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Extensive clonal spread and extreme longevity in saw palmetto, a foundation clonal plant

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Abstract

The lack of effective tools has hampered out ability to assess the size, growth and ages of clonal plants. With *Serenoa repens* (saw palmetto) as a model, we introduce a novel analytical framework that integrates DNA fingerprinting and mathematical modelling to simulate growth and estimate ages of clonal plants. We also demonstrate the application of such life-history information of clonal plants to provide insight into management plans. *Serenoa* is an ecologically important foundation species in many Southeastern United States ecosystems; yet, many land managers consider *Serenoa* a troublesome invasive plant. Accordingly, management plans have been developed to reduce or eliminate *Serenoa* with little understanding of its life history. Using Amplified Fragment Length Polymorphisms, we genotyped 263 *Serenoa* and 134 *Sabal etonia* (a sympatric non-clonal palmetto) samples collected from a 20×20 m study plot in Florida scrub. *Sabal* samples were used to assign small field-unidentifiable palmettos to *Serenoa* or *Sabal* and also as a negative control for clone detection. We then mathematically modelled clonal networks to estimate genet ages. Our results suggest that *Serenoa* predominantly propagate via vegetative sprouts and 10 000-year-old genets may be common, while showing no evidence of clone formation by *Sabal*. The results of this and our previous studies suggest that: (i) *Serenoa* has been part of scrub associations for thousands of years, (ii) *Serenoa* invasion are unlikely and (ii) once *Serenoa* is eliminated from local communities, its restoration will be difficult. Reevaluation of the current management tools and plans is an urgent task.

Keywords: amplified fragment length polymorphisms, conservation genetics, longevity, management plan, minimum branching tree, *Serenoa repens*

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Introduction

Conservation efforts prioritize rare species given their high risk of extinction; yet, there is an increasing realization of the importance of conserving more common foundation species (Ellison *et al.* 2005; Gaston & Fuller 2008). Locally dominant plants are typically foundation species because their physical structure and their ecological and physiological characteristics define the local assemblage, habitat and ecosystem by modulating fundamental ecosystem processes and providing commu-

nity stability for other species (Ellison *et al.* 2005). Yet, because of their abundance, many foundation species do not receive appropriate conservation attention (Gaston & Fuller 2008). As a result, the lack of understanding of life-history traits and ecological importance of foundation species may result in the development of improper conservation management plans. In such cases, there is an urgent need for research that will provide important life-history information before inappropriate management plans become implemented and adversely affect local ecosystems.

Extended longevity and persistence of foundation plant populations are important to long-term community stability and ecosystem resilience against

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environmental and demographic stochasticity as well as global climate change (Steinger *et al.* 1996; Weiher *et al.* 1999; Eriksson 2000; Garcia *et al.* 2008; Morris *et al.* 2008; de Witte & Stöcklin 2010). Because clonal plants are common across most habitats and are in general long-lived (i.e. to thousands of years), age estimation of clonal plants has received considerable interests to understand the life history and population dynamics of clonal plants as well as their role in the community and ecosystem stability (see review in de Witte & Stöcklin 2010). However, because clonal networks typically grow underground and are not permanent, they leave no physical evidence of previous connections. This and the lack of effective study tools have hampered our ability to assess the size, growth and age of clonal plants (Arnaud-Haond *et al.* 2007; de Witte & Stöcklin 2010). As a result, such important life-history information for clonal plants has remained relatively poorly understood (de Witte & Stöcklin 2010). The advent of DNA fingerprinting techniques has facilitated several studies that estimated clone ages by first identifying the extent of genets [i.e. clones: groups of individuals (ramets) with the same genotypes, Harper 1977] and then dividing genet patch size (i.e. diameter or radius) by genet expansion rates (Escaravage *et al.* 1998; Reusch *et al.* 1999; Jónsdóttir *et al.* 2000; Wesche *et al.* 2005). This method is appropriate for clonal plants whose ramets (i.e. genetically identical individuals belonging to the same genets) expand radially or directionally. Yet, many clonal plants expand via more complicated ramet growth patterns. We use *Serenoa repens* (Arecaceae, saw palmetto, hereafter *Serenoa*) as a model species to accomplish two goals: (i) to introduce a novel analytical framework to estimate genet ages of clonal plants in which we identify ramets of individual genets using amplified fragment length polymorphisms (AFLPs), mathematically model the most parsimonious ramet growth patterns and subsequently estimate genet maximum, minimum and average ages by dividing respective distances of modelled clonal networks by field-determined ramet-growth rates and (ii) to demonstrate an application of such life-history information of foundation clonal plants to provide insights into conservation management plans.

As a foundation species, *Serenoa* provides crucial structural and functional services to many Southeastern United States ecosystems (Henderson *et al.* 1995). Its importance to the Florida scrub ecosystem, a biodiversity hotspot, is well recognized (Stein *et al.* 2000). This shrub-sized palm not only defines many south-eastern plant communities but also plays a critical role in controlling succession and habitat heterogeneity in fire-prone ecosystems by facilitating fire with their highly inflammable leaves (Abrahamson 1984b; Schmalzer

2003). Moreover, *Serenoa* extensively provides food (i.e. fruits) for a large number of vertebrates including the state-threatened Florida black bear (*Ursus americanus floridanus*), white-tailed deer (*Odocoileus virginianus*), wild turkey (*Meleagris gallopavo*) and federally threatened gopher tortoise (*Gopherus polyphemus*) (Halls 1977; Maehr & Brady 1984; Abrahamson & Abrahamson 1989; Layne & Abrahamson 2010). *Serenoa* also provides important wildlife cover and is the most commonly used plant by the federally endangered Florida panther (*Puma concolor coryi*) for resting, hiding and denning (Maehr 1997). The remaining populations of the federally endangered Florida grasshopper sparrow (*Ammodramus savannarum floridanus*) are also critically associated with the distribution of *Serenoa* (Delany *et al.* 1985). Over 200 terrestrial vertebrate species, as well as numerous invertebrate species, are reported to utilize *Serenoa* for food, nest sites or refuges (Maehr & Layne 1996). However, because of human development and the expansion of improved pasture for cattle grazing, the extent of *Serenoa* and of Florida's naturally intact *Serenoa*-dominant associations have declined (Peroni & Abrahamson 1985; Weekley *et al.* 2008). For example, Florida Fish and Wildlife Conservation Commission (2005) lists the statewide threat rank of Florida scrub associations where *Serenoa* is a foundation species as 'Very High.'

In addition to *Serenoa*'s ecological importance, the pharmaceutical industry has generated remarkable interest in the medicinal value of its fruits. A national survey conducted in 2002 reported that 2.5 million adults in the United States alone use *Serenoa* fruit extract, mainly by men for the treatment of benign prostatic hyperplasia (Barnes *et al.* 2004). *Serenoa* extract is also used to treat other medical conditions including renal disorders, cystitis, diarrhoea, epididymitis, baldness and bronchitis (see review in Bennett & Hicklin 1998). As a result, there is a high demand for *Serenoa* fruits not only in the United States but also for export to European countries. These fruits are entirely wild-collected (Bennett & Hicklin 1998), and collection from naturally occurring *Serenoa* populations is likely to continue because the slow recruitment of *Serenoa* populations has discouraged the establishment of commercial farms (Abrahamson 1999; Carrington *et al.* 2000; Abrahamson & Abrahamson 2002, 2006, 2009). Given its ecological and economic values, several authors have recently argued for the conservation of *Serenoa* and its associated ecosystems (Maehr & Layne 1996; Bennett & Hicklin 1998; Carrington *et al.* 2000; Tanner & Mullahey 2009), and some restoration protocols have been developed (Schmalzer *et al.* 2002; Makus 2008).

In contrast, some consider *Serenoa* a troublesome plant that can spread and dominate other plant species.

Accordingly, some researchers and land managers have actively investigated the ways to reduce *Serenoa* abundance by prescribed fire and mechanical and chemical treatments, including roller chopping, mowing and herbicide (Lewis 1970, 1972; Tanner *et al.* 1988; Watts & Tanner 2006; Menges & Gordon 2010; Willcox & Giuliano 2010). Such management plans appear to be based on little or no empirical data that document *Serenoa* invasions or marked increases in its ramet densities since European colonization. Furthermore, our understanding of *Serenoa*'s life history including its establishment and generation time as well as its reproductive biology is very limited, which provides a poor basis for the development and implementation of proper management plans.

We do know that *Serenoa* propagates both by sexual reproduction (i.e. seeds and seedlings) and clonal propagation via vegetative sprouts (i.e. young individuals arising from rhizomes) (Fisher & Tomlinson 1973; Abrahamson 1995). However, we know virtually nothing about extent and longevity of *Serenoa* genets. We also lack knowledge of the relative contribution of sexual reproduction vs. asexual propagation in *Serenoa* recruitment. Studying *Serenoa*'s life-history traits has been challenging to date for several reasons. First, *Serenoa*'s prostrate stem is largely subterranean, which makes it difficult to observe. Second, its prostrate stems are not persistent; they decay and leave no physical evidence of previous clonal connections (Abrahamson 1995). Based on the slow growth rate of its stems (0.6–2.2 cm/year) in Florida scrub associations, Abrahamson (1995) suggested that individual *Serenoa* ramets are long-lived with 500-year-old ramets common. Yet, we do not know the longevity of its genets because of temporal and subterranean clonal connections. Finally, the seedlings of *Serenoa* are virtually impossible to distinguish from those of the sympatrically occurring *Sabal etonia* (scrub palmetto, hereafter *Sabal*; Abrahamson & Abrahamson 2009). Because of the extremely slow aerial growth of *Serenoa* and *Sabal* seedlings (3–5 mm/year), the vast majority of monitored seedlings in a 19-year study remained small and remained unidentifiable (Abrahamson & Abrahamson 2009).

If *Serenoa* is highly clonal, then longevity of *Serenoa* genets is far greater than that currently assumed for individual *Serenoa* ramets. Also, despite *Serenoa*'s abundant fruit production, clonal propagation may be its dominant means of propagation. If so, such life-history information would suggest that: (i) *Serenoa* has been part of local vegetative associations for long period (e.g. several thousand year), (ii) rapid change in *Serenoa* abundance and invasion are unlikely and finally (iii) once *Serenoa* is reduced or eliminated from local communities, its restoration would be difficult.

Methods

Study site and sampling procedures

In October 2001, we established a 20 × 20 m study plot in Archbold Biological Station located in the southern portion of the Lake Wales Ridge (LWR) on the Florida peninsula (27°11′N, 81°21′W). Palmettos in this region occur in low-elevation flatwoods, transitional scrubby flatwoods, sand pine scrub and sandhills. Our plot was located in scrubby flatwoods in which *Serenoa* and *Sabal* were codominant cover species, and slash pine (*Pinus elliottii* var. *densa*) is an occasional canopy species. *Sabal* is a non-clonal palmetto that shares many life-history characteristics with *Serenoa*, such as habitat preference, dwarf stature, large underground mass, post-fire resilience and slow recruitment (Abrahamson 1984a, 1995, 2007; Menges & Kohfeldt 1995; Abrahamson & Abrahamson 2009). Both *Serenoa* and *Sabal* flower during May and mature the resulting fruits during September/October, which are consumed and dispersed by wild animals (Abrahamson 1999; Layne & Abrahamson 2010).

A *Serenoa* leaf sample was collected from every adult individual growing within the 20 × 20 m plot that was gridded into 400 1 × 1 m squares. In total, we collected 218 *Serenoa* samples. We also collected 139 field-unidentifiable small individuals that were scattered across the grid. These individuals could be *Serenoa* or *Sabal* seedlings, or *Serenoa* vegetative sprouts. These 139 individuals represented approximately a half of field-unidentifiable small individuals occurring within the 20 × 20 m plot. In addition, we collected a leaf sample of every adult *Sabal* within the same plot (*N* = 55). *Sabal* samples were used to identify the field-unidentifiable small individuals based on multilocus genotypes (MLGs) of adult *Serenoa* and *Sabal*. *Sabal* samples also served as a negative control to examine whether our clone analyses were able to correctly distinguish the genet structures between clonal *Serenoa* and non-clonal *Sabal*. We placed the collected samples into individually numbered tubes and recorded their locations with grid IDs (i.e. 1 × 1m resolution). Tubes were then stored in liquid nitrogen for shipment back to Bucknell University in Pennsylvania, USA, where we transferred the samples to a −20 °C freezer.

DNA extraction and AFLP analyses

We ground the leaf samples using Tissueruptor (QIAGEN Inc.) and extracted DNA using a DNeasy Plant Mini kit (QIAGEN Inc.). AFLPs were generated based on the protocol developed by Vos *et al.* (1995). AFLP

analysis consists of four steps, restriction digestion, adaptor ligation, preselective amplification and selective amplification. We used an AFLP Core Reagent kit (Invitrogen Inc.) for the first two steps and an AFLP Plant Mapping kit (Applied Biosystems Inc.) for the last two steps. After screening of 32 selective primer pairs, we used the three most informative primer pairs of *Eco*RI and *Mse*I for both *Serenoa* and *Sabal* as follows: (i) *Eco*RI: ACT—*Mse*I: CAA, (ii) *Eco*RI: ACG—*Mse*I: CAA and (iii) *Eco*RI: ACA—*Mse*I: CAT. AFLP profiles were obtained using an ABI 3100 genetic analyzer (Applied Biosystems Inc.) and GeneMapper software (Applied Biosystems Inc.). Given our large sample size ($N = 412$) and three primer combinations, we generated AFLP binary matrices using GeneMapper's automated scoring. AFLP automated scoring suffers varied error rates with a trade-off between getting more characters of lower quality or fewer characters of higher-quality depending on parameter settings of the software (Holland *et al.* 2008). We parameterized GeneMapper automated scoring settings so that AFLP profiles consisted of higher quality data: fragment range = 100–350 bp, bin width = 1, binary threshold value for peak height '0' ≤50 relative fluorescent unit (rfu) < 'missing data' ≤100 rfu < '1'. The GeneMapper AFLP guide (Applied Biosystems Inc.) recommends that ambiguous peaks >50 and ≤100 rfu be manually checked. We found that manually classifying those ambiguous peaks to 1 or 0 was often difficult, error-prone, and extremely time-consuming given our large data set. Thus, to minimize scoring errors, we treated them as missing data.

Assignment of field-unidentifiable samples

Using the known *Serenoa* and *Sabal* samples, we assigned the field-unidentifiable individuals as *Serenoa* or *Sabal* (i.e. $K = 2$) by importing the AFLP binary matrix into STRUCTURE (Pritchard *et al.* 2000). We set Markov chain Monte Carlo simulation parameters with a 10^3 burn-in period and 10^4 iterations under the models of recessive alleles (Falush *et al.* 2007), no population admixture and allele frequency independent.

Detection of Serenoa clones

Multilocus genotypes obtained via AFLPs have been widely used for the identification of genets in plants; yet, clonemates (i.e. ramets of the same genet) are often represented by slightly different MLGs because of scoring errors or somatic mutation (Douhovnikoff & Dodd 2003; Arnaud-Haond *et al.* 2007; Lasso 2008; Lazzi *et al.* 2009; Mathews *et al.* 2009; Stölting *et al.* 2010). Using GeneMapper, Holland *et al.* (2008) found mean error rates among replicated AFLP profiles generated from

the same individuals to be between 6% and 18%. Minimizing scoring errors is critical for detecting clones because the ability to distinguish between error-caused and true differences in MLGs dwindles as error rates increase. Using the 'overlay' function of GeneMapper, we projected a single chromatograph with all individuals overlaid and manually selected reliable loci that showed relatively distinct allelic distribution of 'absent (<50 rfu)' and 'present (more than 100 rfu)'. We used these selected loci for further clone analyses. For comparison, we applied the same procedure to the non-clonal *Sabal* samples. Using these loci, we also estimated reproducibility of AFLP profiles by duplicating AFLP profiles of randomly selected *Serenoa* and *Sabal* samples ($N = 10$ each) through repetition of the entire AFLP process. We used an independent t-test to compare scoring error rates between *Serenoa* and *Sabal*.

Most studies of clonal plants using genetic markers set thresholds to allow slightly different MLGs to be clonemates (See review in Arnaud-Haond *et al.* 2007). We set our threshold based on the frequency distribution of pairwise genetic distances (Douhovnikoff & Dodd 2003; Meirmans & Van Tienderen 2004; Arnaud-Haond *et al.* 2007). When the frequency distribution of pairwise genetic distances is multimodal, a threshold can be assigned to the valley between the first and the second peak as the first peak represents clonal individuals that have slightly different MLGs owing to scoring errors or somatic mutations, while the second peak represents closely related individuals (Douhovnikoff & Dodd 2003; Meirmans & Van Tienderen 2004). Meirmans & Van Tienderen (2004) provided the software GENOTYPE and GENODIVE, with which we can set a threshold to assign individuals into clonal and non-clonal genotypes and also can estimate various clonal diversity indices. By importing the AFLP binary matrices into GENOTYPE, we created the frequency distribution of pairwise Dice distances and set a threshold as described earlier. We then assigned all individuals into genets. Pairwise Dice distance was calculated by the equation:

$$\left\{ 1 - \left[\frac{2a}{2a + b + c} \right] \right\} \times 100$$

where a is the number of shared bands, b is the number of bands present in the first individual but not in the second and c is the number of bands present in the second but not in the first. Dice distance, together with its relative Jaccard distance, is commonly used to analyse AFLP data (Douhovnikoff & Dodd 2003; Meirmans & Van Tienderen 2004; Lasso 2008; Mathews *et al.* 2009) as these distance indices do not count shared absence as similarity and thus are suited for

analyses of dominant data. We classified the field-un-identifiable individuals that were assigned to *Serenoa* into seedlings or vegetative sprouts based on whether they had unique genotypes (seedlings) or not (vegetative sprouts). We used a chi-square test to examine the proportional difference between the ratio of vegetative sprout to seedling and the ratio of clonal adults to adults with unique genotypes.

We used GENODIVE to calculate three clonal diversity indices that are commonly used in the literature and recommended for studies of clonal plants (Arnaud-Haond *et al.* 2007): the number of genotypes (*G*), the ratio of *G* to the sample size ($P_d = G/N$) and Nei's (1987) genetic diversity corrected for sample size (D_{corr}). Nei's genetic diversity is also known as the Simpson complement, which varies between 0 and 1 with the greater value suggesting the higher clonal diversity. D_{corr} was calculated by the formula:

$$\frac{N}{N-1}\left(1 - \sum\nolimits_{i=1}^{s} p_i^2\right)$$

where p_i is the observed frequency of the *i*th of *s* genotypes and *N* is sample size. We also used GENODIVE to evaluate whether our sample size was large enough to calculate unbiased D_{corr} via jackknife permutations.

The power of AFLP fingerprints was estimated by calculating the probability of identity [$P_{(ID)}$] which is the probability of two randomly drawn individuals from a population having the same MLGs (Waits *et al.* 2001). We used the following equation to estimate $P_{(ID)}$ for dominant genetic markers such as AFLPs and for a population that probably contains relatives:

$$P_{(ID)} = 1 - \left\{\left(\frac{3}{2}p\right)\left(q^2\right)\right\}$$

where *p* is the frequency of a present band and *q* is the absence of a band (Waits *et al.* 2001). Overall $P_{(ID)}$ was calculated by multiplying the $P_{(ID)}$ of each locus across all loci.

Age estimation of clonal genets

We estimated ages of *Serenoa* genets by dividing distances among clonemates (based on the grid data) by an average stem growth rate of 0.88 cm per year (Abrahamson 1995). This mean was determined by measuring 60 individuals (20 × 3 sites) over 4 years in a scrubby flatwoods near our sampling site. As it is impossible to know the true pattern of clonal networks, we constructed a minimum branching tree (MBT) from each adult ramet designated as a starting point (or a root) of the clonal growth and constructed a directed weighted graph where adult ramets and sprouts were represented by vertices and outgoing edges were drawn from each ramet vertex to all other vertices except to the root ramet vertex. The edges represent possible clonal growth relations. No edges were drawn from sprout vertices. The weight of each edge was set to the Euclidean distance on the grid between the two clonemates represented by the vertices. We then applied the Edmonds' algorithm to the graph to generate the MBT from the root ramet (Karp 1971). The resulting MBT represents the most parsimonious clonal network (in terms of the total amount of clonal growth) from the root ramet with sprouts designated as terminal individuals. We repeated this procedure by designating each adult ramet as a starting root of the clonal growth and created a series of MBTs for each genet. Topologies of MBTs within each genet can vary depending on which ramet was designated as a root. Finally, we calculated a series of maximum distances from each ramet to any of its clonemates. Based on these series of maximum distances, we calculated maximum, minimum and average estimated ages for each genet with multiple ramets. We programmed these procedures using C++ and MATLAB® (the codes are available at http://www.susqu.edu/facstaff/k/kubota/ClonalPlantsMBT.zip).

Results

We obtained AFLP profiles from 215 *Serenoa*, 55 *Sabal* and 139 unidentifiable small individuals (409 of 412 collected samples). Excluding common loci, three primer pairs produced a total of 465 polymorphic loci among these 409 samples. Using these loci, STRUCTURE (Pritchard *et al.* 2000) assigned all unidentifiable individuals to *Serenoa* or *Sabal* with *Q* = 1 (*Q*: the proportion of genomes originating from an ancestral population). Of the 139 field-unidentifiable samples, 56 were assigned to *Serenoa* and 83 were assigned to *Sabal* (Fig. 1). For the detection of genets, we used only samples that had complete AFLP profiles from all three primers, which included 263 of 271 total *Serenoa* samples and 134 of 138 total *Sabal* samples. We used 177 polymorphic loci for the *Serenoa* genet analyses after filtering common loci and ambiguous loci of 493 total loci detected. On average, *Serenoa* samples had 3.83 ± 0.11% (±SE) missing data, thus *Serenoa* had 170.2 ± 0.2 polymorphic loci per sample. Because the frequency distribution of pairwise Dice distances of *Serenoa* samples was multimodal, we set a threshold of 2% dissimilarity and considered pairs with 2% or less dissimilarity to be clonemates (Fig. 2a). In contrast, the frequency distribution of pairwise distances of *Sabal* samples, which was based on 148 polymorphic loci of 439 total loci, was unimodal. There was no evidence of clones in *Sabal*
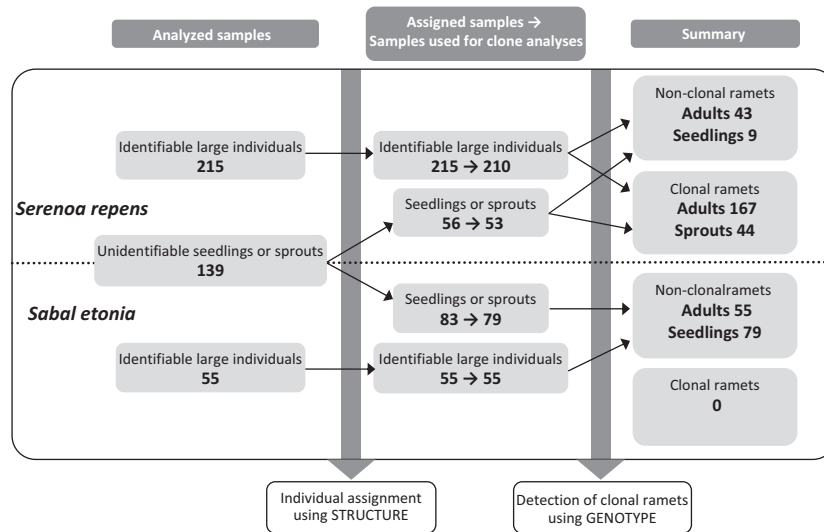
Fig. 1 Sample sizes, the individual assignment and the genet analyses of *Serenoa repens* (facultative clonal palm) and *Sabal etonia* (non-clonal palm). Of 409 samples assigned to either species, 12 samples had amplified fragment length polymorphism profiles from only two primer pairs (instead of three); thus, those samples were removed from the subsequent clone analyses.

(Fig. 2b). On average, *Sabal* samples had $4.87 \pm 0.22\%$ missing data and thus had 141.7 ± 0.3 polymorphic loci.

The average error rates (i.e. dissimilarity) between duplicated samples were $1.2 \pm 0.5\%$ ($N = 10$, range: 0–4%) for *Serenoa* and $2.1 \pm 0.7\%$ ($N = 10$, range: 0–5%) for *Sabal*. There was no difference in error rates between *Serenoa* and *Sabal* ($t = -1.063$, $P = 0.302$), and the grand average for the error rate was $1.7 \pm 0.4\%$. Among 263 *Serenoa* samples, we found 57 genotypes ($G = 57$). Of these, five were clonal genotypes with multiple ramets, while 52 genotypes consisted of single ramets (Table 1, Fig. 3). The ratio of G to the sample size ($P_d = G/N$) and Nei's genetic diversity corrected for sample size (D_{corr}) were 0.22 and 0.65, respectively. The result of Jackknife permutations showed that D_{corr} levelled off before it reached the actual sample size (around $N = 106$), suggesting that our sample size was large enough to calculate unbiased D_{corr}.

Of 53 small *Serenoa* individuals analysed, we identified nine to be seedlings and 44 to be vegetative sprouts based on whether they had unique genotypes (seedling) or not (vegetative sprout) (Fig. 1). There was no difference between the ratio of sprout to seedling and the ratio of clonal adults to adults with single ramets ($\chi^2 = 0.013$, $P = 0.910$). Overall $P_{(ID)}$ for *Serenoa* was 0.0028, suggesting that the probability of two randomly drawn individuals from the population having the same MLGs was <0.3%. Overall $P_{(ID)}$ for *Sabal* was 0.0002.

We found that topologies of MBTs for maximum and minimum age estimation were identical in genets 1, 2, 3 and 4, while genet 5 showed slightly different topologies (Fig. 4a–f). Estimated ages for the five clones ran-

ged from 1227 to 5215 year based on their MBTs (Table 1).

Discussion

Scoring error and frequency distribution of pairwise genetic distances

A recent study using AFLPs found that the frequency distributions of pairwise genetic distances of clonal plant populations can often be unimodal (Lasso 2008). Overlapping peak ranges such as in Fig. 2a in our study and unimodal distributions of pairwise genetic distances in clonal plant populations can occur when differences in MLGs between clonal pairs and between non-clonal pairs overlap owing to scoring errors, somatic mutations of clonal ramets, intensive inbreeding or selfing of non-clonal pairs. Genotyping errors in DNA fingerprinting techniques (e.g. AFLP, RAPD and microsatellite) have been commonly reported (Douhovnikoff & Dodd 2003; Meirmans & Van Tienderen 2004; Lazzi *et al.* 2009; Mathews *et al.* 2009; Schnittler & Eusemann 2010). In particular, use of automated scoring in analysing AFLP profiles accompanies relatively low repeatability with 6–18% error rates (Holland *et al.* 2008; Stölting *et al.* 2010). However, automated scoring is an attractive and often is the only practical option to deal with large data sets (Holland *et al.* 2008). Through our efforts to filter out ambiguous alleles and loci, we minimized the error rate (on average 1.7%), which is much lower than the rates of the above studies, while still providing sufficient power to identify individuals.
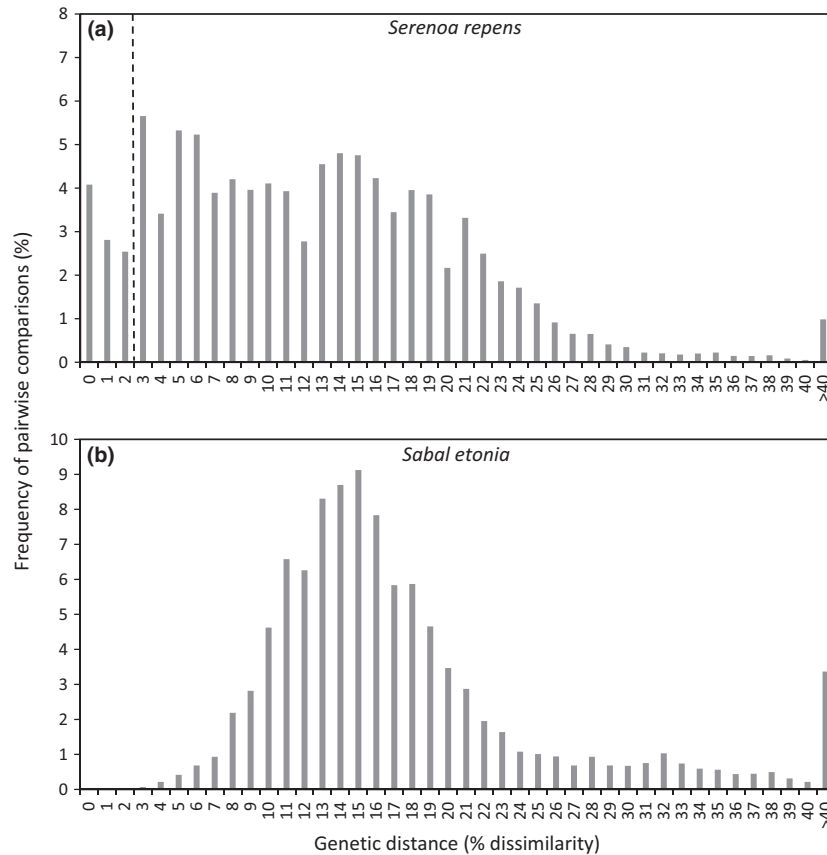
Fig. 2 Frequency distribution of pairwise genetic distances (Dice distance) of *Serenoa repens* (a) (*N* = 263) and *Sabal etonia* (b) (*N* = 134). *Serenoa* distribution is multimodal, and the dotted line assigned between the first and the second peaks represents a threshold. Any pairs with slightly different Multilocus genotypes with 2% dissimilarity or less were considered to be clonemates. *Sabal* distribution is unimodal and shows no sign of clonal spread.

Table 1 Numbers of ramets and sprouts and estimated ages of five clonal genets detected

	Genet 1	Genet 2	Genet 3	Genet 4	Genet 5
No of ramets	148	8	3	4	48
No of sprouts	36	2	0	3	3
Estimated age (year)					
Max	5215	2253	3181	1294	3774
Min	2609	1227	1702	1294	1710
Average	3920	1552	2689	1294	2483

Age estimations were based on minimum branching trees (see Fig. 4).

Yet, even with this minimized error rate, the higher end of its range (5%) is likely to overlap with the minimum dissimilarities between close relatives (Fig. 2a). As minimizing scoring error is critical in the detection of genets, our approach should provide implications for the future studies of clonal plants using AFLP.

Clonal spread and ages of Serenoa

Our analytical framework, which integrates genetic data with clone-network modelling, provides a practical tool to estimate ages of clonal plants. Such life-history information of clonal plants is important to understand not only the life history of clonal plants but also the development of management plans for the foundation clonal species and their ecosystems, given that the long-term community stability of such ecosystems is modulated by the extended longevity of clonal plant populations. We estimated *Serenoa*'s genet ages to be 1227–5215 year which are an order of magnitude older than the previous estimates of *Serenoa*'s ramet longevity based on physical clonal connections (500–700 year, Abrahamson 1995). Yet, these new estimates are still likely conservative for at least four reasons. First and foremost, it is probable that our 20 × 20 m plot did not encompass entire clonal networks. Genets 1 and 5 have extensive spread, much of which may be outside our plot. For this reason, although still conservative, our maximum
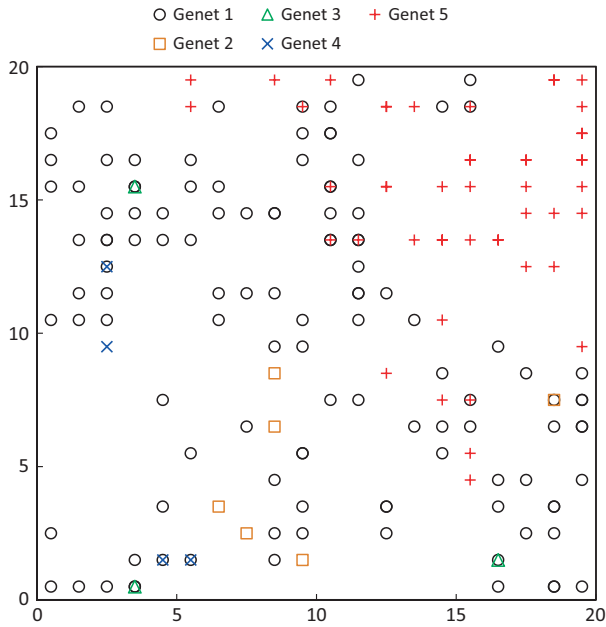
Fig. 3 The clonal spread pattern of five clonal genets of *Serenoa repens* within the 20 × 20 m study plot.

age estimations for genets 1 and 5 represent a more realistic scenario in which the ancestral ramet, from which the clonal spread was assumed to start, was a marginal ramet. In contrast, in the minimum age estimation, an ancestral ramet was identified at the centre of the large genets 1 and 5. Our plot was embedded within a hugely larger *Serenoa* population, and it is highly probable that the origins of the detected genets are outside our plot. Second, our age estimation based on minimum branching trees is conservative as the actual clonal networks are likely less parsimonious. Although *Serenoa* ramets have high survival rates (Abrahamson 1984a; Abrahamson & Abrahamson 2002, 2006, 2009), mortality certainly occurs. Our analyses could not incorporate ramets that died and disappeared; the inclusion of which would have resulted in estimates of greater longevity.

Third, when we estimated the ages of *Serenoa* genets based on the MBTs, we assumed that rhizome growth was continuous. Yet, it is possible that rhizome growth is intermitted when a rhizome produces vegetative sprouts until the sprouts reach certain size. If so, given the slow aerial growth rate of *Serenoa* seedlings (~0.3 cm/year in scrubby flatwoods; Abrahamson 1995), it is probable that numerous decades are required for vegetative sprouts to grow to a size that they can further extend rhizomes. A rough estimate of the time for vegetative sprouts to grow to adult size is ~200 year based on known seedling growth rates and minimum average height of reproductive *Serenoa* (~60 cm; Abrahamson 1995, 1999). Even if we use a conservative

estimate of 100 year for a sprout to become an adult, the maximum age of genet 1 is estimated at ~8000 year when this growth period was added to each adult ramet between the starting and the ending individual of the MBT.

Finally, the threshold that we used to determine clonemates has likely missed some ramet pairs. The frequency distribution of pairwise genetic distances for *Serenoa* shows that the first and second peak ranges overlap, making it impossible to separate ramet pairs from pairs of their close relatives with 100% certainty. For example, when the threshold of 3% dissimilarity (instead of 2%) is used, genets 3 and 4, which were small and spatially well overlapped with genet 1, collapse into clone 1. Indeed, 100% of genet 3 and 75% of genet 4 occur in the same 1 × 1 m quadrates as the ramets of genet 1 (Fig. 3). In contrast, genets 1, 2 and 5, which were larger and relatively spatially exclusive from each other, remain as independent genets. This raises the possibility that genets 3 and 4 are parts of genet 1. The notably high frequencies of the genetic distance classes 3, 5 and 6 in Fig. 2a may also indicate the mixed presence of clonal pairs with non-clonal pairs in the second poorly defined peak. As such, using a higher threshold would increase the risk of considering non-clonal pairs as clonal, while decreasing the probability of misidentifying clonal pairs as non-clones. Thus, our threshold is conservative in terms of detection of clonal pairs, in favour of a low misidentification rate of non-clonal close relatives. In sum, considering all these points discussed earlier, it is reasonable to think that 10 000-year-old *Serenoa* genets may be common in scrubby flatwoods habitats.

What does this remarkable longevity of *Serenoa* genets mean to its ecosystem? Several authors have suggested that extended longevity and persistence of foundation species can enhance community stability and ecosystem resilience against environmental and demographic stochasticity as well as global climate change (Steinger *et al.* 1996; Weiher *et al.* 1999; Eriksson 2000; Garcia *et al.* 2008; Morris *et al.* 2008; de Witte & Stöcklin 2010). Pollen profiles obtained from Archbold's Lake Annie show that *Serenoa* has been part of the LWR ecosystem for at least 37 000 years in spite of the historical climate oscillation (Watts 1975; Grimm *et al.* 1993). In addition to the climatic fluctuation, the Florida scrub ecosystem has evolved with frequent natural fires (Laessle 1958; Brown & Smith 2000). Yet, with its large underground mass, *Serenoa* possesses remarkable resilience to repeated fire and is able to rapidly recover its foliage to its pre-burn level of dominance (Abrahamson 1984b). With its remarkable longevity and resilience, *Serenoa* has played and will play a vital role in providing foundation and stability to their ecosystems.
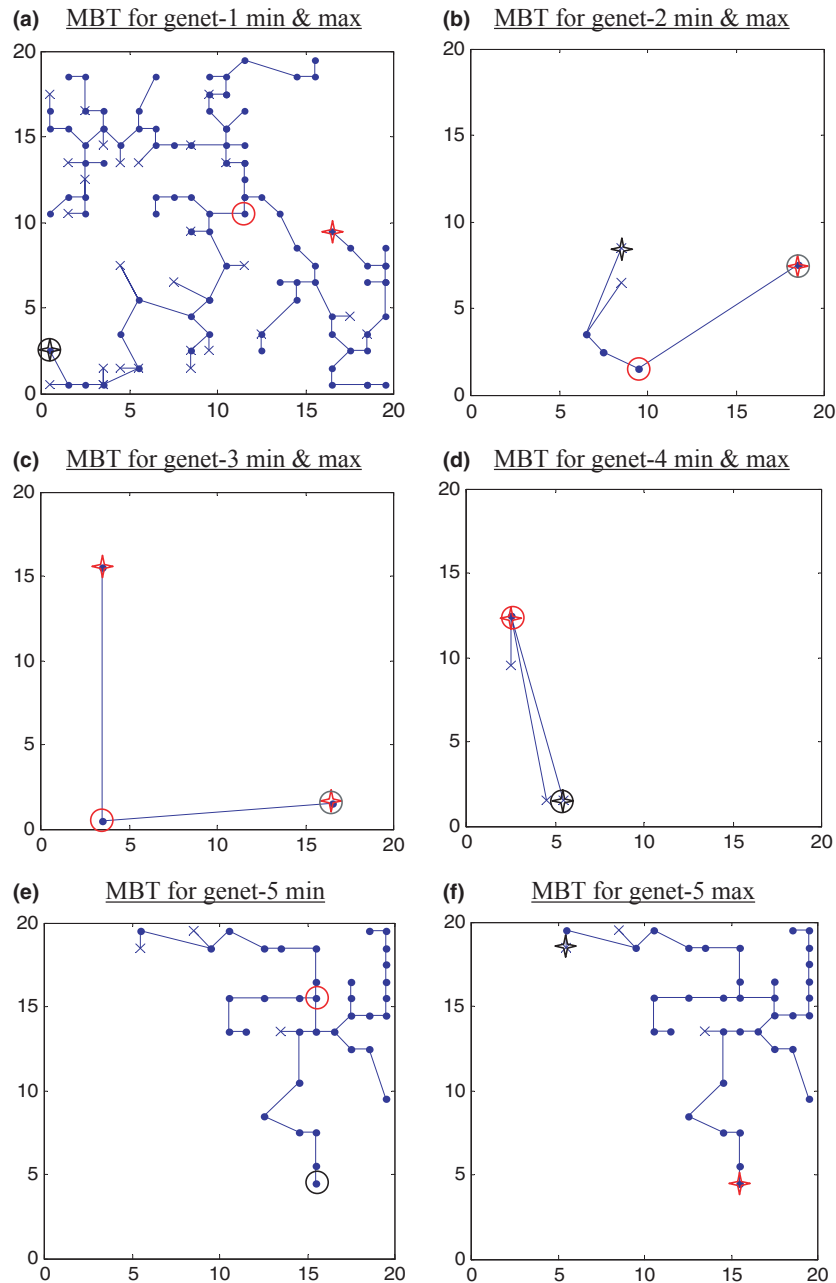
Fig. 4 Minimum branching trees (MBTs) constructed for minimum (Min) and maximum (Max) age estimations of five genets of *Serenoa repens*. Topologies of MBTs for Min and Max were identical in genets 1 through 4 (a–d), while they were different in genet 5 (e and f). Blue filled dots are adult ramets, while blue crosses are sprouts. Circles indicate the pairs of individuals used for minimum age estimations with red as a starting individual and black as an ending individual (see Table 1). Crosses indicate the pairs of individuals used for maximum age estimations with red as a starting individual and black as an ending individual. In (c), both crosses are red, suggesting topologies and maximum age estimation do not change depending on the direction of clonal spread between those two ramets.

It is important to note that rhizome growth rates vary temporarily and spatially, especially depending on habitat types. For example, the average rhizome growth rate in flatwoods, which are adjacent to scrubby flatwoods but topographically lower, was 1.47 cm per year (in stead of 0.88 used in this study) during the same survey period (Abrahamson 1995). Furthermore, *Serenoa* has a wide distribution range across the Southeastern US coastal plain. While the rhizome growth rate used in this study appropriately represents the rates expected in

nutrient-poor Florida scrub associations (Abrahamson *et al.* 1984), nutrient-richer habitat types likely allow *Serenoa* rhizomes to grow faster. For example, *Serenoa* rhizomes grow faster in abandoned citrus groves because of residual nutrients, but only with the absence of competition from exotic grasses such as *Paspalum notatum* (P. Schmalzer, personal communication). Yet, it is difficult to speculate how this variation in growth rate affects genet ages. Populations with greater growth rates may live as long as those with reduced growth rates and achieve larger genet size. To precisely estimate genet ages at a given site, one would want growth rate data that are specific to that site.

Our results also offer insights into the extensive clonal spread and reproductive biology of *Serenoa repens*. Genet 1, for example, was composed of 148 ramets (58% of all *Serenoa* samples) and came close to covering the entire 20×20 m plot, which suggests the possibly much greater extend of genet 1 spread. *Serenoa* seedlings accounted for only 20% of the small *Serenoa* individuals; the remaining 80% were vegetative sprouts. This ratio is consistent with that detected between clonal adults and adults with unique genotypes. This suggests that although seed germination and establishment often fail (Hilmon 1968), once established, *Serenoa* seedlings and vegetative sprouts likely survive at a similar rate (see also Abrahamson & Abrahamson 2009). Despite *Serenoa*'s abundant flower and fruit production, our data suggest that *Serenoa* is highly clonal and that asexual propagation is the predominant mode of propagation in Florida's scrubby flatwoods and possibly in many other ecosystems with mature *Serenoa* populations. Comparison of adult to seedling ratios for *Serenoa* and *Sabal* further emphasizes the limited role of recruitment via seedlings in *Serenoa*. The adult to seedling ratio was 1–0.04 in *Serenoa*, whereas in *Sabal* it was 1–1.44. Even with the inclusion of sprouts, the adult to seedling and sprout ratio in *Serenoa* was 1–0.25. Despite the smaller adult-to-seedling and sprout ratio in *Serenoa*, *Serenoa* exhibits greater dominance in scrubby flatwoods than *Sabal*, which suggests the potential importance of *Serenoa*'s longevity in its population dynamics and community assembly.

Recommendations for Serenoa *management plans*

Our previous work has demonstrated *Serenoa*'s extremely slow seedling recruitment as well as growth rate (Abrahamson 1995; Abrahamson & Abrahamson 2009). The present study reveals the prevalence of *Serenoa*'s clonal propagation, limited seedling dispersal or establishment and its extraordinary longevity. These findings collectively suggest that: (i) *Serenoa* has been part of its ecosystem for a remarkably long period, (ii) rapid changes in *Serenoa*'s abundance or its invasion into new sites is unlikely and finally (iii) once *Serenoa* is reduced or extirpated at a site, its reestablishment and the recovery of a naturally functioning ecosystem will take considerable time and will be challenging to accomplish.

A few attempts to restore *Serenoa*-dominant association have been made to date. Schmalzer *et al.* (2002) reported that abandoned agricultural lands, which were originally part of scrub, did not return to scrub vegetation through natural succession even though these lands are adjacent to intact scrub vegetation. Instead, exotic grasses such as guinea grass (*Panicum maximum*) together with cabbage palms (*Sabal palmetto*) and muscadine (*Vitis rotundifolia*) dominated. Accordingly, restoration of scrub vegetation on this fallowed farmland was attempted by eliminating invasive plants and planting horticulturally grown foundation species including *Serenoa* and several oak. During 8 year of monitoring, scrub vegetation was poorly restored. In particular, *Serenoa* grew very slowly and *Serenoa* cover remained low (<1%). Although this is one case study, with the knowledge provided by our study, we now have a mechanistic understanding of the challenges in restoring previously *Serenoa*-dominant association, and we can predict that such restoration failures may be common. Despite an increasing appreciation of *Serenoa*'s ecological significance (Maehr & Layne 1996; Bennett & Hicklin 1998; Carrington *et al.* 2000; Tanner & Mullahey 2009), a recent paper still argues that *Serenoa* is one of the shrubs whose encroachments and invasions degrade ecosystems and recommend growing season roller chopping as an effective method to reduce *Serenoa* (Willcox & Giuliano 2010). We argue for a careful reevaluation of such management, and the development of proper management practices relative to *Serenoa* is an urgent task.

With *Serenoa* as a model, the current study introduces a novel analytical framework that integrates DNA fingerprinting and mathematical modelling of clonal plants to estimate genet ages and also demonstrates the application of such life-history information to the proper management planning. *Serenoa* genets exhibit the extraordinary spread and astonishing longevity (i.e. thousands of year) as well as limited seedling recruitment in scrubby flatwoods. These findings provide important insights into the conservation management plans for vegetative associations where this ecologically important foundation species occurs. With its dominance and remarkable persistence, *Serenoa* defines, regulates and protects communities and ecosystems against environmental stochasticity. Yet, *Serenoa* is vulnerable to human actions that destroy and fragment associations. Once reduced or eliminated, the reestablishment of *Serenoa* populations will be challenging.

Careful reconsideration of the current management practices is essential to safeguard *Serenoa*-dominant associations and their dependant biodiversity.

Acknowledgements

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Data accessibility

AFLP binary data of *Serenoa repens* and *Sabal etonia*: DRYAD entry doi:10.5061/dryad.6th24.