High variation in clonal vs. sexual reproduction in populations of the wild strawberry, *Fragaria virginiana* (Rosaceae)

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**INTRODUCTION**

Many plant species are capable of both sexual and asexual reproduction. How such plants partition their reproductive effort between clonal and sexual growth is a fundamental question in plant ecology and evolution. The extent of clonal growth can impact species' responses to disturbance (Moola and Vasseur, 2009) as well as vegetation structure, species composition and diversity (Song and Dong, 2002). Advantages to sexual reproduction include increased offspring diversity and greater dispersal potential for seeds compared with ramets. Clonal propagation limits gene flow through pollen and seed, and the absence of genetic variation and recombination limits opportunities for adaptive evolution (Eckert, 2001). However, clonal growth offers increased reproductive assurance if mates or pollinators are limiting and avoids the costs of sexual reproduction (flower and pollen production and the cost of meiosis). Because local ecological and demographic conditions provide the framework that determines the trade-offs among different reproductive modes, the relative rates of vegetative and sexual reproduction will probably change across a species' range, and spatial patterns of ramet and genet distribution will vary (Parks and Werth, 1993; Eckert, 2001).

Differences in the predominant reproductive mode are generally presumed to manifest across broad spatial, ecological or temporal scales, and several studies have shown variation in clonal reproduction rates within a species. Clonal reproduction may increase at higher altitudes, as shown for the subapline forb *Rutidosis leiolepis* (Young et al., 2002), or at the periphery of a species range, as shown for *Decodon verticillatus* (Dorken and Eckert, 2001). Disturbance may increase clonal reproduction, as shown for the woodland herb *Anemone nemorosa* (Rusterholz et al., 2009) or, alternatively, increase sexual reproduction, as in eelgrass (*Zostera marina*), a marine angiosperm (Reusch, 2006). Invasive species are often characterized by changes in reproductive modes associated with colonization and invasive spread (Barrett et al., 2008). In a recent literature review, alien species were found to have significantly higher rates of asexual reproduction than natives (Silvertown, 2008). In some cases, invasion involves clonal spread and rapid establishment of monocultures of single genets, such as the invasive water hyacinth *Eichhornia crassipes* in Southern China (Li et al., 2006) and the invasive Japanese knotweed *Fallopia japonica* in Britain (Hollingsworth and Bailey, 2000).

Studies of clonality were previously hampered by an inability to distinguish between individual genets and individual ramets (Arnaud-Haond et al., 2007). In recent years, the application of highly variable nuclear microsatellites markers provides the opportunity to identify and map distinct genets precisely, and to characterize the distribution of individual plants propagated by either ramets or seeds. In this study, we examine the clonal structure of three neighboring populations.
of wild strawberry, *Fragaria virginiana*, in northeastern Illinois. *Fragaria virginiana* is a perennial herb native to North America and grows in open meadows and woodland edges as well as in more disturbed areas such as abandoned agricultural fields. It has a gynodioecious sexual system, with the proportion of females and hermaphrodites varying in different habitats (Ashman and Diefenderfer, 2001). Plants reproduce by both seeds and vegetative stolons. We assay multilocus microsatellite genotypes to identify and map genets of *F. virginiana* at each site. Our results suggest that reproductive patterns may vary significantly on a relatively small spatial scale, and depend on localized differences in demographic history or habitat.

**MATERIALS AND METHODS**

The study area was located in and around the Chicago Botanic Garden (CBG) in northern Cook County, Illinois. *Fragaria virginiana* Mill. populations were located in two remnant oak woodlands in various stages of degradation. The first *F. virginiana* study site, Green Bay Road (42°09’06.3″N, 87°46’66.2″W) was located within CBG along a dirt trail adjacent to woodland with a dense overstorey of both native and invasive tree species. The second site, McDonald Woods (42°09’03.6″N, 87°47’11.8″W), was located 630 m away, also within CBG and the same woodland tract. At this site, management in the form of fire and invasive species removal is used to maintain native species diversity and an open understory. The third study site, Chipilly Woods (42°07’89.3″N, 87°48’15.5″W), was located in a railway right-of-way adjacent to unmanaged remnant woodland <3 km from the two CBG populations. This site contained little woody vegetation; that present was in the form of low shrubs. Prior to European settlement in the region, this site was part of the same continuous woodland habitat as the two CBG sites but today is separated by suburban development and a major freeway.

The three sites had fairly linear populations of *F. virginiana*, two along pedestrian trails and the third along a railway line. A total of 95 leaf samples were collected from the three sites in summer 2003. Samples were collected via transect at each locality, 29–35 individuals per site. The length and sampling density of transects varied with the density of vegetation so as to provide similar numbers of specimens. Transects were run through the longest axis of each population. At the Green Bay Road site, an 8.5 m transect ran from east to west, and samples were taken every 25 cm. The Chipilly Woods transect ran 9.0 m south to north, and samples were similarly taken every 25 cm. The McDonald Woods transect ran 16.4 m from east to west, and samples were taken every 50 cm. Morph (female or hermaphrodite) was noted for all flowering ramets, but some ramets were not flowering at the time of sampling. Due to the linear nature of this sampling scheme, clone sizes were computed using only the on-transect distance between the furthest members of the genetically identical group.

Leaf samples were preserved by desiccation using DriRite crystals (W.A. Hammond Drierite Co. Ltd, Xenia, OH). Approximately 20 mg of leaf material was pulverized by grinding in liquid nitrogen and DNA extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA).

Cytogenetic studies show *F. virginiana* to be octoploid, and the species is reported to be an allopolyploid (Bringham, 1990) but shows disomic inheritance of DNA microsatellite loci (Ashley et al., 2003). The microsatellite loci used for clone identification were previously developed for *F. virginiana* (Fvi08b, Fvi09 and Fvi11; Ashley et al., 2003) or for cultivated strawberry, Fragaria × ananassa Duch. (ARSFL-4) (Nourse et al., 2002) and ARSFL-099 (Lewers et al., 2005). Forward primers were synthesized with 5' M13 tails to allow for labelling with fluorescent M13 primers (Schuelke, 2000). All amplifications were carried out in 20 µL reactions containing 1 µg µL⁻¹ bovine serum albumin (BSA), 0.04 mM forward primer, 0.16 mM M13 dye, 2 µL of PCR buffer, 1 mM dNTPs and 0.08 U of Taq, with slightly varying concentrations of MgCl₂, reverse primers and genomic DNA. Genotypes were resolved on an ABI 3730 DNA analyzer (Applied Biosystems Inc., Foster City, CA). Allele scoring was carried out using the Genemapper software (Applied Biosystems Inc.).

Descriptive statistics including allele frequencies, gene diversity, F-statistics and probability of identity (PI) were calculated using GenAIEx 6.2 (Peakall and Smouse, 2006). PI represents the probability of two individuals sharing the same multilocus genotype by chance (rather than because they are clones) and was calculated for each of the three populations for individual loci and all loci together. Genotype data were used to assess the number of different multilocus genotypes in the plot. The proportion of distinguishable multilocus genotypes (PD) was calculated as described by Ellstrand and Roose (1987) as G/N, where G is the number of distinct genotypes and N is the number of individuals sampled.

Additional analyses were carried out using GenClone 2.0 (Arnaud-Haond and Belkhir, 2007). Two additional diversity indices were calculated (Parker, 1979): (1) D, the complement of the Simpson index corrected for finite samples (Pielou, 1969; Peet, 1974); and (2) a genotypic evenness measure, E (Fager, 1972). Power tests for clone detection were carried out using jackknife resampling simulations on each set of specimens. The average number of genotypes detected using progressively smaller subsets of the sampled loci or individuals is used to examine the sensitivity of genotype detection to reductions in sampling effort. Analogous to rarefaction curves, if the detection power is greatly impacted by a small reduction in either the number of loci or the number of individuals sampled, it is inferred that the sampling effort was insufficient. Sensitivity to loci reduction implies that the genetic data are insufficient to distinguish all genotypes (genets) and sensitivity to reductions in the number of individuals sampled implies that additional sampling would find additional genotypes (Arnaud-Haond et al., 2005).

Spatial autocorrelation analysis was also performed to describe and compare the spatial structure of the three populations. Each transect was divided into distance classes, each one-tenth of the total transect distance. Since the original transect length was chosen to produce approximately even numbers of specimens collected at each locality and therefore varied with the population density of *F. virginiana*, the size of these distance classes were allowed to vary proportionally to avoid variation in statistical power among sites.
The spatial autocorrelation statistic, $f_{ij}$, summarizes the incidence of homology between alleles $p_i$ and $p_j$, across all loci, between two individuals, $i$ and $j$. This value is then averaged across all individuals within a particular distance class. A value of zero is expected for a population at Hardy–Weinberg equilibrium, a positive value indicates a higher than expected degree of homology and a negative value indicates a lower than expected degree of homology (Loiselle et al., 1995).

Once clones were identified, a second data set was prepared in which each multilocus genotype was only represented once. This culled data set was used for calculation of $F_{ST}$ values and for principal components analysis (PCA) in order to analyse genetic differentiation among genets and sites. The software GenAIEx 6-2 was used for these analyses. Visualization and plotting of statistical output were performed using PAST version 1-89 (Hammer et al., 2001).

**RESULTS**

The five microsatellite loci were highly polymorphic and provided excellent resolution for genet identification. At each locus, each individual had only one or two alleles, consistent with previous reports of diploid segregation of microsatellites in this octoploid species (Ashley et al., 2003). The number of alleles per locus ranged from six for ARSFL-99 to 11 for FV/9. The Green Bay Road Site had the lowest allelic diversity, with a mean of 3.6 alleles per locus, and the Chipilly Woods site had the highest, with a mean of 5.8 alleles per locus. PI was extremely small at each site, <0.0003 (Table 1). The simulation-based clone detection power tests also confirmed that the five loci were sufficient for the detection of all unique genotypes among the individuals sampled. The simulated genotype detection difference between the fourth and fifth loci ranged from an average of <0.0001 additionally detected genotypes at the Green Bay Road site to an average of 0.067 additional genotypes at the McDonald Woods site. The tests of sampling effort indicated that the average simulated genotype detection difference between $n$ and $n-1$ ranged from <0.001 additional genotypes at the Green Bay Road site to 0.021 additional genotypes at the Chipilly Woods site. These results suggest that even at the Chipilly Woods site, approx. 48 more individuals would need to be sampled to find an individual possessing a new genotype. Thus we are confident that any two individuals sharing the same genotype across these loci are derived from clonal recruitment and that our sampling intensity fully characterized the genotypic diversity of these transects.

The three populations differed in their reproductive mode and spatial genetic structure (Table 1). The Green Bay Road site had very low genotypic diversity, and was comprised of only two large clones (Fig. 1A), both hermaphrodites. These two nearly equally sized clonal groups (4.19 and 3.65 m across) showed the lowest levels of spatial segregation of the three sites, with strong boundaries and no overlap. Genotypic evenness, $E$, was very high (0.999).

The McDonald Woods site had six multilocus genotypes comprised of three multiramet clones and three genotypes that were each found in a single ramet (Fig. 1B). The multiramet individuals were all hermaphrodites, but the gender of the single ramet clones could not be determined because they were not flowering at the time of sampling. The clones at McDonald Woods varied the most in size among the three sites, with multiramet clones ranging in size from 1.45 to 9.05 m (Table 1), and $E$ was 0.648. The autocorrelation analysis (Fig. 2) showed that McDonald Woods had an intermediate level of spatial organization, with significantly positive $f_{ij}$ values extending to 3.1 m and significantly negative $f_{ij}$ values beyond 8.6 m (Fig. 2).

The Chipilly Woods site had the highest genotypic diversity and was the least spatially structured of the three sites. The specimens collected at this location were identified as 19 distinct multilocus genotypes, 14 of which were represented by a single ramet (Fig. 1C), and $E$ was 0.563. Of the five observed clones, one was relatively large, with 13 sampled ramets extending 4 m along the transect, while all of the remaining clones consisted of pairs of ramets ranging in size from 25 to 75 cm along the transect. One of these was the only confirmed female in our study. The presence of a number of small clones and several single genets results in the weakest spatio-genetic structure of the three sampled populations (Table 1 and Fig. 2). Significant, high levels of $f_{ij}$ were found up to 1.6 m and significantly low levels of $f_{ij}$ beyond 3.7 m. The heterogeneous nature of this site is reflected in the lowest (still significantly non-zero) rate of change in observed probability of co-ancestry over distance of the three populations (Fig. 2).

Although the three sites were within 3 km of each other, they were genetically differentiated. Each site had private alleles not found at the other two sites, ranging from four for Green Bay Road to 11 for Chipilly Woods. Pairwise $F_{ST}$ values were 0.131 for the two closest sites, McDonald Woods and Green Bay Road. $F_{ST}$ was 0.181 between Chipilly Woods and McDonald Woods, and 0.121 for the

**Table 1. Genetic and clonal diversity by site**

<table>
<thead>
<tr>
<th>Site (n)</th>
<th>PI</th>
<th>Genotypes</th>
<th>Large clones</th>
<th>PD</th>
<th>$D$</th>
<th>Mean clone size ± s.d. (m)</th>
<th>Autocorrelation slope, mean (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green Bay Road (31)</td>
<td>5.9 x 10^{-5}</td>
<td>2</td>
<td>2</td>
<td>0.0645</td>
<td>3.92 ± 0.382</td>
<td>-0.1036 (−0.1257 to −0.0891)</td>
<td></td>
</tr>
<tr>
<td>McDonald Woods (29)</td>
<td>1.1 x 10^{-4}</td>
<td>6</td>
<td>2</td>
<td>0.207</td>
<td>5.15 ± 3.80</td>
<td>-0.0397 (−0.0482 to −0.0313)</td>
<td></td>
</tr>
<tr>
<td>Chipilly Woods (35)</td>
<td>2.1 x 10^{-4}</td>
<td>19</td>
<td>1</td>
<td>0.543</td>
<td>1.19 ± 1.58</td>
<td>-0.0244 (−0.0311 to −0.0193)</td>
<td></td>
</tr>
</tbody>
</table>

$n$ is the sample size, PI is the cumulative probability of identity for all five loci, genotypes are the number of multilocus genotypes found, large clones are those identified in ≥5 ramets, PD is the proportion of distinguishable multilocus genotypes, $D$ is the complement of the Simpson index, clone size is calculated along the transect axis, and the autocorrelation slope is the regression of the likelihood of genetic identity against pairwise distance.
Chipilly Woods vs. Green Bay Road comparison. For this analysis we included each multilocus genotype only once. This reduced the sample size to six for McDonald Woods and to two for the Green Bay Road site, so that population level values such as $F_{ST}$ may not be reliable. We therefore used an individual-based PCA to examine genetic structure (Fig. 3). The first two principal components explained 54.9% of the variation in the data (34.9% and 20.0%, respectively). Multilocus genotypes were clearly segregated by site, with all Chipilly Woods samples having negative values for the first principal component, and, with one exception, the McDonald Wood genotypes having high positive values for the first principal component. The two Green Bay Road site genotypes were intermediate.

**DISCUSSION**

In this study we found that wild strawberry, *F. virginiana*, undergoes both clonal and sexual reproduction at our study sites in northeastern Illinois. The five DNA microsatellite loci used for our analysis of clonal structure provided excellent resolution for identifying clones. Microsatellites provide higher levels of resolution for clonal identification than other markers including allozymes and amplified fragment length polymorphisms (AFLPs), yet in Silvertown’s (2008) recent review of 356 studies of population structure in clonal plants, only 23 (9%) used nuclear microsatellites, and most of those studies examined clonality at a single site. Our results revealed that even sites in close proximity may have strikingly different clonal structure. At the Green Bay Road...
site clonal recruitment predominates, and all ramets were derived from one of two large clones. In contrast, the Chipilly Woods site had much higher clonal diversity, with 19 distinct multilocus genotypes, with most represented by a single sampled ramet.

The genotypic structure of clonal plant populations such as *F. virginiana* will be determined by the balance between the loss or spread of established clones and recruitment of new genotypes through sexual reproduction. The observed differences in clonality among our sites may reflect underlying fine-scale heterogeneity in ecological factors that can influence this balance, such as resource availability, disturbance frequency and intensity, herbivory, pollination and seed dispersal. It is interesting to note, however, that these sites are <3 km apart, and two are separated by only 630 m; prior to European settlement (circa 1850), they were part of a single, large continuous tract of forest. It is possible that dramatic landscape alterations over the past 150 years may have shifted ecological processes that balance sexual and asexual reproduction from site to site. Although our study cannot distinguish among these two scenarios (as well as other possibilities), our results indicate that it will be difficult to extrapolate patterns of genotypic structure of clonal plants to larger spatial scales based on patterns at one or a few sites, at least until the processes underlying clonal diversity are better understood.

Some authors have suggested a temporal component to sexual vs. clonal reproduction. Since colonization of new or disturbed habitats will primarily be through seed, sexual reproduction will be favoured during colonization, gradually replaced by clonal reproduction as habitat patches mature (Piquot et al., 1998). In a recent review of asexual reproduction in clonal plants, Silvertown (2008) found a significant decline in G/N with population age, indicating that the absence of disturbance favours asexual over sexual reproduction. Reusch (2006) tested effects of disturbance in eelgrass (*Z. marina*) and found that it increases genetic diversity of clones at intermediate disturbance levels. If similar processes hold for *F. virginiana*, it would suggest that the Green Bay Road site is the oldest and/or least disturbed population and the Chipilly Woods is the youngest or most disturbed. It is possible that mowing and burning by the railway to maintain an open right-of-way has contributed to the high diversity and seed recruitment at Chipilly Woods.

Our results agree with previous reviews that have shown that clonal plant species generally do not have reduced levels of genetic diversity as measured by allelic diversity or heterozygosity (Ellstrand and Roose, 1987; Hamrick and Godt, 1996). Genotypic diversity over all three sites was considerable. The growth pattern of *F. virginiana* was one in which solid clumps of the same genetic individual predominate at sites where clonal growth is frequent. Single multilocus genotypes were not scattered within sites nor were any shared among sites. Local genotypes (restricted to a single population) as well as multiclonal populations, as we report here, are prevalent in clonal species (Ellstrand and Roose, 1987; Widén et al., 1994).

The range in clonal diversity among our sites was striking. Our value for the mean proportion of distinguishable genotypes (PD) values ranged from 0.0645 to 0.543 among sites (Table 1), spanning much of the range of values for clonal plant species reported in previous reviews (Ellstrand and Roose, 1987; Widén et al., 1994; Diggle et al., 1998). Such a wide range in genotypic diversity among sites is not uncommon in clonal species (Arnaud-Haond et al., 2007). Together, Ellstrand and Roose (1987) and Diggle et al. (1998) include 22 studies of clonal plants where D values are available for multiple populations. Of these, 16 (73%) report ranges in these
values that are large, as great as the range we found in *F. virginiana* (Table 1), and includes species that have varying modes of asexual reproduction. Many of these studies have been conducted on larger spatial scales than ours, but extensive variation in genotypic diversity among adjacent sites has also been reported in other clonal plant species. Interestingly, in a study of a closely related species, *Fragaria chiloensis*, microsites within 900 m had *D* values that ranged from 0.00 to 0.80 (Alpert *et al.*, 1993). In the alpine perennial *Polygonum viviparum*, *D* values ranged from 0.30 to 0.89 at three adjacent sites (Diggle *et al.*, 1998). These studies, together with the findings reported here for *F. virginiana*, indicate that clonal and sexual reproduction show considerable plasticity in many plant species, and fine-scale variation in clonal diversity may be quite common.

Although *F. virginiana* is a gynodioecious species, our populations were dominated by hermaphrodites. Among the genets that were flowering at the time of sampling (22 of 27), only one was identified as a female. Sex ratios in *F. virginiana*, as well as many other gynodioecious plant species, have been associated with environmental harshness, with females more prevalent on drier or nutrient-poor sites (Ashman, 1999). Theory suggests that hermaphrodites produce fewer fruits and seeds under limiting resources, and are functionally ‘more male’ at low resource sites where females are more common (reviewed in Ashman, 2006). The low proportion of females suggests that conditions that have been reported to co-vary negatively with sex ratio, such as water or nutrient stress, are not promoting reproductive specialization at our sites.

In populations such as the Green Bay Road site, where clonal growth predominates, effective population size is much lower than census population size. Hermaphrodites are capable of self-pollination in *F. virginiana* and, when opportunities for outcross pollination are diminished, inbred offspring may suffer fitness declines from inbreeding depression. Inbreeding depression for traits including vegetative size and flowers per plant have been shown for *F. virginiana* in controlled crosses (Botham *et al.*, 2009). It may be that once a population is dominated by one or a very few clones, opportunities for sexual recruitment are further limited by inbreeding.

As mentioned above, all three sites were part of a single forest tract prior to European settlement, and two sites, McDonald Woods and Green Bay Road, are still within a continuous remnant forest. Yet all three sites are well differentiated genetically, as evidenced by private alleles, high *F*<sub>ST</sub> values, and PCA (Fig. 3), and no genets were shared between sites. With the exception of one genet from McDonald Woods, individuals within sites clustered together in the PCA (Fig. 3), further indicating that genetic diversity is largely partitioned among sites. Geographic distance does not predict genetic distance; the two geographically most distant populations, Chipilly Woods and Green Bay Road, actually fall closer together on the PCA plot than the contiguous Green Bay Road and McDonald Woods sites. These high levels of genetic differentiation suggest that gene flow among sites is extremely limited. *Fragaria virginiana* is pollinated by small solitary bees, butterflies and flies (Ashman and Diefenderfer, 2001), and pollinators show a preference for hermaphroditic over female flowers (Ashman, 2000). We do not know what barriers to pollen movement are operating, especially between the two CBG sites. Strawberry fruits would probably be eaten by a variety of birds and small mammals, but the genetic differentiation we found among sites suggests that seed dispersal by frugivores is also limited. We did not follow fruit set in our populations, but fruit set by hermaphrodites is reportedly low in this species (Ashman, 2003; Case and Ashman, 2007), so the absence of females may limit opportunities for gene flow via seeds.

In summary, fine-scale variation in reproductive mode will have important consequences for the ecology and evolution of clonal plants. Using microsatellites to identify genets, we found fine-scale variation in reproductive mode for *F. virginiana* among three nearby sites, which ranged from primarily sexual recruitment to primarily clonal recruitment. We also found fine-scale genetic differentiation, suggesting that gene flow among sites is extremely limited.

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**LITERATURE CITED**


