ABSTRACT

In established white clover (Trifolium repens L.) populations, seedling recruitment is rare and plants spread by clonal growth, which could lead to low intraspecific genetic diversity. To assess how populations of white clover maintain high genetic variability in grazed swards during a growing season, we examined spatial and temporal changes in white clover clones by identifying genotypes by RAPD (random amplified polymorphic DNA) profiles. Trifoliate leaf samples were taken from plants at up to 37 specific sampling points in each of four 2.8-m² permanent quadrats in a grazed pasture from April to October 1997. RAPD profiles (genotype) based on 28 markers were determined for genomic DNA prepared from leaf samples. The number of sampled clones per quadrat ranged from zero to 10 and the sampled members per clone ranged from two to 34 over the growing season. Some clones were found more than once. During the study, numbers of clonal members increased or decreased, clones were found on one to three dates, and clones were found in one to two quadrats. The clonal diversity index D (probability that any two sampled plants have different genotypes) ranged from 0.89 to 0.99 for the eight dates, showing that genotypic diversity of populations was affected by number of clones and number of clonal members. We report here the first use of dominant RAPD markers to document dynamic changes of white clover population structure. These data show that white clover clones undergo dynamic spatial and temporal variability at a local scale, which accounts for much of the genetic diversity within field populations.

As a plant produces more stolons and stolon branches, it expands in area and is made up of clonal members. Because of decay of older stolons and environmental disturbance, a clone frequently fragments into smaller clones. Therefore, physically separate, but genetically identical clonal plants may be found at the subhectare (local) and hectare (field) scales. In theory, any clone could potentially become a dominant genotype, producing patches covering a large area within grassland (Cahn and Harper, 1976; Harberd, 1963), and thus reduce the genetic variability within the white clover population.

Despite the potential for one clone to dominate a white clover population, the reported area of clonal patches in the field is from several centimeters to several meters in maximum width. Cahn and Harper (1976) did not find high genetic variation and local domination by one or more clones. The magnitude of genetic variation among phenotypically distinct clones sampled in a 50-yr-old grassland in North Wales was comparable to that expected between clonal “populations taken from distinctly different environments” (Burdon, 1980). Gustine and Huff (1999) also found high genetic variation within and among white clover populations at 18 locations in three northeastern U.S. states using RAPD markers.

Previously, Gustine and Huff (1999) found few white clover clones when they sampled plants at 10-m intervals and they found dramatic changes in genetic makeup of populations within a period of 6 wk. Since white clover clones occur at much smaller scales, we wanted to investigate clonal dynamics at a scale of 20 to 30 cm and we wanted to know whether white clover populations change over much shorter time intervals of 2 to 4 wk. RAPD profiles have been used to characterize genetic variability, clonal structure, and population structure in several plant species (Buso et al., 1998; Huff et al., 1998; Palacios and Gonzales-Candelas, 1997; Sydes and Peacock, 1998). Our objective was to quantify spatial and temporal changes in genetic variation at the local scale over a growing season in a rotationally stocked sward.

MATERIALS AND METHODS

White clover populations used in this study formed part of a managed permanent pasture, in the ridge and valley physiographic region of central Pennsylvania (40°38’ N, 77°38’ W; 231 m asl). The grazed sward, on a Hagerstown silty clay loam (fine, mixed mesic Typic, Hapludalf; USDA, 1981), was not treated with chemical fertilizers. The cumulative rainfall during March through October was 711 mm (data from the Lewistown, PA, weather station, 9.7 km to the south), which was 8 mm below normal. The average temperature for that time was 14.8°C, 0.8°C below normal (NOAA, 1997). The field capacity for the Hagerstown soil was 0.16 to 0.24 m³ m⁻³ of

Abbreviations: PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA.

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Fig. 1. The sampling grid design used for collecting white clover samples from permanent quadrats. PIN, permanent steel stakes in the pasture paddocks used to place the steel grid. The numbered positions on the concentric circles and the center indicate where leaves were collected, if present.

White clover was not seeded at the time grazing began in 1991; the populations studied in 1997 presumably developed from the viable seed pool of naturalized clover. The grassland consisted of mostly naturalized white clover, tall fescue (Festuca arundinacea Schreb.), quackgrass (Elytrigia repens L.), and blue grass (Poa pratensis L.), with about 20% of the forage in weedy species. In each of 8 yr of grazing, 60 dairy cows (Bos taurus) grazed the pasture on a 14-d rotation from April through October on 14 paddocks of 0.4 ha each.

Four permanent 2.8-m² quadrats were established in two paddocks. Two quadrats were in a flat, well-drained paddock (Quadrats 1 and 2) and two were in a second paddock (Quadrats 3 and 4) about 100 m away in a drainage swale. The location and orientation of each quadrat in a paddock was defined by a pair of 30-cm-long steel pins driven into the ground flush with the soil surface, 1.67 m center to center. Because of differences in drainage, soil moisture was measured at each sampling date, but soil moisture differences were not found among quadrats and sampling dates.

A 1.67- by 1.67-m portable steel grid subdivided with steel rods into 110 15.2- by 15.2-cm subquadrats (Fig. 1) was positioned to the west over the pins of each quadrat at sampling. The grid was used to locate sampling points defined by the center of the quadrat (sampling point no. 19) and numbered sampling points on four concentric circles with radii of 22.8, 45.6, 68.4, and 85.6 cm, respectively (Fig. 1). The grid was in place only during each date of sampling. Leaf samples if present were collected for RAPD analyses at 37 sampling points (Fig. 1) in the quadrats on eight arbitrarily selected dates: every 2 wk during rapid sward growth (28 April, 15 and 28 May, 10 June) and every 4 to 6 wk during slower sward growth (24 July, 19 August, 16 September, and 16 October). If a selected leaf was connected to a stolon at a sampling point, a sample of one to four trifoliolate leaves was taken from that stolon. Sometimes more than one stolon was present at a sampling point, but the leaf selected determined which stolon was sampled. If no stolon was within 2 cm of the sampling point, a sample could not be taken. Thus, we often collected fewer than 37 samples from the quadrats. Only 18 and 19 samples were collected from the Quadrats 3 and 4 on 28 April, respectively, but 21 to 37 samples were collected on the other dates and quadrats. To characterize the vegetation in the quadrats, floristic composition and bare soil areas (sward gaps) were visually estimated in 10 subquadrats that were not sampled for white clover.

Leaf samples were placed on ice after collection, returned to the laboratory, and their fresh weights recorded. The samples were placed in 2 mL microcentrifuge tubes, frozen, and lyophilized. Genomic DNA was prepared from 25 to 100 mg of plant tissue as described by Gustine and Huff (1999); 100 mL of ROSE buffer (Steiner et al., 1995) was used for each 25 mg (fresh weight) of powdered tissue. Polymerase chain reactions (PCR) of genomic DNA, gel electrophoresis, and ethidium bromide staining were performed according to Gustine and Huff (1999). Gels were documented with a Kodak DC120 digital camera and bands detected with Kodak 1D Image Analysis Software (v. 2.1, Eastman Kodak Co., Scientific Imaging Systems, Rochester, NY). Genomic DNA in prepara-
tions that did not yield usable RAPD profiles was precipitated in 2 M NaCl to remove polysaccharides (Sambrook et al., 1989). Purified DNA from this procedure provided usable RAPD profiles. Because of the time required to perform RAPD analyses, a single DNA extraction was done for each sample; however, PCRs were performed at least twice on each sample and only repeatable bands were scored.

The three primers (OPA08, OPB14, and OPH12; Operon Technologies, Inc., Alameda, CA) used by Gustine and Huff (1999) produced 28 distinct polymorphic bands (markers) that were reproducibly scorable by three people. Marker fragment bp size was determined by comparison with the 100-bp ladder from Life Technologies (Gaithersburg, MD). Polymorphic fragment sizes ranged from 0.275 to 1.45 kb in length and they were scored as present (1) or absent (0). RAPD profiles of polymorphic markers were compared by the RAPD distance v. 1.04 freeware package (Armstrong et al., 1996), which listed samples with identical RAPD profiles (genotypes). For this report, two or more white clover samples with identical RAPD profiles were defined as clonal; samples with a RAPD profile that was found only once were defined as nonclonal.

The clonal diversity index (D) of Ayres and Ryan (1999) was calculated for white clover populations according to their equation, $D = 1 - \sum [r_i (r_i - 1) / R (R - 1)]$, where $r_i$ is the number of members sampled from Genotype $i$, $R$ is the number of different genotypes sampled from the quadrat, and genotype is determined by the RAPD profile. Since this index is the complement of the Simpson (1949) diversity index, $D$ is the probability that any pair of white clover samples have different RAPD profiles. It is also a measure of the effect of clonal plants on genetic diversity in a population. When $D = 1.0$, no clones are present in the population, and genetic diversity is high. When $D = 0$, clones present in the population have many members sampled, and genetic diversity is lower.

Repeated measures ANOVA was applied to soil moisture data, number of sampled clones per quadrat, number of sampled clones per area of sward gaps, number of members per sampled clone, and number of genotypes per quadrat.

**RESULTS**

We collected 954 (Table 1), or 81%, of the maximum number of white clover leaf samples that could have been taken during the study (had there been a stolon with a trifoliate leaf at each of the 37 sampling points in the four quadrats). Over the eight sampling dates, 122 sampled clones were identified from the four quadrats (Table 1). The number of polymorphic DNA markers defining a sampled clone ranged from 2 to 17 for all sampling dates, except for 34- and a 27-member clones in September and October (Table 1), which were each identified by a single polymorphic marker. Of the 954 samples taken, 571 were represented in the 122 sampled clones and the remaining 383 samples did not match any other RAPD profile. Thus, there were 505 genotypes (122 clones + 383 nonclonal samples) identified in the study.

Table 1 summarizes the numbers of samples, genotypes, and sampled clones taken from the four quadrats during the growing season. The clonal diversity index $D$ was calculated for the combined quadrat populations for each date (Table 1). Some of the individual quadrats had very low $D$ values on some sampling dates. In Quadrat 1, $D$ was 0.38 in October and in Quadrat 2, $D$ was 0.27 in September and October (data not shown). These populations had 20 or more members sampled from a clone. In 25 instances, population $D$ values for populations in individual quadrats were greater than 0.8 and the number of members in a quadrat were generally less than 10 (data not shown). The $D$ values for combined populations were lower in September and October (Table 1) than those for the previous sampling dates, and corresponded to sampled clones with the most members.

The number of sampled clones per quadrat varied from 0 to 15 (data not shown) with a mean of 3.8 (SE = 1.9). The clones contained 2 to 34 members per clone with a mean of 4.1 (SE = 4.3). The number of nonclonal samples per quadrat ranged from 1 to 28, with a mean of 11.8 (SE = 8.3). Statistical analysis did not reveal significant differences in these variables among quadrats and dates nor were changes in these variables associated with white clover cover (data not shown). These variations during the study were not consistent over quadrats or dates.

In five of the 122 sampled clones, at least one member was found more than once on two or more dates in the same quadrat (Fig. 2, 3A, and B). Seven clones had at least one member found more than once on the same or different dates in different quadrats (Fig. 3B, C, and D, and Fig. 4). Sampled clones were also found at non-successive sampling dates in six instances. None of the 12 clones that were found more than once remained in place from one date to the next; however, in one instance the same genotype was found at the same sampling point on two different dates (Fig. 2B). The number of clonal members both increased (Fig. 2D, Fig. 3A, and Fig. 3B) and decreased (Fig. 2B), and numbers of clonal members that changed on the second date, changed in the opposite direction on the third date (Fig. 2A and Fig. 3B). On the basis of the positions of sampled members in Fig. 2 to 4 and the quadrat dimensions in Fig. 1, the physical distances covered by white clover sampled clones were about 15 cm for a clonal member by itself (Fig. 3 and 4) to 140 cm in length for 14 clonal members (Fig. 2D).

The white clover cover was highly variable within each quadrat at all sampling dates of the study and

<table>
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<tr>
<th>Date</th>
<th>Samples</th>
<th>Genotypes</th>
<th>Sampled clones</th>
<th>Largest clone Members</th>
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<td>94</td>
<td>59</td>
<td>11</td>
<td>14</td>
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<tr>
<td>15 May</td>
<td>101</td>
<td>49</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>28 May</td>
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<td>15</td>
<td>15</td>
</tr>
<tr>
<td>10 Jun</td>
<td>137</td>
<td>104</td>
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<td>24 Jul</td>
<td>108</td>
<td>89</td>
<td>12</td>
<td>6</td>
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<td>19 Aug</td>
<td>125</td>
<td>53</td>
<td>24</td>
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<td>16 Oct</td>
<td>133</td>
<td>24</td>
<td>13</td>
<td>27</td>
</tr>
</tbody>
</table>

† Clonal diversity index (Ayres and Ryan, 1999) is the complement of the Simpson diversity index (Simpson, 1949), $D = 1 - \sum [r_i (r_i - 1) / R (R - 1)]$, where $r_i$ is the number of clonal members sampled from genotype $i$ and $R$ is the number of genotypes sampled from the quadrats. The index value is the probability that any two samples will have different genotypes.

‡ Two or more samples with identical genotypes.

§ Number of members in the largest clone.
ranged from 19 to 69% (data not shown). At Quadrats 1 and 2, white clover cover for 28 May and 10 June was 44 to 69%, compared with 19 to 30% for the other sampling dates. Similarly, white clover cover ranged from 27 to 55% on 28 May and 10 June at Quadrats 3 and 4, compared to 8 to 37% for the other dates. The mean value for sward gap areas in quadrats ranged from 0 to 49%; however, the values recorded for subquadrats were highly variable and the area of sward gaps sometimes was as high as 95% within a subquadrat. Few seedlings were observed when leaf samples were collected, and none were white clover.

**DISCUSSION**

We report here the first use of dominant RAPD markers to quantify temporal and spatial genotypic changes in white clover population structure. Our results are consistent with previous reports of white clover genetic variation in pastures. Earlier work established the ge-
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Fig. 4. Location of clonal plants in Quadrats 2, 3, and 4 and the dates sampled. Symbol description as in Fig. 2. Clonal members were found in more than one quadrat.

netic variability of this species on a regional (Gustine and Huff, 1999) and pasture scale (Burdon, 1980). In this study, we characterized white clover genetic variation and clonal diversity in 2.8-m² quadrats at the local scale. We assessed spatial and temporal genetic changes within these white clover populations and followed changes in physical position of clonal members and of sampled clones (Fig. 2 to 4). We did not attempt to sample every leaf in a quadrat because analyzing that number of samples would have been unmanageable. Although this would have shown the exact distribution of each white clover clone in the area, complete defoliation would have defeated one of our objectives—to examine temporal changes in populations in a grazed sward.

One weakness of our sampling design was that if two or more stolons were present at a sampling point on two or more sampling dates, there was no way to assure that the same stolon was sampled every time. Because of this, we may have underestimated the number of clonal members and/or the areas covered by sampled clones. Even so, our data indicated major changes in the number of clonal members, in the surface area covered by clones, and in the location of clones during the growing season.

Our results showed that when sampled clones reappeared in a quadrat, positions of members in the quadrat had changed and the number of members in the clone had changed (Fig. 2–4). The distances covered by white clover sampled clones were not very large and ranged from 15 to 140 cm. In one case, identical RAPD profiles were found twice at the same sampling point in a quadrat (Fig. 2B). The clonal changes we observed were continuous through time.

Periods of dormancy or simply not sampling the clone could account for intermittent appearance of sampled clones. In fact, Harberd (1963) observed that dormant white clover plants under severe drought conditions returned to normal growth when soil moisture was restored. This may suggest that water stress is one factor controlling white clover dormancy.

The temporal and spatial changes we found for sampled clones and changes in the number of clonal members could have been driven by several factors, including (i) a combination of stolon extension and production of new ramets (exploration), (ii) death of ramets and physical damage to stolons leading to fragmentation, and (iii) a process of alternating growth and dormant periods. These factors were partly reflected in the finding that white clover cover and sward gaps in the quadrats were highly variable during the growing season. With these dynamic changes in place, the genotypic composition of the population would have appeared to change rapidly over a growing season, whether sampling was repeated at the same point or done randomly as did Gustine and Huff (1999). Some of these factors could be controlled by dynamic biotic and abiotic environmental processes, which influenced growth and dormancy of stolons. Examples of these processes include changing climatic conditions and edaphic properties encountered during stolon exploration, and regrowth following herbivory (Chapman, 1983).

Nonclonal genotypes were found at each sampling date and in each quadrat. They could have originated from previously unsampled clonal plants, previously dormant stolons, or from newly established seedlings. Thus, under rotational stocking, identifiable clonal patches and nonclonal samples were continuously changing, presumably because of responses of stolon growing tips to changing environmental factors, sam-
plling of different clones, and seedling recruitment. Consequently, the number of clonal members and the genotype of a leaf collected at given spot in grassland would have changed often throughout the growing season, but primarily through the dominant means of white clover persistence—clonal growth. Our data show that white clover clones undergo spatial and temporal variability at a local scale, which accounts for much of the genetic diversity within populations.

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REFERENCES


