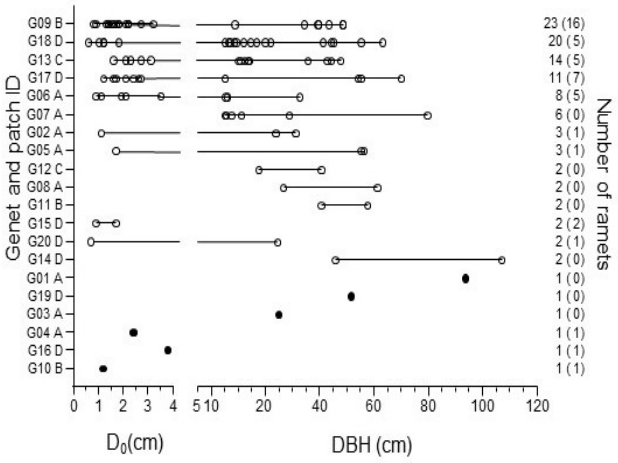


Figure 2
Size distribution and number of ramets of each genet of *Cryptomeria japonica* in a study plot. Closed and open circles denote genets with a single ramet and multiple ramets, respectively. D_0 indicates the diameter at the base of a small-sized ramet with a diameter at breast height (DBH) less than 5 cm. The number in parentheses of the number of ramets indicates the number of small-sized ramets.

Clonal reproduction was detected in all four sampling patches (Figures 2 and 3). No multilocus genet was shared among the sampling patches; in fact, every genet was unique to a single sampling patch (Figures 2 and 3). We found solitary genets in three of the four sampling patches. The *G*/*N* ratio in each patch varied from 0.12 to 0.32, with an average of 0.18. The *E* value ranged from 0.13 to 0.56. Genet G09 had the largest number of ramets (at least 23). Multiple genets formed mutually exclusive patches (Figure 3). Genet G18 occupied the largest area, with a maximum diameter of about 10.6 m. We identified the largest ramet from each genet, which are considered to be putative founders; other ramets likely derive from layering.

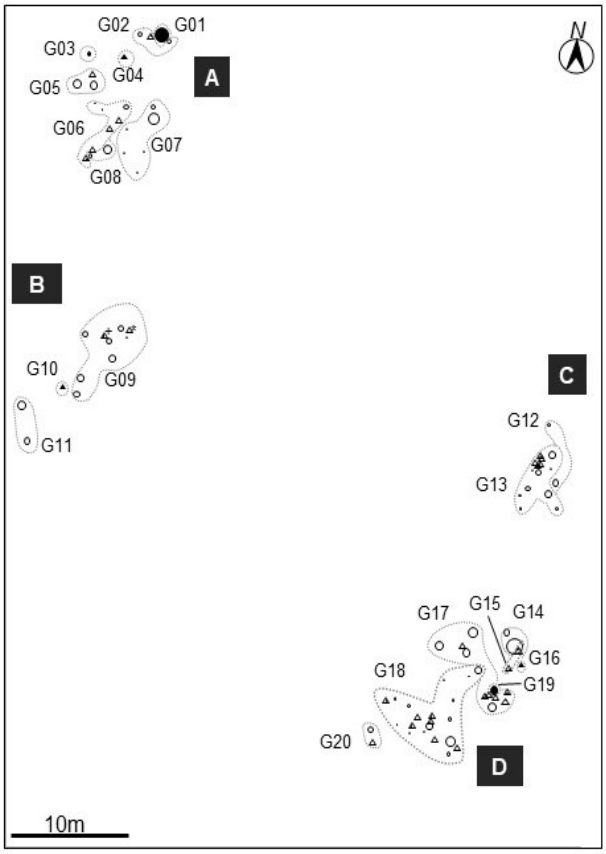


Figure 3
Spatial distribution of ramets of *Cryptomeria japonica* in a study plot. Genets were aggregated in four patches (A–D). Areas enclosed by dotted lines indicate genets. Triangles denote small-sized ramets of <5 cm in diameter at breast height (DBH), and circles denote large ramets of ≥5 cm in DBH. The size of the circles reflects the DBH of ramets. Closed and open symbols denote genets with a single ramet and multiple ramets, respectively. Triangles with * and + indicate a cluster of 5 and 10 small-sized ramets, respectively.

The effective population size estimated using the COLONY 2.0.3.3 software was 23 (90 % confidence interval, 13–45) under random mating versus 20 (90 % confidence interval, 11–42) under nonrandom mating. The number of estimated parents was 17 from 20 genets. We estimated the family structure among genets with the use of combined data from five runs using the likelihood method. We found 3 full-sib relationships and 15 half-sib relationships (Figure 4). The full-sib genets were all found within the same sampling patch (patch B and patch D). Two parent–offspring relationships were identified in patch D (G15 and G18 and G15 and G20).

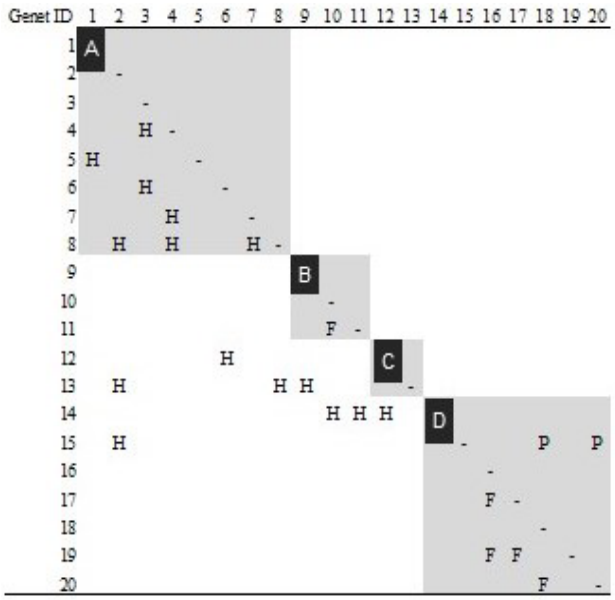


Figure 4
Estimated kinship between genets of *Cryptomeria japonica* in a study plot. Shaded rectangles denote patches. Kinship is categorized as follows: P, parent–offspring; F, full-sib; H, half-sib.

Discussion

The size structure of the ramets revealed an inverse J-shaped distribution for all of the ramets of the four sampling patches, suggesting that continuous recruitment of new ramets occurs in this species. However, potential seedling recruitment was limited at the study site (3/46) (Figure 2). Additionally, the total *E*, including small-sized ramets in the <5 cm DBH class, was low (0.52) compared with that of previous studies (e.g., 0.751–1.000; Kimura et al., 2013a). These results suggest that new ramets were recruited by clonal reproduction rather than as seedlings. Hirayama and Sakimoto (2005) reported that more than 70 % of *C. japonica* seedlings died within 1 year. It is difficult for *C. japonica* seedlings to establish themselves in the understories of forests covered by *C. japonica* leaves. In addition, the number of intermediate-sized ramets (around 10 cm DBH) at the present study site was small (Figures 1 and 2), suggesting that most new ramets die while they are still small. Many understory *C. japonica* ramets die of pressure from heavy snow (Hirayama and Sakimoto, 2003).

Every genet was unique to a single sampling patch in the present study, and genets formed mutually exclusive patches (Figure 3), suggesting a phalanx strategy of clonal reproduction (Jonsson, 1995). We have identified similar genet structures in several other *Cryptomeria* populations (Taira et al., 1997; Moriguchi et al., 2001; Hirayama and Sakimoto, 2008; Kimura et al., 2013a; Doğan et al., 2017). Sprouting is an efficient means of regaining biomass and reducing genet mortality following a disturbance (Bond and Midgley, 2001). Our results suggest that clonal reproduction may be the most important strategy for establishing and maintaining a genet in areas where there is

frequent disturbance, such as in areas of heavy snowfall. Restriction of the establishment of seedlings of *C. japonica* to fallen logs and stumps was also reported in natural forests on Sado Island (Tomizawa and Maruyama, 1993) and in Akita Prefecture (Ota et al., 2002), both of which are snowy regions. Since these sites are not covered by understory vegetation, the light environment is relatively good. When covered with moss at these sites, seedlings do not dry out. At the present site, several seedlings (e.g., G16 and G10) had become established on the mossy butt swell of the canopy *C. japonica* trees. Simulation studies have revealed that a seedling recruitment rate of 3 % is sufficient to maintain genetic variation and clonal diversity (Watkinson and Powell, 1993). Sibling relationships were also attributed to seedling establishment. It is thought that despite the limited light and water, the mossy butt swell of a tree is more suitable for seedling establishment than the understory. Such mossy habitats are probably cryptic safe sites for *C. japonica* in snowy regions. Temporal and spatial differences in safe site among sampling patches probably influenced the number of ramets per genet in the present study. For example, parent–offspring and full-sib genets were all found in the same sampling patches (patch B and patch D, respectively; Figure 4). This suggests that the timing of the establishment of each genet differed among sampling patches.

In summary, the difficulty of seedling establishment and the importance of early clonal reproduction in *C. japonica* were demonstrated in the present study by analyzing small-sized ramets. *C. japonica* is genetically divided into two groups (Takahashi et al., 2008; Tsumura et al., 2012), and a higher frequency of clonal reproduction was reported in the Japan Sea side group than in the Pacific Ocean side group (Kimura et al., 2013a). This trend may be the result of adaptation to environmental factors such as disturbance caused by snow pressure. The importance of clonal reproduction is likely to vary from region to region, as this species has a wide distribution range and diverse habitat. In the future, more information will be needed to clarify the role of clonal reproduction in canopy tree species.

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