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RESEARCH ARTICLE

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The oldest known clones of *Salix herbacea* growing in the Northern Apennines, Italy are at least 2000 years old

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Abstract

Premise: Dominant in many ecosystems around the world, clonal plants can reach considerable ages and sizes. Due to their modular growth patterns, individual clonal plants (genets) can consist of many subunits (ramets). Since single ramets do not reflect the actual age of genets, the ratio between genet size (radius) and longitudinal annual growth rate (LAGR) of living ramets is often used to approximate the age of clonal plants. However, information on how the LAGR changes along ramets and how LAGR variability may affect age estimates of genets is still limited.

Methods: We assessed the variability of LAGR based on wood-section position along the ramets and on the duration of the growing season on three genetically distinct genets of *Salix herbacea* growing in the Northern Apennines (Italy). We compared genet ages estimated by dividing genet radius by the LAGRs of its ramets.

Results: LAGR increased significantly from the stem apex to the root collar; indicating that ramet growth rate decreased with time. Furthermore, a difference of ca. 2 weeks in the onset of the growing period did not impact LAGR. Considering the high LAGR variability, we estimated that the three genets started to grow between ~2100 and ~7000 years ago, which makes them the oldest known clones of *S. herbacea* even considering the most conservative age estimate.

Conclusions: Our findings indicate that analyzing ramets at the root collar provides an integrative measurement of their overall LAGR, which is crucial for estimating the age of genets.

KEYWORDS

bud scars, clonal growth, dwarf shrub, genet size, growth rings, population persistence, relict population, snowbed willow, stem longitudinal growth

In cold biomes, challenging environmental conditions can reduce the probability that plants will reproduce sexually. Clonality offers an alternative way to reproduce, ensuring the survival of the population (de Witte and Stöcklin, 2010) with the primary outcome an increase in the size of individual clonal plants or "genets" (Vallejo-Marín et al., 2010). Genets can have an advantage due to their physiological integration (i.e., the ability to share resources among subunits or ramets), particularly in habitats characterized by a heterogeneous availability of resources (e.g., Hutchings and Wijesinghe, 1997). Genets can reach remarkable sizes in their marginal populations due to the peculiar reproductive dynamics that may affect the edges of a species' distribution, such as the prevalence of clonal growth over sexual reproduction induced by geographic isolation and habitat fragmentation (e.g., Cristóbal et al., 2014; Macaya-Sanz et al., 2016). Southern European peninsulas host the southernmost outposts of many clonal plants and, among the most fragile, are the disjunct

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populations of arctic-alpine species (Abeli et al., 2018). Investigations aimed at estimating the age of clonal plants in such peculiar ecological conditions are scarce, but a recent study on the remnant populations of the dwarf shrub *Salix herbacea* in the Northern Apennines started to shed light on the size that genets can reach in extreme ecological and demographic conditions (Carbognani et al., 2019). Using molecular markers, the aforementioned study defined the clonal structure of these populations and found genets up to 60 m in diameter. Such genet size is one order of magnitude larger than the largest previously reported in core populations (Reisch et al., 2007; Stamati et al., 2007; de Witte and Stöcklin, 2012).

Genets are made of many subunits called ramets (Ally et al., 2008). The roots of the ramets spread into the soil, while the rhizomes (horizontal subterranean stems) grow close to the soil surface and produce aerial stems. While growing in length, the stems of the ramets often split due to trampling, decay, landslide, and rockfall. This process originates new independent ramets, which can spread and root (de Kroon and van Groenendael, 1997; Hutchings and Wijesinghe, 1997; Vallejo-Marín et al., 2010). Each ramet dies after a few years or decades, but the continuous process of ramet segmentation and regeneration allows the whole genet to spread wider and persist longer than single ramets (Harper, 1977; Clarke, 2012).

Estimating the age of clonal plants can be challenging due to their growth pattern, thus direct and indirect methods have been previously applied. The longevity of sufficiently young genets, where the oldest ramet is still alive, can be determined using direct methods, like counting the growth rings of the ramets and cross-dating them. However, in genets of hundreds of years, no single ramet reflects the actual age of the entire genet. Therefore, an indirect method has been used to estimate the age of these clonal plants (Suvanto and Latva-Karjanmaa, 2005; de Witte and Stöcklin, 2012), whereby the size of the genet (i.e., genet radius) is divided by an estimate of its annual rate of horizontal growth (i.e., annual growth of horizontal stems). This indirect method (from now on "linear method") has been previously applied to determine the age of clonal trees, shrubs, herbs, and grasses (de Witte and Stöcklin, 2010; Thomas, 2013) (Appendix S1). In the case of the linear method, the age estimate is a range between a minimum and a maximum age, with consideration of the variability of the measures based on living material (which can be assessed) and the uncertainties over the lifetime of very old clones (which can only be assumed).

Estimating the size of the genet and the longitudinal annual growth rate (LAGR) of the ramets is not straightforward due to several factors. The size of the genets might be miscalculated due to inappropriate field sampling strategies (Arnaud-Haond et al., 2007) or uncertainties in the inference from molecular tools (Arnaud-Haond et al., 2007; de Witte and Stöcklin, 2010). On the other hand, the LAGR of the ramets might be dependent on factors such as climate, time (particular year), age of the ramet and of the genet, disturbance, successional age of the vegetation (de Witte and Stöcklin, 2010). The frame is even more complex when the study target (clone) is extremely old, implying an extreme variability in the climatic conditions that the plant has experienced and survived. Nevertheless, de Witte and Stöcklin, (2012) found that the horizontal growth of four arctic-alpine dwarf shrubs was not affected by climatic variability among regions, suggesting that the LAGR of living ramets of a genet might not differ significantly from the LAGR of the ramets that were growing in the past (with different climatic conditions). Although the LAGR of ramets living in the past can only be inferred, the LAGR of living ramets can be precisely estimated. Previous studies have made such estimates by marking growing stems in the fields and measuring growth increment by returning to marked stems over time for several years (de Witte et al., 2012) or, in other cases, by measuring the distance between leaf scars on the apical part of the ramets (Callaghan et al., 1989; Johnstone and Henry, 1997; Rayback and Henry, 2006; Rayback et al., 2011, 2012; Buizer et al., 2012; Weijers et al., 2012, 2013). However, stem growth may vary greatly between life stages, and longitudinal growth may substantially decrease as ramet gets older (Wijk, 1986a; Myers-Smith et al., 2015). Thus, inferring the LAGR from the stem longitudinal increments of the last few years might underestimate the temporal variation of the LAGR.

In this study, we estimated the age of three genets of *Salix herbacea* L. (Salicaceae) of large dimension growing close to the southernmost limit of the species distribution, in the Northern Apennines (Italy). They were genetically characterized by Carbognani et al. (2019), which allowed us to define confidently their dimension. Furthermore, *Salix herbacea* is a prostrate dwarf shrub, presenting ramets that are growing mainly horizontally. Thus, the longitudinal growth (i.e., LAGR) of the ramets gives information on the horizontal growth of the shrub, which is a key variable to assess the age of the genets. For this reason, first, we assessed the variability of the LAGR of the living ramets, due to the position of the sampling along the ramets and the duration of the growing season. Second, we computed the age of the genets by dividing the radius of the genet by the LAGR of their ramets. The age thus obtained allows discussion of the current methodologies applied to estimate the age of clonal plants while providing new insights into the long lifespan that clonal plant species can reach.

MATERIALS AND METHODS

Study species

Salix herbacea is a dioecious dwarf shrub with an extensive ramifying system of rhizomes (stems growing horizontally beneath the soil surface), that grow and often split due to trampling, decay, or landslide and so originating new independent ramets, which spread and root. A ramet can be divided into roots and stems. The stems can differentiate into rhizome(s) and aerial stems, the latter extending only a

few centimeters above ground (Wijk, 1986b). The aerial stems and horizontal stems (i.e., rhizomes) lack anatomical differences, so we include both types when referring to "stem". The bud scales at the apex of stems leave scars on the stem (Wijk, 1986b).

Study system

In the Northern Apennines (Italy), *Salix herbacea* L. (Salicaceae) occurs only on the north face of Mt. Prado (2054 m a.s.l.) (Figure 1A, B) and Mt. Cimone (2165 m a.s.l.), where local climatic and geomorphologic conditions favor extended snow cover (Ferrarini, 1969, 1974; Petraglia and Tomaselli, 2007). The Mt. Prado population extends for 1.5 ha (Figure 1C), whereas the Mt. Cimone population covers only 0.16 ha. The spatial genetic structure of the Mt. Prado population was determined using a 5 × 5 m grid sampling scheme and genotyping 347 randomly selected ramets from the samples collected according to the sampling strategy described in detail by Carbognani et al. (2019), genotyped through a carefully selected set of 11 nuclear microsatellite markers (nSSRs). The Monte Carlo procedure used to evaluate the power of the marker set showed that the genotypic resolution reached a plateau at 11 nSSRs (with 98, 97, and 94% of the total genets detected, on average, by using 10, 9, and 8 nSSRs, respectively). The probability of a second encounter with an identical genet via sexual reproduction was always very low ($P_{SEX} < 0.001$) for the Prado population (Carbognani et al., 2019). In the Prado population, although 145 genets were detected, and one third of the population was characterized by a majority of unique genets (i.e., genotypes detected in only one of the sampled ramets), three extremely large genets, one male (genet 44) and two females (genet 147 and genet 015), were identified (Figure 1C). The three individuals occupy an area of ~1 ha on the northern slope of Mt. Prado, immediately below the mountain summit

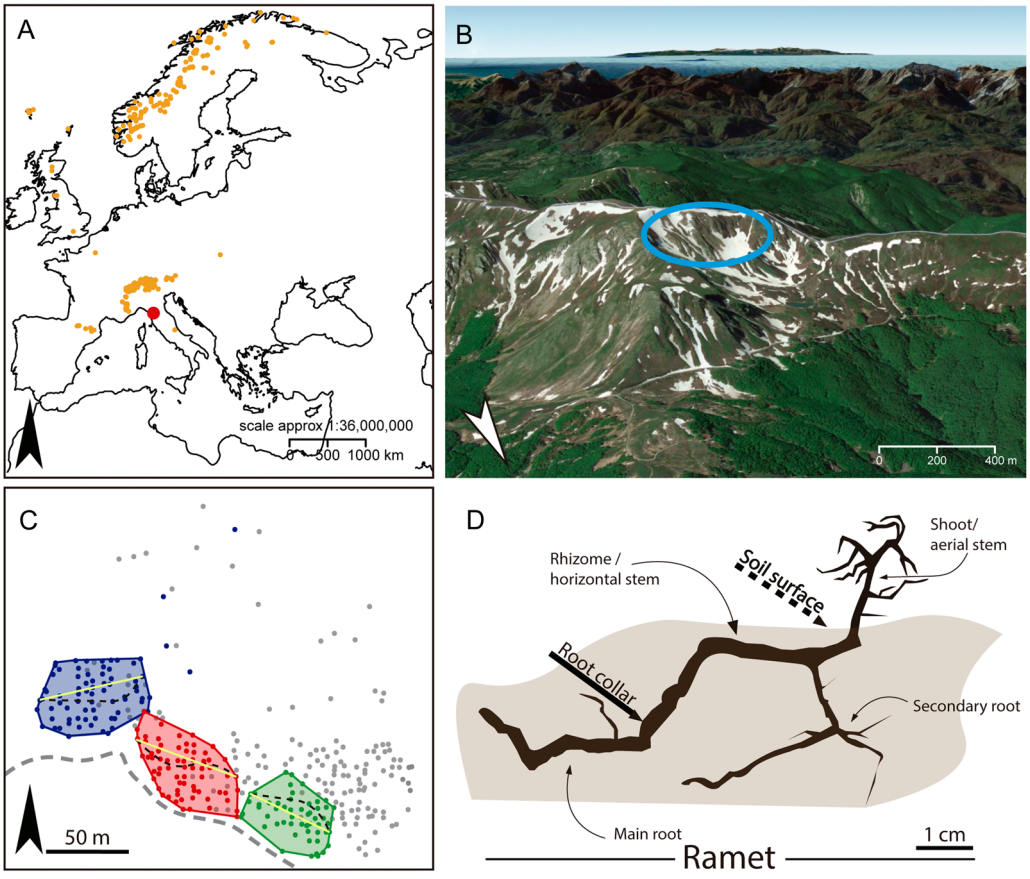


FIGURE 1 Distribution and morphology of *Salix herbacea*. (A) Distribution in Europe, with arctic and alpine distribution range in northern Europe and mountainous regions of central and southern Europe, where it is confined to the mountain tops to avoid the high temperatures of these latitudes. Indeed, the relict populations in the Pyrenees (Spain) and Apennines (Italy) (red dot marks Mt. Prado [44°14′ N,10°24′ E]) are located at the southern limit of the distribution of the species. Orange dots mark occurrence sites obtained from the open-access GBIF data set (https://www.gbif.org/species/5373083). (B) Location of the *Salix herbacea* population on Mt. Prado (2054 m a.s.l.). Image from Google Earth. (C) Distribution of the three main genets. Blue: genet 44 (diameter of 63.26 m); red: genet 15 (diameter of 63.83 m); green: genet 147 (diameter of 53.31 m). The grey dots mark the distribution of the rest of the population of *Salix herbacea* in Mount Prado as described by Carbognani et al. (2019). The grey dashed line represents the crest of Mount Prado; the black dashed lines within the three genets represent the contour line passing through the middle of each genet. The yellow lines within the three genets represent the diameter used to estimate the radius of each genet. (D) Morphology of a ramet of *Salix herbacea* showing aboveground (i.e., shoots) and belowground (i.e., rhizomes and roots) parts of the ramet.

(Figure 1B, C), where glacial cirques and deposits originated during the Würmian period (Losacco, 1982; Carton and Panizza, 1988). A temporal gradient of snowmelt due to differences in winter snow accumulation and wind action is visible within about 25 m downslope from the mountain ridge. The spatial extent of the three large clones covers this gradient, allowing us to sample ramets, within each clone, throughout a phenological gradient spanning about 2 weeks during bud burst of *S. herbacea*.

Sampling

In September 2016, six ramets for each of the three target genets were sampled. We sampled the full stem of the ramets (aboveground and belowground parts) until reaching the main root (Figure 1D). To do so, we scratched the soil surface following the stem growing horizontally (belowground) until an impediment (a rock) would not allow us to follow. With this technique, we were able to reach the root collar (that represents the base of the stem of the ramets) and the main root of all ramets. We decided to sample an additional ramet for each genet, which was chosen among the ramets with larger stem diameter that we could spot in the area. These three extra ramets have been used for maximum ramet age estimation.

Estimating genet size and its radius

The genet radius is frequently estimated as half of the genet diameter due to the impossibility of directly identifying the original growth point of the genet (Steinger et al., 1996; Reusch et al., 1999; Jónsdóttir et al., 2000; Suvanto and Latva-Karjanmaa, 2005). In our study area, we first defined the convex hull (i.e., the smallest polygon containing all the genetically identified points belonging to a specific genet) for each genet (Figure 1C). Secondly, we drew the contour lines in the map and chose the one that halved the convex hull of each genet. We considered the direction of the contour lines to avoid biases due to environmental constraints (south exposure, slope) that might have affected the natural growth and shape of the genets. Then, for each genet, we selected the two points where the contour line crossed the convex hull and computed the linear distance between the two points. We considered this distance as the most conservative estimation of the diameter of each genet and estimated the radius as half the diameter estimate. We are aware that the genets could have started to grow from a different starting point (not corresponding to the center of the convex hull) and not grown linearly; however, due to the dimension of the genets and the lack of any residual material that could be used as a reference, we opted for this conservative measure, not considering the diameter perpendicular to the contour lines (i.e., along the slope of the mountain), because it could have been biased for two reasons. First, on the top of the mountain, environmental constraints do not allow the clones to grow beyond the ridge, on a south exposure where the temperatures are too high and there is no snow cover. Second, on the other side (toward the valley), the slope of the mountain might have facilitated some ramets to detach and go downhill. For these reasons, we considered that plant growth along that diameter was not age dependent; instead, for age computation, we relied on the radius aligned to the contour lines, which should not have been biased by environmental constraints or slope. Genet 15 presented a diameter of 63.83 m for a radius of 31.92 m. Genet 44 presented a diameter of 63.26 m, and a radius of 31.63 m. Finally, genet 147 presented a diameter of 53.31 m and a radius of 26.66 m.

Bud scar and ring correspondence

To account for the presence of missing rings in cross sections along the ramets, we first defined (for each ramet) the points corresponding to each anatomical cross section (Figure 2A), and before cutting the ramets, we counted the number of bud scars present in the aboveground part of the stem between the apex and each determined point (Figure 2B). This technique was previously used to determine the age of *Salix* stems (Palmer and Miller, 1961) and to estimate the LAGR of ramets of other shrubs (e.g., Rayback et al., 2011, 2012; Weijers et al., 2012, 2013). Secondly, we cut the cross sections (as detailed in the next section), counted the number of growth rings, and compared the number of bud scars with the number of growing rings.

LAGR variability along the stem

To consider the LAGR variation along the stems, we applied a serial sectioning technique (Kolishchuk, 1990) to samples from six locations along stems until reaching the root collar (Schweingruber and Poschlod, 2005; Myers-Smith et al., 2015). The root collar of the ramet was identified by looking for pith in the stem and its absence in roots (Eamesand& MacDaniels, 1947; Evert, 2006) (Figure 2A, C, D). In the case of multiple stems or roots, we chose the main stem and root based on thickness and length and removed all the others. We then cut seven cross sections per ramet using a sliding microtome (Reichert, Vienna, Austria); we collected five stem cross sections at a fixed distance from the stem apex (A = 3 mm (from the apex), B = 7 mm, C = 15 mm, D = 30 mm, E = 70 mm), one stem section next to the root collar (F = variable distance), and one root cross section. The six stem cross sections were then used for further analysis to compute the LAGR, while the root cross section was just used to verify that we reached the root collar. Furthermore, we counted the number of rings on the root section (as close as possible to the root collar) and compared it with the number of rings counted in the stem section closer to the root collar. We found good correspondence in all samples indicating that we had chosen the "main" stem and root. The growth rings were identified after staining with a 1:1 aqueous solution of

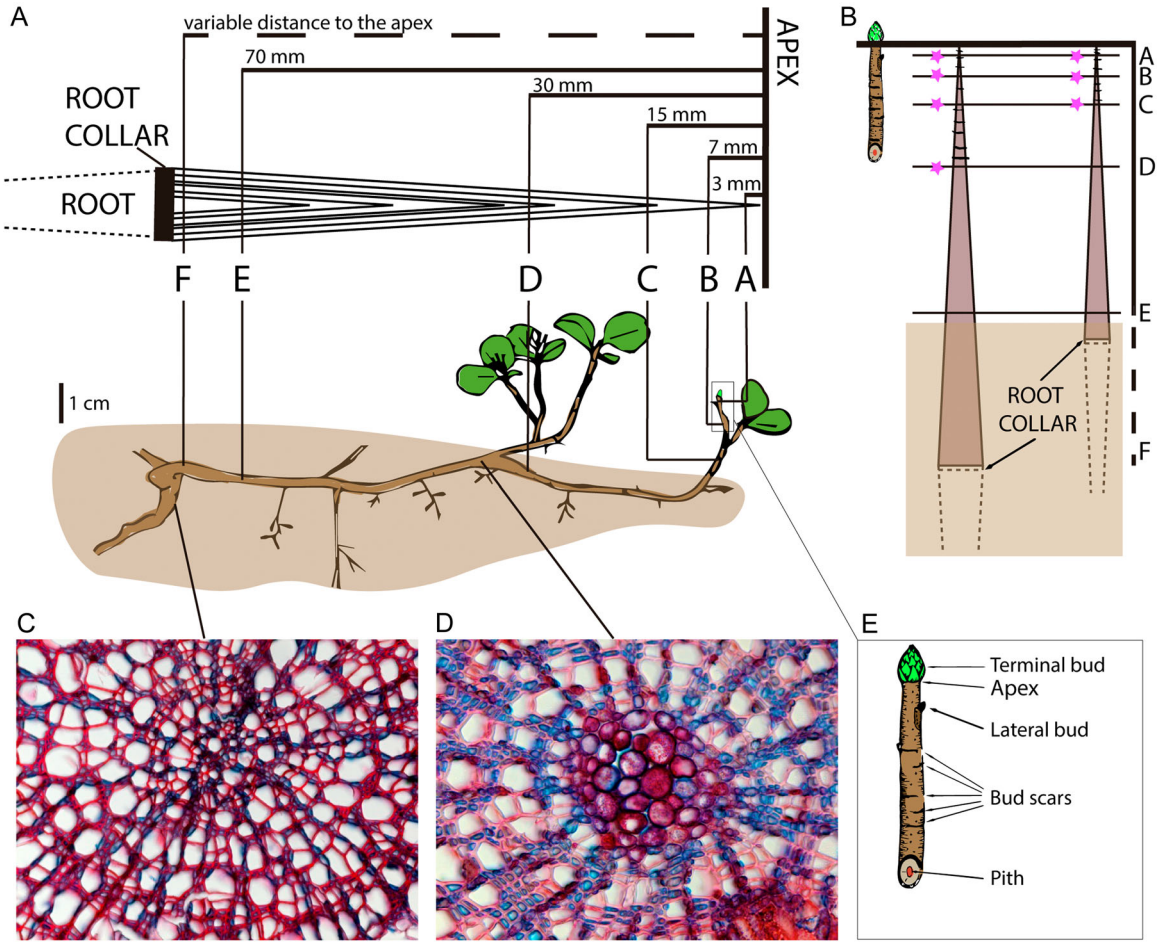


FIGURE 2 Serial cross section and bud-scar count methods used in the present study on ramets of *Salix herbacea*. (A) Serial sectioning technique (on top) and morphology of a ramet (on the bottom), with belowground roots and rhizomes and aboveground shoots. We sampled at six locations along the main stem (main rhizome + main shoot) until the root collar of the ramet. Distance from the apex: section A = 3 mm, B = 7 mm, C = 15 mm, D = 30 mm, E = 70 mm, F = variable. (B) For each of the six ramets of each genet (in Figure 2B only two ramets are shown), we counted the number of bud scars (short horizontal lines on the stems) between the apex and the point corresponding to the cross section (long horizontal lines across the figure). When we could count the bud scars and the rings, we had an observation (pink stars in the figure) that could be used in the analysis. The length of the ramets and the soil level are shown as an illustration only and do not correspond to real data. Root (C) and stem (D) anatomical cross sections of a ramet. The pith (central part, with a different cell distribution compared to the surrounding cells) is present only in the stem. (E) The apex corresponds to the base of the terminal bud; however, each shoot might have several lateral buds. The terminal bud leaves bud scars. Thus, the stem becomes distinctly segmented where each segment corresponds to the annual increment in length (Wijk, 1986b).

safranin (1%) to astra blue (0.5%). After dehydration with ethanol, sections were permanently mounted on microscope slides using Eukitt (BiOptica, Milan, Italy).

We calculated the LAGR (in mm yr^{-1}) for each section point along the stem (n) by dividing the distance of the cross section from the stem apex (y, in mm) and the number of growth rings (x) in the cross section (1):

$$\text{LAGR} = \frac{y_n}{x_n}. \quad (1)$$

LAGR variability and snowmelt timing

To consider LAGR variability due to the differences in the duration of the growing season, we sampled three of the six ramets for each genet close to the ridge in areas with an earlier snowmelt and the other three in adjacent areas at lower elevation with a later snowmelt.

Statistical analyses

Statistical analyses were implemented in the R software environment (version 4.2.3, R Core Team, 2023). The function lme in the package nlme (Pinheiro et al., 2023) was used to run linear mixed-effect models (LMMs), and the function lsmeans in the package emmeans (Lenth, 2023) to obtain estimated marginal means for the LLMs. The function cld in the package multcomp (Hothorn et al., 2008) was used for post hoc multiple comparisons, and the function cor.test in the R

package stats (R Core Team, 2023) was used to obtain correlation coefficients.

Bud scar–ring relationship

To check the relationship between the number of scars and the number of rings we used a LMM for each genet, considering the number of rings as dependent variable and the number of bud scars as explanatory variable. The ramet identity was included as random factor because for each ramet we performed different sections where the buds and the rings were counted. We also computed the correlation between bud scars and rings using the Pearson correlation method.

We had six cross sections (where we counted the number of rings) for each ramet of each genet. However, we did not always have the corresponding information on the number of bud scars for each of the six sections of each ramet because bud scars are visible only on the aboveground part of the stem. Thus, the final number of observations for each ramet (i.e., points along the stem giving information on both the number of buds and rings) depended on the morphology of each ramet (i.e., when the majority of the stem was buried, we could count the bud scars only for section A, B, and C, but when the stem was mostly aboveground, we could count bud scars until section D or E). Furthermore, for some ramets, we could count the bud scars only on the apical part of the aboveground part of the ramets because in the rest of the stem we could not clearly distinguish them (Figure 2B, E). For genet 15, we had six ramets with a total of 20 observations, for genet 44, six ramets with 16 observations, and for genet 147, six ramets with 13 observations.

LAGR variability along the stem

To analyze the effects of the position of the sections along the ramets on LAGR, we used a LMM considering the three genets together, with LAGR as the response variable. The distance of the section from the apex (six-level predictor, from A to F) was considered as a categorical explanatory variable, while the genet and ramet identity were included as random factors to take into account that for each genet were considered multiple ramets and that we had multiple sections for each ramet. Since the variances for the sections were heterogeneous, in the model we included a variance structure (i.e., varIdent), allowing for a different variation of LAGR across the different sections.

To identify which sections (A, B, C, D, E, F), differed significantly within each genet, we applied the LMM described above for each genet (excluding the random factor "genet"), and then a post hoc Sidak test.

LAGR variability and snowmelt timing

To analyze the effects of snowmelt timing on the LAGR of the ramets of the three genets (nine ramets in early-snowmelt patches and nine in the late-snowmelt), we used a LMM with the LAGR of section F as the response variable, snowmelt location (two-level factor: early and late) as a fixed effect, and genet as a random factor.

Age estimation of ramets and genets

We estimated the age of the ramets by counting the number of growth rings visible at the stem base of each ramet. We estimated the age of the genets by dividing the radius of the genet by the mean LAGR and the 95% confidence interval (CI) of section F of each genet. The CI is the mean LAGR ± the margin of error, with alpha = 0.05. We also report the age estimation obtained using section C for comparison purposes only.

RESULTS

The LAGR increased significantly from the stem apex, section A (mean: 1.94 mm yr^{-1}) to the root collar, section F (mean: 7.88 mm yr^{-1}) throughout the 18 sampled ramets belonging to the three genets (Figure 3; Appendix S2). The 2-week difference in the onset of the growing period did not impact the LAGR (Appendix S3). The LAGR of section F, considered for final clonal age estimation, was estimated counting the rings found in section F and considering the total length of the ramet. Although the variability of the LAGR corresponding to section F was higher compared to the other sections (because section F did not have a fixed distance from the apex), we chose it for final clonal age estimation because it can be considered as a more integrative measurement of the ramet overall LAGR. Nevertheless, due to the high LAGR variability, we did not provide a precise age of the genets; conversely, we estimated a range between a minimum of ~2100 and a maximum age of ~7000 years.

Bud scar and ring correspondence

The comparison of the number of bud scars and of rings in the apical part of the ramet revealed an almost perfect correspondence in the three genets both when considering the result of the ANOVA of the LLMs (genet 15: $F_{1,13} = 448$, $P < 0.001$, genet 44: $F_{1,11} = 118.8$, $P < 0.001$, genet 147: $F_{1,6} = 60.2$, $P < 0.001$) and when using Pearson's correlation analysis (genet 15: $P < 0.001$, $r = 0.98$; genet 44: $P < 0.001$, $r = 0.94$, genet 147: $P < 0.001$, $r = 0.92$) (Appendix S4).

LAGR

LAGR variability along the stem

The longitudinal annual growth rate (LAGR) of the ramets of the three genets was significantly affected by the position of the

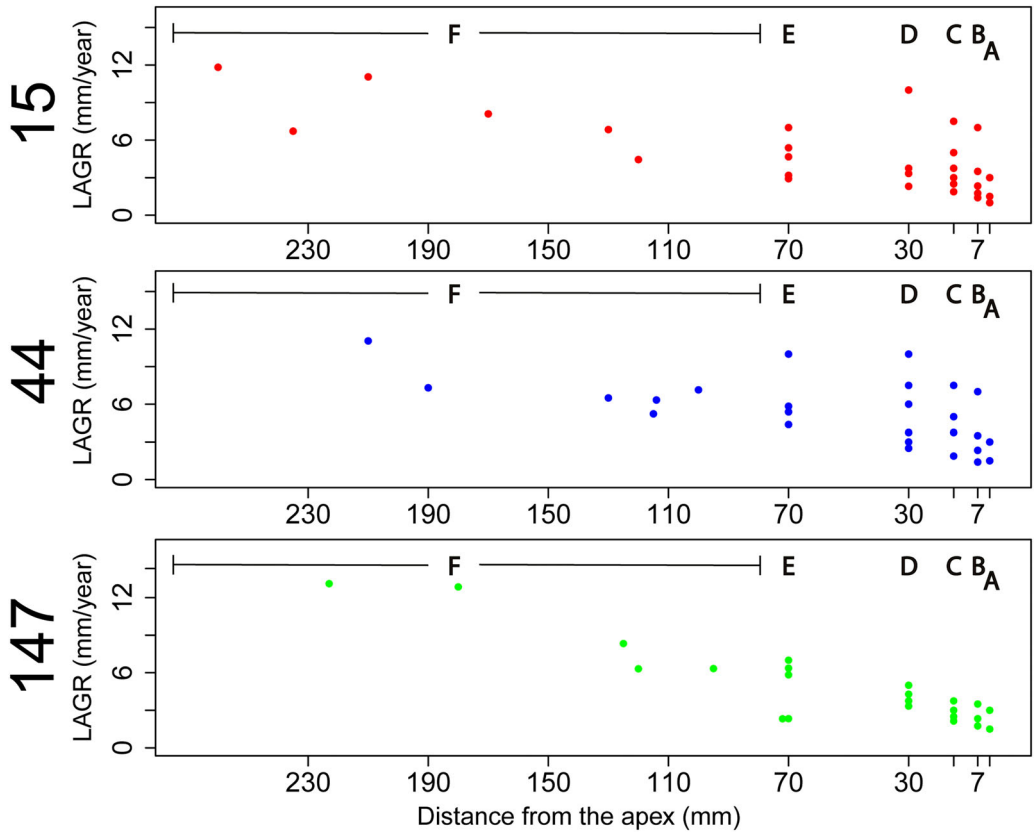


FIGURE 3 Longitudinal annual growth rate (LAGR, mm yr^{-1}) from the root collar (F, on the left) to the stem apex (A, on the right) of the three genets. Each graph includes data for six ramets per genet. Distance from apex: section A = 3 mm, B = 7 mm, C = 15 mm, D = 30 mm, E = 70 mm. The distance for F is variable because it represents the closest cross section to the root collar of each ramet and depends on the maximum length of the ramet.

section along the stem ($F_{1,5} = 30.177$, $P < 0.001$, see Figure 3 and Appendix S2). The mean LAGR of the different cross sections within each ramet and the results of the post hoc test are reported in Table 1. The LAGR decreased from the stem base to the apex in all the ramets of the three genets. In particular, for genet 15, the mean LAGR of the sections close to the stem base (root collar, corresponding to section F) ranged between 4.4 and 11.8 mm yr^{-1}, decreasing to between 2.9 and 7 mm yr^{-1} at 70 mm from the apex (section E) and dropping to between 3 and 1 mm yr^{-1} at 3 mm from the apex (section A). For genet 44, the LAGR values of the sections close to the stem base (section F) ranged from 5.2 to 11 mm yr^{-1}, decreasing to between 4.3 and 10 mm yr^{-1} at 70 mm from the apex (section E) and dropping to less than 3 mm yr^{-1} at 3 mm from the apex (section A). For genet 147, the LAGR values of the sections close to the stem base (section F) ranged between 3 and 13.1 mm yr^{-1}, decreasing to between 2.3 and 7 mm yr^{-1} at 70 mm from the apex (section E) and dropping to between 3 and 1.5 mm yr^{-1} at 3 mm from the apex (section A).

LAGR variability and snowmelt timing

The LAGR of ramets growing in early- and late-snowmelt patches did not differ significantly (Appendix S3). Consequently, the LAGRs of ramets belonging to the same genets have been averaged (Table 1).

Age estimation

The age of the 18 ramets of the three genets ranged between 14 and 35 years, while the extra ramet in genet 15 was 70 years old, 40 years old in genet 44, and 38 years old in genet 147. The age of genet 15, considering the LAGR corresponding to section F, ranged between 2874 and 6119 years, between 3380 and 6126 years for genet 44, and between 2115 and 6971 years for genet 147, while the age of genet 15 considering section C, ranged between 5240 and 17,890 years, genet 44 between 4922 and 16,584 years and genet 147 between 7286 and 11,992 years (Table 2). Our level of certainty is 95% for all the estimated age ranges, which represent the variation in the estimated mean age for each genet.

DISCUSSION

Our results show that LAGR varied significantly throughout the ramet lifespan. When we considered the overall LAGR from the stem apex to the root collar level (represented by

TABLE 1 Mean longitudinal annual growth rate (LAGR) of the sections within the ramets of each genet (3 genets × 6 ramets × 6 sections = 108 sections) followed by the result of the post hoc test (comparing the mean among the different sections of each genet). Each ramet was sectioned 6 times with a distance from the stem apex of 3 mm (section A), 7 mm (section B), 15 mm (section C), 30 mm (section D), 70 mm (section E), >70 mm (section F). Section F has not a fixed position, depending on the stem length.

	Genet 15		Genet 44		Genet 147	
Section ID	LAGR ± SD (mm yr^{-1})	Post hoc	LAGR ± SD (mm yr^{-1})	Post hoc	LAGR ± SD (mm yr^{-1})	Post hoc
A	1.83 ± 0.93	a	2.25 ± 0.82	a	1.75 ± 0.61	a
B	3.05 ± 2.06	a	3.34 ± 1.96	ab	2.33 ± 0.64	b
C	3.93 ± 2.05	b	4.16 ± 2.15	abc	2.94 ± 0.68	c
D	4.48 ± 2.76	bc	5.46 ± 2.92	cd	4.18 ± 0.69	d
E	4.63 ± 1.49	abc	5.96 ± 2.08	bcd	5.81 ± 1.76	cd
F	8.16 ± 2.80	c	7.26 ± 1.99	d	8.21 ± 4.18	bcd

TABLE 2 Genet age estimation obtained dividing the radius of each genet by the mean longitudinal annual growth rate (LAGR) of section C and section F.

				AGE (yr)		
GENET	RADIUS (m)	LAGR mean ± SD (mm yr^{-1})		Lower bound (95% CI)	Mean	Upper bound (95% CI)
15	31.917	C	3.938 ± 2.05	5239.956	8105.798	17890.48
	31.917	**F**	**8.161 ± 2.80**	2873.676	**3910.796**	6119.256
44	31.630	C	4.167 ± 2.15	4922.150	7591.259	16584.39
	31.630	**F**	**7.261 ± 1.99**	3380.080	**4356.404**	6125.830
147	26.656	C	2.940 ± 0.68	7286.827	9065.328	11992.30
	26.656	**F**	**8.213 ± 4.18**	2115.150	**3245.513**	6970.792

section F), the values were two times larger than those obtained using the LAGR of the last few years (represented by section C in the analysis), thus highlighting the crucial role of the position of the section chosen to determine the LAGR (and consequently to estimate genet age). Interestingly, when de Witte and Stöcklin (2012) similarly estimated the LAGR for early pioneer populations of *S. herbacea*, the LAGR was between 7 and 10 mm, similar to what we report here for section F (see Table 1). In addition to LAGR variation along the stem of the ramet, another issue may bias LAGR estimates: missing growth rings. We considered this issue by counting the bud scars at different points along the apical part of the stem and comparing it with the number of growth rings counted on the corresponding points. The almost perfect correlation gave us proof that the ramets presented an annual growth. In any case, some rings could have been miscounted, particularly in sections D, E, and F (the closest to the root collar). Thus, the growth over 2 or more years could have been counted as the growth for 1 year, resulting in an overestimate of the growth rate (and underestimate of the genet age). Indeed, Appendix S4 shows an indication of missing rings (e.g., genet 147) because some counts of bud scars indicate fewer rings. In other cases, we counted more rings than scars (e.g., genet 44), meaning that either we counted one growth ring as two rings, or we missed a bud scar. Or both a ring and a bud scar could be missing, meaning that the ramet did not grow at all for 1 year. In this case, we would not be able to detect the bias. Nevertheless, the anatomical approach allowed us to count at the stem base of a ramet (belonging to genet 15) the unprecedented number of 70 growth rings, which almost doubled the age of 43 years recorded in the oldest known ramet of *S. herbacea* (Schweingruber and Poschlod, 2005b).

The impressive size of the three genets of Mt. Prado amplifies the uncertainty correlated to sizing the genet and, consequently, estimating its age. In fact, these genets have experienced changes in climatic conditions, snow cover, and disturbance intensity which affected their growth dynamics. Massively extended clones can also differ in the LAGR within the same ramet and between ramets of the same clone, for instance, due to a difference in the growing season or heterogeneous topography (Little et al., 2016; Wheeler et al., 2016). In the present study, we considered the possible effect of the length of the snow-free period on ramet growth, and within each genet, we analyzed the LAGR of ramets in an early- or late-snowmelt patch. We expected that ramets growing in early-snowmelt patches, having a 2-week shorter snow cover, would grow longitudinally more than ramets growing in the late-snowmelt patches (Ferrari and

Rossi, 1995). However, we found that LAGR in early and late-snowmelt patches did not differ significantly. This finding must be interpreted with caution due to the small sample size considered. However, our result suggests an interaction between morphology (slope and topography) and atmospheric factors (precipitation and wind) that might affect the LAGR of the ramets. Indeed, on Mount Prado, ramets growing in early-snowmelt patches are located closer to the ridge, where they are exposed to the wind and the soil is shallower and rockier. Conversely, the ramets growing in late-snowmelt patches are in a more sheltered position, where the soil is more developed. Thus, the ramets growing in the late-snowmelt patches might compensate for the shorter vegetative season with a more protected location and better soil conditions. Similarly, de Witte and Stöcklin (2012) found that horizontal growth in four arctic-alpine clonal plants was not affected by climatic variability across geographical regions, despite a difference of as much as 50 days in growing-season length.

We estimated the age of three genets that are seven times larger than the largest genet of *S. herbacea* that has been used for age estimation so far (Stamati et al., 2007), which makes us think that the sampling methods previously used might have biased the real dimension of the genets. A potential limiting factor might have been the choice of the distance between samples and the total sampled area. Indeed, choosing a centimeter (Reisch et al., 2007; Stamati et al., 2007) or meter scale (Carbognani et al., 2019) in grid/transect design will highly affect the resulting minimum and maximum genet dimension obtained. In particular, the sampling approach of Carbognani et al. (2019) is suboptimal to depict fine-scale clone perimeters but is ideal to define the size of large clones. Nevertheless, previous studies on clonal plants were, in most cases, not aimed at inspecting entire populations and, therefore, identifying large clones, nor at examining populations at the limit of their ecological niche.

Our age estimations (considering section F) suggest that the three genets started to grow between ~2000 and ~7000 years ago. These populations persist so far south because they avoid the higher temperatures of these latitudes by growing at rather high altitudes on the northern slope (Figure 1B). According to Baroni et al. (2018), during the Local Last Glacial Maximum (LLGM) more than 100 mountains in the Northern Apennines, including Mt. Prado, were covered by glaciers, reaching the most advanced position between 18,000 and 21,000 yr BP. These dates show that the top of Mt. Prado could not have been colonized by *S. herbacea* before 14,000–16,000 yr ago. Interestingly, the maximum age estimation we obtained, using section F, refers to a period when the glacier on the top of Mt. Prado had disappeared, making the colonization of that area by *S. herbacea* possible.

Methods to estimate the longevity of clonal plants are continuously improving and combining molecular and dendrochronological tools is a promising approach to refine our knowledge about the age of large clones in clonal plant populations. Nonetheless, methods to measure clonal plant age have inherent uncertainties (Arnaud-Haond et al., 2007; de Witte and Stöcklin, 2012), that should be carefully taken into account during the process. Possible caveats related to the age estimation effort presented here are linked to the nature and analysis of genetic data presented by Carbognani et al. (2019) and to the linear method used to simplify the complex clonal growth dynamic of the genets of *Salix herbacea*.

The sizing of genets might be imprecise due to the low genetic accuracy of the marker set used; more details are presented in the aforementioned study (Carbognani et al., 2019), but in summary, several indicators show that the genet detection was highly accurate and that the genet-size estimate was conservative. As anticipated, the genotypic resolution was high, reaching a plateau close to the number of genetic markers used, and the probability of a second encounter with an identical genet via sexual reproduction was always <0.001. The sizing of genets was based on the identification of multilocus genotypes (MLGs) instead of multilocus lineages (MLLs), which are composed of multiple MLGs differentiated by a few somatic mutations. All the ramets of the study population were sexed, and the three large clones studied here were coherently all constituted by ramets of the same sex (genet 15: all female ramets; genet 147: all female ramets) with the only exception of genet 44, a male individual, for which three of the 64 sexed ramets had female flowers. Although this determination of male and female ramets in the same genet could be potentially related to an error in the assignment, the size of this genet did not change after removing the three female ramets. Finally, in the range-core alpine population analyzed by Carbognani et al. (2019) in the Gavia Pass (Rhaetian Alps, 46°21′ N, 10°29′ E), every sampled ramet was a different MLG, which is also generally the case in the eastern part of the Prado population, thus further indicating the high genetic accuracy of the marker set used. Moreover, we are aware that the linear method used to estimate the age of the three genets (dividing the genet radius by the LAGR of the sampled ramets) is an extreme simplification of the complex clonal growth dynamic. The method excludes any physiologic characteristic (branching of the daughter ramets, ramet death rate) and any biotic (human and animal trampling on the site, presence of diseases) or abiotic (landslides, rock fall, temperature) factors that may influence the expansion of the ramets. For instance, horizontal elongation of the shoots is unlikely to have remained constant (i.e., in the same direction) throughout their life with no natural impediments or obstacles altering their morphology and growth path (as we can see in Figure 1D). Thus, the genets might have taken longer to reach their actual dimension than what we estimated and are probably older than what we assessed. These uncertainties could have been solved with radiocarbon dating, which is a good method for dating clonal shrubs (e.g., Vasek, 1980). Unfortunately, in our study, we could not use this technique because we did not have any remaining parts (roots, root collar or stems) of the first ramet (for each genet) from which the genets growing on Mt Prado originated (the ramets last on average less than 50 years before deteriorating).

For more precise age estimates, we think that future studies on clonal growth dynamics should include in the

sampling design information about rhizome growth direction, ramets density per square meter, ramet LAGR (considering the variability along the ramet), generation time (time necessary for stem renewal), ramet branching angle, death rate of new ramets, presence of possible natural (landslide, snowpack, rocks) or anthropic (trampling on the site) disturbances that can influence the death rate and position of the genet centroid and dimension of the genet (avoiding linear sampling). A prerequisite for the inclusion of such factors is, of course, a working understanding of how they influence the pattern of growth of the plant.

CONCLUSIONS

Here we estimated the ages of the oldest known clones of *S. herbacea* and highlighted the importance of analyzing ramets at the root collar to obtain a more integrative estimation of their LAGR throughout their lifespan. The extreme longevity of the three genets of *S. herbacea* is an indicator of the long population persistence in the Northern Apennines (Italy). Nevertheless, determining the age of extremely old and widespread alpine clonal plant species is still challenging because the morphology and growth dynamics of such species are difficult to investigate and understand.

AUTHOR CONTRIBUTIONS

A.C., G.C., M.C., A.Pe., and A.Pi. conceived the ideas, designed the methodology, and collected the data; G.C. and M.C. analyzed the data; G.C. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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DATA AVAILABILITY STATEMENT

The data set generated during and/or analyzed during the current study is available in CORA (https://doi.org/10.34810/data784).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Indirect age estimation of clonal plants (genet size/longitudinal annual growth rate).

Appendix S2. Variation of the longitudinal annual growth rate (LAGR) from the apex of the ramets (on the x axis, corresponding to the letter "A") to the root collar (corresponding to the letter "F") of the three genets.

Appendix S3. Results of the LLM considering the LAGR (section F) of the 18 ramets of the 3 genets, according to the snowmelt time.

Appendix S4. Relationship between the number of bud scars (on the *x*-axes) and the number of growth rings (on the *y*-axes) counted along the ramets.

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