Inter-simple sequence repeat (ISSR) variation in three populations of *Gaura neomexicana* ssp. *coloradensis* (Onagraceae), F.E. Warren Air Force Base, Cheyenne, Wyoming

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INTER-SIMPLE SEQUENCE REPEAT (ISSR) VARIATION IN THREE POPULATIONS OF GAURA NEOMEXICANA SPP. COLORADENSIS (ONAGRACEAE), F.E. WARREN AIR FORCE BASE, CHEYENNE, WYOMING

Dorothy E. Tuthill and Gregory K. Brown

ABSTRACT—We investigated genetic variation within and between 3 populations of Gaura neomexicana ssp. coloradensis, a federally listed threatened species, using inter-simple sequence repeats. The data matrix included 24 individuals scored for 88 bands, with 3.4% missing data. Cluster analysis showed that members of the 3 populations are very similar and are intermixed in the phenogram. Principal coordinate analysis indicated that members of the Crow Creek population are different from the Diamond Creek and "unnamed drainage" populations. This could be the result of historic differences, current changes in vegetation at the Crow Creek site, or an artifact of limited sampling. In any case, for management purposes it is best to preserve the dwindling population at Crow Creek as well as the thriving populations at the other 2 sites.

Key words: Gaura neomexicana ssp. coloradensis, inter-simple sequence repeats, ISSR, populations, variation, management, cluster analysis, principal coordinate analysis, DNA quality.

Colorado butterfly plant (Gaura neomexicana Wooton ssp. coloradensis [Rydb.] Raven and Gregory) is a federally listed threatened species endemic to north central Colorado, southeastern Wyoming, and a small, adjacent portion of western Nebraska. Currently, 16 extant populations are known, a decrease from 26 locations recorded in the past (Fertig 2001). Two of the largest populations are within the F.E. Warren Air Force Base (WAFB) in Cheyenne, Wyoming, and are managed within the Colorado Butterfly Plant Research Natural Area.

The subspecies is restricted to drainage bottoms, low alluvial terraces, and abandoned stream channels with short, sparse vegetative cover. The plants do not thrive where shrubs such as Salix spp. or noxious weeds like Cirsium arvense (L.) Scop. or Euphorbia esula L. have become dominant. Annual surveys of the Colorado butterfly plant have been conducted on WAFB since 1984, so that distribution, size, and trends within the populations there are well understood (Fertig 2001 and citations within). In addition, the U.S. Air Force has sponsored research on weed management (Floyd 1995a, Hollingsworth 1996, Munk 1999, Heimstra and Fertig 2000), population genetics (Brown 1999, 2000), and demographic structure and survivorship (Floyd 1995b, Floyd and Ranker 1998) of the Colorado butterfly plant.

The 3 populations selected for study are located on, and designated as, Crow Creek (CC), Diamond Creek (DC; a tributary of Crow Creek), and "unnamed drainage" (UD; Fig. 1). The first 2 populations are nearly contiguous at the confluence of the 2 streams, while the 3rd site contains an ephemeral stream that drains into Crow Creek, somewhat downstream of the other 2 populations. Diamond Creek and "unnamed drainage" populations were sampled and numbered from west to east along the drainages, but the Crow Creek population was sampled from just a single site, the only apparent remaining population in the Crow Creek area. The 3 populations combined extend approximately 3 km from north to south and 2 km east-west and may represent remnants of a single continuous population that existed before settlement. This area, and Crow Creek in particular, are also within the range of Freibie’s jumping mouse (Zapus hudsonius preblei), another federally listed threatened species. The purpose of this study was to determine the extent of inter- and intra-site variation in
METHODS AND MATERIALS

Previous experience (Brown 1999) had shown the procurement of PCR-quality DNA from *Gaura* to be problematic, due to the presence of mucilage that co-precipitated with the DNA. Therefore, the initial portion of this study focused on ways to eliminate or reduce the amount of mucilage through modification of tissue-collection methods, DNA-extraction methods, and post-extraction cleanup. All leaf samples for this preliminary study were collected from the "unnamed drainage" site, 1 September 1999. Tables 1 and 2 contain complete descriptions of the method modifications.

Once the trials were complete, we collected samples for the major part of the study. For each of the 3 populations, we collected 50 leaf samples, each from a different plant, on 7 September 1999. Leaves were immediately placed in silica gel, stored at room temperature for 24 hours, then frozen (option 1, Table 1). DNA extraction followed the standard 2X CTAB protocol (Doyle and Doyle 1987), followed by cleanup with Qiagen DNeasy plant kit (Qiagen, Inc., Valencia, CA). Cleanup was repeated as necessary until no more mucilage was apparent in the sample. Typically this required 1 or 2 applications.

The 25 μL PCR reactions consisted of 1X buffer (Sigma; 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.0001% gelatin), additional 50 nmoL MgCl₂, 8 nmoL dNTPs, 6 nmoL primer, 10 μg BSA, 5 μL saturated betaine, 1 U Taq polymerase, and 2 μL DNA. Thermal cycling parameters were initial denaturing for 2 minutes at 96°C, followed by 40 cycles of 30 seconds at 96°C, 45 seconds at 44°C, and 90 seconds at 72°C, with a final elongation at 72°C for 10 minutes. Primers were purchased from the University of British Columbia, Nucleic Acid-Protein Service Unit. We tried many primers and selected 12 based on the number of scorable bands generated (Table 3). A number of the primers, including all based on repeats of AT, failed to produce any bands.
TABLE 1. Leaf tissue-collection methods attempted for *Gaura neomexicana* ssp. *coloradensis*. All collection trials used plants from the "unnamed drainage" population, FE, Warren AFB. Only healthy, undamaged leaves were used. Each trial was replicated twice. All leaves were collected midday, 1 September 1999.

<table>
<thead>
<tr>
<th>Method</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Leaves were placed in a clean 50-mL plastic, screw-top centrifuge tube. Tube was filled with 200-mesh silica gel (desiccant), lid was firmly attached, and tube placed in a styrofoam ice chest to keep cool and out of direct sunlight. After leaves were fully dried (ca. 24 hours), tubes were placed in a lab freezer (-20°C) and held there until extraction.</td>
<td></td>
</tr>
<tr>
<td>2. Leaves were placed in a clean 50-mL plastic, screw-top centrifuge tube. Tube was filled with 95% ethanol containing 100 mM EDTA, lid was firmly attached, and tube placed on wet ice in ice chest. Immediately upon return to lab, tubes were transferred to a lab freezer (-20°C) and held there until extraction.</td>
<td></td>
</tr>
<tr>
<td>3. Leaves were placed in a field press while in the field. Immediately upon return to lab, press was placed on plant drier and kept there until leaves were fully dry (ca. 36 hours). Dried leaves were kept at room temperature in a lab cabinet until extracted.</td>
<td></td>
</tr>
<tr>
<td>4. Leaves were placed in a small, sandwich-sized zip-type plastic bag. The bag was closed and placed on wet ice for transport back to the lab. Immediately upon return to lab, leaves were extracted for DNA.</td>
<td></td>
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<tr>
<td>5. Leaves were placed in a small, sandwich-sized zip-type plastic bag. The bag was closed and placed on wet ice for transport back to the lab. Immediately upon return to lab, bags were transferred to a lab freezer (-20°C) and held there until extraction.</td>
<td></td>
</tr>
<tr>
<td>6. Leaves were placed in a small, sandwich-sized zip-type plastic bag. The bag was closed and placed on dry ice for transport back to the lab. Immediately upon return to lab, leaves were extracted for DNA.</td>
<td></td>
</tr>
<tr>
<td>7. Leaves were placed in a small, sandwich-sized zip-type plastic bag. The bag was closed and placed on dry ice for transport back to the lab. Immediately upon return to lab, bags were transferred to a lab freezer (-20°C) and held there until extraction.</td>
<td></td>
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<tr>
<td>8. Leaves were ground to a fine powder in liquid nitrogen while in the field, and the powder transferred to a clean 50-mL plastic, screw-top tube containing 10 mL of 2X CTAB extraction buffer. Tube was immediately placed on dry ice for transport back to the lab. Immediately upon return to lab, the extraction was completed.</td>
<td></td>
</tr>
<tr>
<td>9. Leaves were ground to a fine powder in liquid nitrogen while in the field, and the powder transferred to a clean 50-mL plastic, screw-top tube containing 20 mL of 2X CTAB extraction buffer. Tube was immediately placed on dry ice for transport back to the lab. Immediately upon return to lab, the extraction was completed.</td>
<td></td>
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</table>

Initially, all 150 samples were subjected to PCR with 3 different primers. Because the level of variation seen in these analyses was more or less uniform across the 3 populations, and because of logistic problems associated with analysis of that number of samples, we decided that a random sample of 8 individuals from each population would adequately sample variation. The reduced sample size allowed us to evaluate more primers, and therefore more polymorphism, in the given time frame.

ISSR reactions were visualized following electrophoresis on 1.5% agarose stained with ethidium bromide and photographed on a UV transilluminator. We scored bands directly from the gel photos as present (1) or absent (0) for each sample (data matrix available from the authors). Missing data points were scored as 9. NTSYS-pc 2.0 (Rohlf 1997) was used for cluster analysis with Dice similarity coefficient and principal coordinate analysis (PCO), also with Dice similarity.

RESULTS

Of the 9 tissue-collection methods tested, none successfully eliminated the mucilage. In fact, methods that included leaf grinding in the field (options 8, 9, Table 1) resulted in increased mucilage levels. The method that resulted in the least amount of mucilage being released involved placing each leaf into a 50-mL screw-top tube, filling the tube with desiccated silica gel, capping the tube, storing it at room temperature until dry followed by storage at -20°C until DNA extraction. Likewise, modifications of the basic 2X CTAB extraction protocol were not successful. The system that worked best was the standard protocol followed by cleanup with the Qiagen DNeasy plant kit.

We scored a total of 88 bands, with the number of bands per primer ranging from 5 to 14 (Table 3). Failed reactions resulted in missing data for only 72 of 2112 matrix cells (3.4%).
Table 2. Trial modifications of the standard 2X CTAB DNA extraction protocol (Doyle and Doyle 1987) to improve the quality of DNA yield in Gaura neomexicana ssp. coloradensis. Judgement on improved DNA quality was based on results visible in gel photograph, compared with DNA extraction using unmodified 2X CTAB protocol.

<table>
<thead>
<tr>
<th>Modified step</th>
<th>Improved DNA quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Increase CTAB detergent from 2% to 6%.</td>
<td>None observed</td>
</tr>
<tr>
<td>2 Reduce ratio of tissue to extraction buffer volume by 50%.</td>
<td>None observed</td>
</tr>
<tr>
<td>3 Grind leaf tissue in ice-cold extraction buffer, not in liquid nitrogen.</td>
<td>None observed</td>
</tr>
<tr>
<td>4 Omit freezing of extraction buffer/ground leaf slurry prior to incubation.</td>
<td>None observed</td>
</tr>
<tr>
<td>5 Insert DNAzol ESP purification treatment of DNA-mucilage pellet after first DNA precipitation.</td>
<td>None observed</td>
</tr>
<tr>
<td>6 Soak DNA-mucilage pellet in 8mM NaOH for 24 hours; process supernate.</td>
<td>None observed</td>
</tr>
<tr>
<td>7 Insert a 2nd chloroform-isoamyl alcohol partition of aqueous phase.</td>
<td>None observed</td>
</tr>
<tr>
<td>8 Substitute ammonium acetate for sodium acetate in the 2nd precipitation.</td>
<td>None observed</td>
</tr>
<tr>
<td>9 Replace entire CTAB protocol with Norgen extraction protocol.</td>
<td>None observed</td>
</tr>
<tr>
<td>10 After standard CTAB extraction, clean DNA-mucilage sample with DNeasy plant kit® following instructions.</td>
<td>Positive, but not universally so</td>
</tr>
</tbody>
</table>

Only 3 samples (UD41, UD42, UD50) had missing data points, and most of these (46 of 72) were from individual UD50.

Cluster analysis indicated a high degree of similarity across the 3 populations (Fig. 2). All samples, with the single exception of UD50, were at least 80% similar, and in many cases individuals were more similar to members of a different population than to members of their own population. UD50 is very different; this may be a reflection of the many missing data points or an indication of true differences in the genome. Disregarding the 46 missing data points, this individual was unique in 9 of the remaining 42 characters.

Prinicipal coordinate analysis (Fig. 3) provided a different picture of variation among
the 3 Gaura populations: UD41 and UD50, again, are unique, but the remaining individuals fall into 2 groups, 1 containing all members of the Crow Creek population plus DC49 and the other containing the rest of the "unnamed drainage" and Diamond Creek members.

DISCUSSION

The high degree of similarity displayed in the cluster phenogram is not surprising, given the spatial proximity and presumably close genetic relationship of the populations at the 3 sampling sites. Both analyses indicate that, genetically, individuals from the Diamond Creek and "unnamed drainage" populations are indistinguishable, although both populations contain "genetic outliers" (DC49, UD41, UD50). The evidence suggests that these 2 populations are members of a single gene pool, which could result from contemporary cross-pollination or be an artifact, if these populations are remnants of a once larger, continuous population.

The PCO separation of the Crow Creek population from the other 2 populations is intriguing, since this site is currently nearly contiguous with the Diamond Creek site. However, the Crow Creek site has recently become dominated by willow (particularly Salix exigua Nutt. and S. eriocephala Michx. var. ligulifolia [Ball] Dorn; Fertig 2001), and this has been responsible for a significant decrease in the size of the Gaura population at that site. In fact, from 1986 (the 1st year of monitoring) through 1990, the Crow Creek site population accounted for approximately 40% of the total Gaura count for the 3 sites; in 2001 it accounted for only 12% of the total (Heidel 2002). There may be some strong selective pressures on plants establishing at that site, compared to plants recruited into the other 2 sites. This may account for the observed genetic divergence of the Crow Creek population from the Diamond Creek and "unnamed drainage" populations.

However, historic differences between the sites could also be responsible for the separation. The soil is moister at the Crow Creek site, and plants there have been observed to flower and set seeds later in the season than elsewhere (W. Fertig personal communication). This temporal difference in flowering could lead to the distinct genetic structure. In fact, we may be seeing only the remains of a once greater distinction, if the Crow Creek population is currently interbreeding with the now-adjacent Diamond Creek population.

Finally, the separation could be an artifact of the limited sample size. A larger sample may reveal genotypes that bridge the gap between Crow Creek and the other sites. One Diamond Creek plant, DC49, is genetically
more like Crow Creek individuals than others of Diamond Creek or the “unnamed drainage” (Fig. 3). It is possible that more individuals within the Diamond Creek population are genetically related to DC49.

From a management perspective, it will be necessary to preserve at least 2 of the 3 populations, 1 of which must be Crow Creek, to maintain maximum diversity within the taxon. The Diamond Creek and “unnamed drainage” populations are currently thriving, and there is no apparent need to choose between them. However, the “unnamed drainage” population harbors some unique alleles (in UD41 and UD50) that are not present in the other 2 populations. For this reason the “unnamed drainage” population is an obvious candidate for preservation.

The Crow Creek population represents a genetic composition shared apparently by only a small number of Diamond Creek individuals and not at all by members of the “unnamed drainage” population. Unfortunately, this population is threatened by encroachment of willow and the need to manage for Preble’s jumping mouse, which prefers such densely vegetated riparian areas (Dark-Smiley and Keinath 2002). However, the conflicting habitat preferences of the 2 threatened taxa may not be a management problem. Population segments of the Colorado butterfly plant persist in patches as small as 20 m² (Heidel 2002), and jumping mice home ranges are several orders of magnitude larger than that and often encompass many patches of open riparian habitat (Dark-Smiley and Keinath 2002). Therefore, it should be possible to manage several small patches of Colorado butterfly plant habitat in the Crow Creek area without substantially affecting habitat quality for the other threatened taxon.

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