

Development of STS and CAPS markers for identification of three tall larkspurs (*Delphinium* spp.)

Xiaomei Li, Dale R. Gardner, Michael H. Ralphs, and Richard R.-C. Wang

Abstract: One cleaved amplified polymorphic sequence (CAPS) and nine sequence tagged site (STS) markers were developed for identifying tall larkspur (*Delphinium* spp.) plants in three species based on the DNA sequence of known species-specific RAPD markers. Four STS markers were used for identification of *Delphinium occidentale*, three STS markers for *Delphinium barbeyi*, and one CAPS and two STS markers for *Delphinium glaucum*. One hundred sixty-six individual plants collected at 19 locations in the western U.S.A. were tested using the STS and CAPS markers. Over 95% of the *D. occidentale* plants contained all four *D. occidentale* specific STS markers, whereas the remaining plants contained three of the four STS markers. Approximately 97% of *D. barbeyi* plants contained all three *D. barbeyi* specific STS markers, and the rest had two of the three STS markers. A small percentage of *D. barbeyi* plants contained one *D. occidentale* specific STS marker. Hybrid populations were characterized as having more *D. occidentale* specific than *D. barbeyi* specific STS markers, suggesting that the three hybrid populations are composed not of F₁ hybrid plants of the parental species but of segregating offspring of different generations from original hybrids. This set of STS and CAPS markers for larkspur species should be useful in classification of unknown plant materials and the identification of hybrid populations.

Key words: poisonous plants, RAPD, molecular marker, PCR.

Résumé : Un marqueur CAPS (« Cleaved Amplified Polymorphic Sequence ») et neuf marqueurs STS (« Sequence Tagged Site ») ont été mis au point pour distinguer trois espèces du genre *Delphinium* à partir de la séquence d'ADN de marqueurs RAPD connus comme étant spécifiques de chacune de ces espèces. Quatre marqueurs STS ont permis d'identifier le *Delphinium occidentale*, trois marqueurs STS sont spécifiques du *Delphinium barbeyi* et la combinaison d'un marqueur CAPS et de deux marqueurs STS permet d'identifier le *Delphinium glaucum*. Cent soixante-six plantes, provenant de 19 sites situés dans l'Ouest des États-Unis, ont été examinées avec ces marqueurs CAPS et STS. Plus de 95 % des plantes du *D. occidentale* possédaient les quatre marqueurs STS spécifiques tandis que les autres individus possédaient trois des quatre marqueurs. Environ 97 % des plants du *D. barbeyi* possédaient les trois marqueurs STS spécifiques du *D. barbeyi* et les autres montraient deux de ces trois marqueurs. Un faible pourcentage des plants du *D. barbeyi* montrait un des marqueurs STS spécifiques du *D. occidentale*. Des populations hybrides possédaient plus de marqueurs STS spécifiques du *D. occidentale* que de marqueurs spécifiques du *D. barbeyi*. Cela suggère que les trois populations hybrides sont composées non pas d'hybrides F₁ issus immédiatement de ces parents, mais plutôt de descendants en ségrégation appartenant à diverses générations. Ces marqueurs STS et CAPS devraient s'avérer utiles pour la classification de plantes inconnues et l'identification d'hybrides au sein du genre *Delphinium*.

Mots clés : plantes vénéneuses, RAPD, marqueur moléculaire, PCR.

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Introduction

Larkspurs (Ranunculaceae *Delphinium* spp. L.) containing toxic norditerpenoid alkaloids (Pfister et al. 1993) are divided into tall and low larkspur according to their growth characteristics (Knight and Pfister 1997). Distributed widely in the western U.S.A., *Delphinium barbeyi* Huth, *Delphinium glaucum* S. Watson, and *Delphinium occidentale* S. Watson are three main species of tall larkspurs that pose a serious

problem for grazing cattle. Losses from fatal poisoning average 5%, and at times exceed 15% in a single year where larkspur is abundant (Nielsen and Ralphs 1988; Pfister et al. 1997).

There is evidence that tall larkspur plants differ in toxicity (Ralphs et al. 1997) and morphology (Ewan 1945). The concentration of alkaloids in *Delphinium* spp. (Manners et al. 1995) and their toxicity to cattle (Pfister et al. 1994; Nation et al. 1982) merits different management recommendations

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Table 1. Accessions of three species of tall larkspur and hybrid of *Delphinium occidentale* × *D. barbeyi* collected from 19 locations in the western U.S.A.

| Code No. | Species | Location | No. samples |
|--------------|--|----------------------|-------------|
| O1 | <i>D. occidentale</i> | Logan, Utah | 10 |
| O2 | <i>D. occidentale</i> | Mendon, Utah | 5 |
| O3 | <i>D. occidentale</i> | Clifton, Idaho | 5 |
| O4 | <i>D. occidentale</i> | Park Valley, Utah | 10 |
| O5 | <i>D. occidentale</i> | Oakley, Idaho | 10 |
| O6 | <i>D. occidentale</i> | Jackson, Wyo. | 10 |
| O7 | <i>D. occidentale</i> | Alpine, Wyo. | 10 |
| O8 | <i>D. occidentale</i> | Franklin, Idaho | 3 |
| B1 | <i>D. barbeyi</i> | Cedar City, Utah | 10 |
| B2 | <i>D. barbeyi</i> | Salina, Utah | 10 |
| B3 | <i>D. barbeyi</i> | Manti, Utah | 10 |
| B4 | <i>D. barbeyi</i> | Montrose Colo. | 8 |
| B5 | <i>D. barbeyi</i> | Crested Butte, Colo. | 12 |
| B6 | <i>D. barbeyi</i> | Yampa, Colo. | 10 |
| G1 | <i>D. glaucum</i> | Sonora, Calif. | 3 |
| G2 | <i>D. glaucum</i> | Placerville, Calif. | 10 |
| H1 | <i>D. occidentale</i> × <i>D. barbeyi</i> hybrid | Yampa, Colo. | 10 |
| H2 | <i>D. occidentale</i> × <i>D. barbeyi</i> hybrid | Salina, Utah | 10 |
| H3 | <i>D. occidentale</i> × <i>D. barbeyi</i> hybrid | Fairview, Utah | 10 |
| Total | | | |
| 19 | | | 166 |

(Ralphs et al. 1997; Pfister et al. 1997). At certain growth stages, especially the early stage (seed and plantlet), morphological differences among the three tall larkspur are subtle. It would be helpful to genetically distinguish among the three species of tall larkspur and their hybrids for making selective management recommendations to prevent livestock poisonings.

Fifty species-specific random amplified polymorphic DNA (RAPD) markers had been previously identified for *D. occidentale*, *D. glaucum*, and *D. barbeyi* (Li et al. 2002). Some of these markers have been cloned and sequenced. Inexperienced persons may have difficulty using the RAPD technique in routine assays for species identification because a RAPD marker is only one of many amplified DNA fragments resulting from the PCR that is based on a single primer 10-bp in length. Sequence-tagged-site (STS) markers (Tragoonrung et al. 1992, Komatsuda et al. 1998) are PCR-based markers generated by a pair of primers (~20-bases-long) that are designed according to known DNA sequences. Ideally, only one DNA fragment of a specific length (STS marker) will be amplified from the template DNA containing the target sequence. Therefore, STS markers are more reproducible and specific than the original RAPD marker. The development of a set of STS markers for each *Delphinium* species would provide researchers with a simple PCR assay to identify larkspur samples in their collections. Unfortunately, some designed STS primers could lead to the loss of the initial polymorphism. CAPS (cleaved amplified polymorphic sequence; Konieczny and Ausubel 1993) markers can be obtained by enzymatic restriction of STS sequences that seem to be non-polymorphic. In this paper, we report on the development of a set of STS-CAPS markers useful for identifying three tall larkspur species and their natural interspecific hybrids.

Materials and methods

Plant materials

One hundred sixty-six individual plants from 19 accessions collected in 1998 from the Rocky Mountains region of the western U.S.A. were used in this study. Among them, 63 *D. occidentale* plants were collected from eight locations in Utah, Idaho, and Wyoming; 13 *D. glaucum* plants were collected from two locations in California; and 60 *D. barbeyi* plants were collected from six locations in Utah and Colorado. Another 30 plants, collected from three accessions in Colorado and Utah, are putative interspecific hybrids between *D. occidentale* and *D. barbeyi* (Table 1). Specific epithets of taxa included in this study follow Welsh and Ralphs (2002).

Sample collection and template DNA preparation

Young leaves of individual plants were collected and immediately placed in a sample bag and stored on ice. After 1–2 days, the leaves were freeze dried and stored in plastic bags at room temperature. The individual plant genomic DNA was extracted from 100 mg of dry leaves using the OmniPrep kit (GenoTech, Inc., St. Louis, Mo.), quantified by a TKO100 DNA fluorometer (Amersham Pharmacia Biotech, Piscataway, N.J.), and diluted to a final concentration of 5 ng/μL and stored at 4°C.

RAPD assay

The optimized reaction mixture (25 μL) contained 13.3 μL sterile ddH₂O, 2.5 μL 10× buffer, 2 μL of 8mM dNTP, 2 μL of 10 μM primer (Operon Technologies, Alameda Calif.), 3 μL of 25 mM MgCl₂, 0.2 μL (2 U) Stoffel fragment (Perkin Elmer, Norwalk, Conn.), and 2 μL template DNA (concentration 2.5 ng DNA/μL) and was covered by 30 μL of min-

Table 2. Primers used for PCR amplification of STS and CAPS markers for larkspur species identification.

| Primer | Species identified | STS/CAPS ^a marker | Sequence (5'→3') | Length (bp) | PCR methods ^b |
|--------|-----------------------|------------------------------|----------------------------|-------------|--------------------------|
| 18F | <i>D. occidentale</i> | S-G12-O ₅₃₆ | GCAGAACCGGTGTATCCTGT | 20 | P32 |
| 18R | <i>D. occidentale</i> | | CAGCTCACGAGAGATGGTGGG | 21 | |
| 36F | <i>D. occidentale</i> | S-K08-O ₂₄₅ | GAACACTGGGTTCTAGATATTTGGTT | 26 | P34 |
| 36R | <i>D. occidentale</i> | | TGTGAGCCTCTGTGGAAATG | 20 | |
| 37F | <i>D. occidentale</i> | S-O07-O ₃₇₀ | GCACTGACCACAGGATTTGTT | 21 | P34 |
| 37R | <i>D. occidentale</i> | | GAGCTAGGTGACCCAAGGTG | 20 | |
| 40F | <i>D. occidentale</i> | S-H03-O ₇₀₁ | TGTGAGATAATACAAATGCCACC | 23 | P38 |
| 40R | <i>D. occidentale</i> | | GTCCACCACCACAAGGAACT | 20 | |
| 27F | <i>D. barbeyi</i> | C-E-17-B ₄₃₄ | CTACTGCCGTGGCTGCTAAT | 20 | P38 + <i>AluI</i> |
| 27R | <i>D. barbeyi</i> | | CTGAGGATTTCTTCGGGGTT | 20 | |
| 30F2 | <i>D. barbeyi</i> | S-K01-B ₄₂₉ | CTCTCATTCATGGGGTGTCC | 20 | P37 |
| 30R2 | <i>D. barbeyi</i> | | CCAAAAAGAGGTCAACCGAC | 20 | |
| 31F2 | <i>D. barbeyi</i> | S-K13-B ₅₀₆ | TTGTACCCTGCAAAAATCACC | 21 | P34 |
| 31R | <i>D. barbeyi</i> | | AGGCTGAAAGTACCACCTG | 20 | |
| 43F | <i>D. barbeyi</i> | S-F13-B ₅₃₇ | AAGATTTACGTCCTGAATG | 20 | P38 |
| 43R | <i>D. barbeyi</i> | | AATCACTGGGCTTTGACCAT | 20 | |
| G12GF | <i>D. glaucum</i> | S-G12-G ₆₃₃ | CAGCTCACGATGTATAAGAC | 20 | P14 |
| G12GR | <i>D. glaucum</i> | | GGAACGATAAACGACG | 16 | |
| 33F | <i>D. glaucum</i> | C-K08-G ₄₅₇ | ACTGGGGTCAATATGATGGC | 20 | P38 + <i>AluI</i> |
| 33R2 | <i>D. glaucum</i> | | CTGACCCGAGTCACCTCTTG | 20 | |
| 48F | <i>D. glaucum</i> | S-K01-G ₆₂₉ | CGTCCCCTAATATAGTGTGTTGG | 23 | P38 |
| 48R | <i>D. glaucum</i> | | GGAAGCCTCGTCTGTCGCCT | 20 | |

^aSTS (with prefix S-) and CAPS (with prefix C-) markers derived from RAPD markers (with random primers from Operon Technologies) for *Delphinium occidentale* (O), *D. barbeyi* (B), and *D. glaucum* (G); the length (in bp) for the marker is in subscript.

^bSee Table 3 for programs used for PCR amplification; *AluI* was used for generating CAPS markers.

eral oil. PCR was performed with the GeneAmp PCR System 9700 (Perkin Elmer) for 40 cycles of 93°C for 1 min, 35°C for 1 min, and 71°C for 2 min. The reaction products were then stored at 4°C.

Separation of PCR products

The amplification products were separated in a 2% agarose (agarose for the separation of GeneAmp PCR products, Perkin Elmer) gel that contained 0.5 µg ethidium bromide in 1× Tris-borate-EDTA (TBE) buffer. DNA fragments were visualized and photographed under UV light. Their sizes were estimated by comparing them with 100-bp DNA size markers composed of 1500, 1200, and from 1000 to 100 bp in decrements of 100 bp (New England BioLabs, Beverly, Mass.).

Cloning of RAPD markers for Southern hybridization and DNA sequencing

The DNA of RAPD markers identified for each species was recovered from low-melting agarose gels and cloned into the pCR 2.1 vector of the TA Cloning Kit (Invitrogen, Carlsbad, Calif.). The plasmid DNA from clones was labeled with digoxigenin by PCR-labeling methods and used as probe in Southern blot hybridization. The DNA hybridization was detected by an antibody of digoxigenin (anti-digoxigenin alkaline phosphatase), in the presence of chromogenic substrates composed of 4-nitro blue tetrazolium chloride (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) toluidine salt in the NBT-BCIP solution (Boehringer Mannheim (now Roche Applied Science), Indi-

anapolis, Ind.). Clones with correct inserts were selected based on their specificity in hybridization with target RAPD marker bands. Insert DNA of the correct clones containing species-specific RAPD markers were sequenced with an ABI 373A DNA sequencer (Applied Biosystems, Foster City, Calif.).

Primer design and PCR amplification for STS

STS primer pairs (Table 2) were designed for each sequenced species-specific RAPD marker using the Primer3 program (Rozen and Skaletsky 1997; available from <http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>). The selected new primer sites may or may not partially overlap with the original RAPD primer sites. PCR was carried out in a reaction mixture (25 µL) containing 12.375 µL sterile ddH₂O, 2.5 µL of 10× buffer, 2 µL of 8 mM dNTP, 2 µL of 10 µM of each designed primer pair, 2 µL of 50 mM MgCl₂, 0.125 µL (2 U) *Taq* polymerase (Gibco BRL, Rockville, Md.), and 2 µL of template DNA (2.5 ng/µL), and was covered by 30 µL mineral oil. STS markers were optimally amplified under various PCR programs (Table 3).

CAPS analysis

In the case where STS primer pairs produced fragments of the same length from different species of larkspur, 5 µL of the PCR product was digested by a restriction endonuclease that has (a) restriction site(s) in the sequence of an STS marker. The enzyme-digested PCR product was then separated in a 3% agarose gel containing ethidium bromide in 1× TBE.

Table 3. PCR programs used in conjunction with primers listed in Table 2 to generate STS and CAPS markers for larkspur species identification.

| Programs | Pre-heating | Heating | Annealing | Extension | Cycles | Holding |
|----------|-------------|--------------|---------------|-------------|--------|------------------|
| 14 | 95°C (7min) | 95°C (1min) | 55°C (1min) | 72°C (2min) | 40 | 72°C(7min), 4°C |
| 32 | 95°C (7min) | 95°C (45sec) | 60°C (1min) | 72°C (2min) | 30 | 4°C |
| 34 | 95°C (7min) | 95°C (45sec) | 60°C (1min) | 72°C (2min) | 35 | 72°C (7min), 4°C |
| 37 | 95°C (7min) | 95°C (45sec) | 62.5°C (1min) | 72°C (2min) | 35 | 72°C (7min), 4°C |
| 38 | 95°C (7min) | 95°C (45sec) | 60°C (1min) | 72°C (2min) | 40 | 72°C (7min), 4°C |

Table 4. Frequency (%) of RAPD (with prefix OP), STS (with prefix S-), and CAPS (with prefix C-) markers in eight accessions of *Delphinium occidentale*, six accessions of *D. barbeyi*, two accessions of *D. glaucum*, and three populations of putative *D. occidentale* × *D. barbeyi* hybrids.

| Markers ^a | <i>D. occidentale</i> | <i>D. barbeyi</i> | <i>D. glaucum</i> | Hybrids |
|---------------------------------|-----------------------|-------------------|-------------------|---------|
| OPO07 ₄₄₃ (AF441760) | 100 | 16.7 | 0 | 90 |
| S-O07-O ₄₀₀ | 87.3 | 3.3 | 0 | 90 |
| OPG12 ₆₀₁ (AF441761) | 100 | 0 | 0 | 77 |
| S-G12-O ₅₃₆ (G73229) | 93.7 | 6.7 | 0 | 87 |
| OPK08 ₃₈₀ (AF441762) | 100 | 0 | 0 | 90 |
| S-K08-O ₂₄₅ (G73225) | 100 | 0 | 0 | 90 |
| OPH03 ₇₁₂ (AF441763) | 92 | 0 | 0 | 20 |
| S-H03-O ₈₀₀ | 100 | 0 | 0 | 33 |
| Mean of RAPD | 98.0 | 4.2 | 0 | 69 |
| Mean of STS | 95.3 | 2.5 | 0 | 75 |
| OPK01 ₄₉₃ (AF441764) | 0 | 100 | 0 | 13 |
| S-K01-B ₄₂₉ (G73226) | 0 | 100 | 0 | 47 |
| OPK13 ₅₅₃ (AF441765) | 0 | 95 | 0 | 17 |
| S-K13-B ₅₀₆ (G73227) | 0 | 100 | 0 | 13 |
| OPF13 ₅₅₃ (AF441766) | 0 | 97 | 0 | 50 |
| S-F13-B ₅₃₇ (G73230) | 0 | 90 | 0 | 17 |
| Mean of RAPD | 0 | 97.3 | 0 | 27 |
| Mean of STS | 0 | 96.7 | 0 | 26 |
| OPG12 ₆₅₇ (AF441767) | 0 | 0 | 100 | 0 |
| S-G12-G ₆₃₃ (G73228) | 0 | 0 | 100 | 0 |
| OPK08 ₅₉₂ (AF441768) | 0 | 0 | 100 | 0 |
| C-K08-G ₄₅₇ (G73231) | 0 | 0 | 100 | 0 |
| OPK01 ₆₅₄ (AF441769) | 0 | 0 | 100 | 0 |
| S-K01-G ₅₈₀ | 0 | 0 | 100 | 0 |
| Mean of RAPD | 0 | 0 | 100 | 0 |
| Mean of STS | 0 | 0 | 100 | 0 |

Note: GenBank accession No. is provided in parentheses for each DNA marker sequence.

^aRAPD (with prefix OP followed by the random primers from Operon Technologies), STS (with prefix S-), and CAPS (with prefix C-) markers for *Delphinium occidentale* (O), *D. barbeyi* (B), and *D. glaucum* (G); the length (in bp) for the marker is in subscript.

Results and discussion

Identification, cloning, and sequencing of species-specific RAPD markers for larkspur

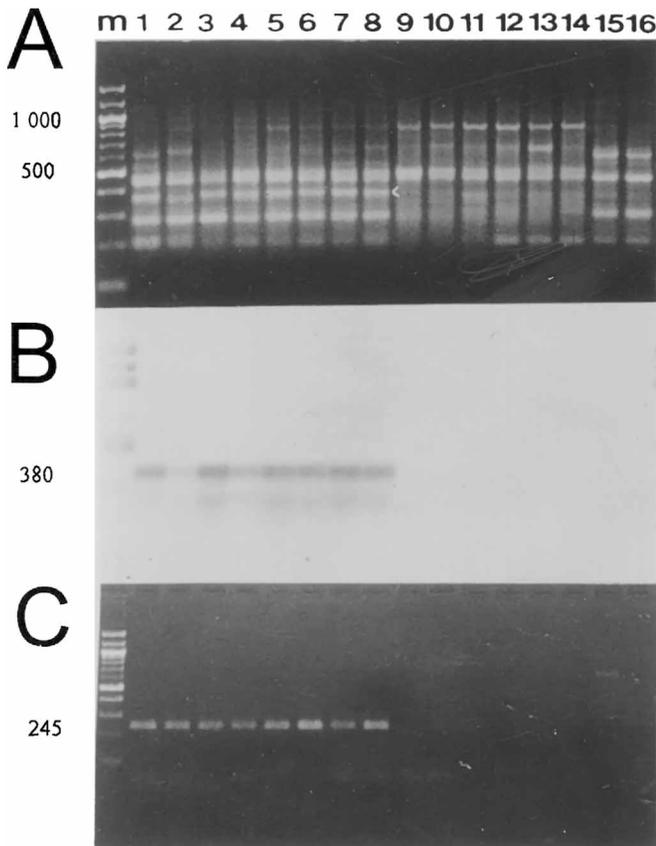
The bulked DNA for each accession was used to screen and select the species-specific RAPD markers, and then the DNA of individual plants was tested for species specificity. We had previously identified 50 species-specific RAPD markers (Li et al. 2002). Twenty-one of these markers were

successfully cloned and sequenced, including five *D. occidentale* specific markers, nine *D. barbeyi* specific markers, and seven *D. glaucum* specific markers. We found these markers to be sufficient to distinguish among the three tall larkspur species.

Design of STS primer pairs for PCR assay of three larkspur species

Eleven STS primer pairs were designed from the sequenced

Fig. 1. RAPD profile (A), Southern blot hybridization (B) of RAPD marker OPK08-O₃₈₀, and converted STS marker S-K08-O₂₄₅ (C) for *D. occidentale* (lanes 1 to 8), *D. barbeyi* (lanes 9 to 14), and *D. glaucum* (lanes 15 and 16). Lane M contains the 100-bp DNA ladder ranging from 1500 (top), 1200, and 1000 to 100 bp (bottom) with 1000 and 500 bp bands in double intensity.



RAPD markers. Each STS primer was designed within the range of 16–26 bp (Table 2). These STS primer pairs were tested using bulked DNA of each accession of the three species (8 *D. occidentale*, 6 *D. barbeyi*, and 2 *D. glaucum*). Only those primer sets producing polymorphism were then used for testing species specificity and the number and size of PCR products in each of the 166 individual samples. Ideally, only one single fragment of specific length was amplified from the specific species having the target sequence. For each primer set, a different PCR program was optimized for ideal STS amplification products (Tables 2 and 3). The annealing temperature and number of PCR cycles had the greatest effect on the number and intensity of PCR products, i.e., lower annealing temperatures produced more PCR products whereas more PCR cycles produced more intense bands.

With the 11 designed primer sets, four *D. occidentale* specific, three *D. barbeyi* specific, and two *D. glaucum* specific STS markers were obtained (Table 4; Figs. 1 and 2). Ideally, an STS primer pair would amplify one DNA sequence of the specified length from the target species but not from other species. This situation was achieved for 6 of the 11 primer

sets listed in Table 2. All three STS primer sets used for identification of *D. barbeyi* produced the target STS marker for this species (Figs. 2a, 2b, and 2c); however, one of those primer sets produced a shorter fragment from the two non-target species (Fig. 2b) and a few *D. occidentale* plants might produce a faint band of S-K01-B₄₂₉ in contrast to a strong band amplified from all *D. barbeyi* individuals (Fig. 2c). With the primer set 40F and 40R, all three *Delphinium* species had PCR products, but *D. occidentale* had one product of approximately 800 bp that was longer than expected (701 bp) and longer than those from *D. barbeyi* or *D. glaucum* (Fig. 2f). The expected STS marker S-O07-O₃₇₀ was present in both *D. occidentale* and *D. barbeyi*, but an unexpected marker, S-O07-O₄₀₀, was present only in *D. occidentale* (Fig. 2g). Two of the three STS primer sets designed for *D. glaucum* produced polymorphic STS markers from the target species although one of the two was not the expected length. Instead of the S-K01-G marker being 629 bp as expected, the polymorphic marker for *D. glaucum* was approximately 580 bp (Fig. 2h). The primer set 48F and 48R must have amplified a polymorphic band for *D. glaucum* from another site in its genome, instead of amplification from the OPK01₆₅₄ site. This may also account for the production of S-H03-O₈₀₀ and S-O07-O₄₀₀. These three unexpected STS products have not been sequenced.

Conversion of a species-specific RAPD marker to a CAPS marker

Some of the PCR products amplified from STS primer sets did not exhibit the polymorphism between and (or) among species revealed by RAPD markers, e.g., STS-K08-G₅₃₇ (Fig. 3a). This situation necessitated the development of a CAPS marker to reveal interspecific polymorphism. Three restriction enzymes, *AluI*, *MnII*, and *RsaI*, were used to digest the PCR products amplified using bulked DNA from each of 16 locations as templates. Only the PCR product digested by *AluI* produced a polymorphic band of the expected size (457 bp) based on the restriction map of the sequenced RAPD marker (Table 4 and Fig. 3b). This CAPS marker is *D. glaucum* specific and only the 13 plants from two *D. glaucum* accessions produced this CAPS marker. The other CAPS marker, C-E17-B₄₃₄ (Table 2), was only present in four of six accessions of *D. barbeyi*. Therefore, it is not universally useful for identifying *D. barbeyi*.

A set of STS and CAPS kit for tall larkspurs identification

Nine STS markers (four for *D. occidentale*, three for *D. barbeyi*, and two for *D. glaucum*) and one CAPS marker (Table 4) were used to identify the 166 individual plants composed of three tall larkspur species and three populations of *D. occidentale* × *D. barbeyi* hybrids. Ninety-five percent of the *D. occidentale* plants had all four *D. occidentale* specific STS markers, and 5% had three of the four STS markers. Ninety-seven percent of *D. barbeyi* plants had all the three *D. barbeyi* specific STS markers, and 3% had two of the three STS markers. Only 2.5% of *D. barbeyi* plants had a single *D. occidentale* specific STS marker. The three hybrid populations had none of the *D. glaucum* specific markers, but had more *D. occidentale*

Fig. 2. Profiles, in a 2% agarose gel, of STS markers (a) S-F13-B₅₃₇, (b) S-K13-B₅₀₆, and (c) S-K01-B₄₂₉ for *D. barbeyi*; (d) S-K08-O₂₄₅, (e) S-G12-O₅₃₆, (f) S-H03-O₈₀₀, and (g) S-O07-O₄₀₀ for *D. occidentale*; (h) S-K01-G₅₈₀, (i) S-G12-G₆₃₃, (j) non-polymorphic S-K08₅₃₇, and (k) C-K08-G₄₅₇ for *D. glaucum*. The markers were amplified from single-plant DNA samples of (1) *D. occidentale*, (2) *D. barbeyi*, and (3) *D. glaucum* for each primer set. The polymorphic markers are indicated with circles. Lane M contains the same 100-bp DNA ladder as in Fig. 1.

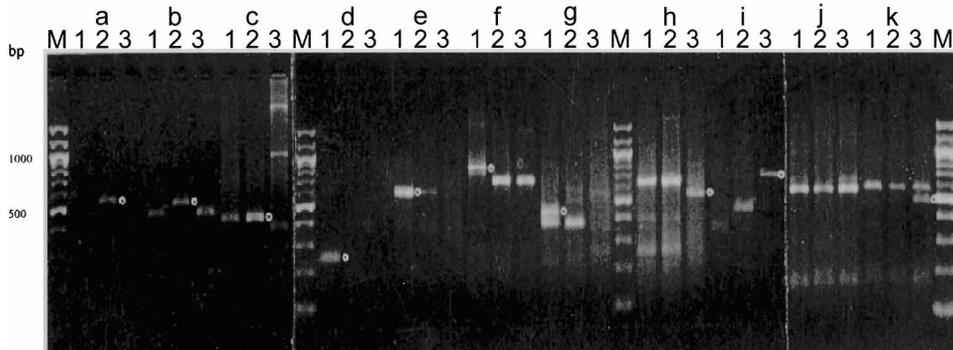
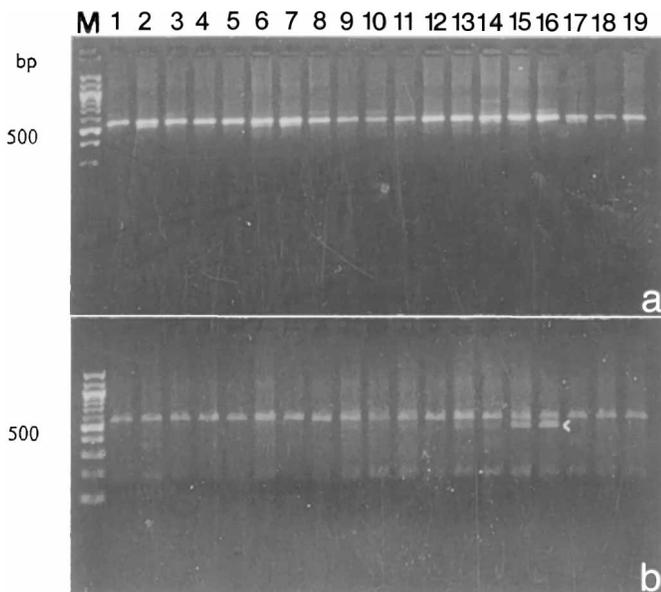


Fig. 3. *D. glaucum* specific CAPS marker (C-K08-G₄₅₇) generated from OPK08-G₅₉₂. (a) STS PCR products (S-K08₅₃₇) amplified by designed new primer pairs exhibited no interspecific polymorphism. (b) After digestion by *AluI*, the STS products revealed the interspecific polymorphism with a specific band (457 bp) for *D. glaucum*. Bulk template DNA was used for each accession of *D. occidentale* (lanes 1 to 8), *D. barbeyi* (lanes 9 to 14), *D. glaucum* (lanes 15 and 16), and *D. occidentale* × *D. barbeyi* hybrids (lanes 17 to 19). Lane M contains the same 100-bp DNA ladder as in Fig. 1.



specific STS markers (H1 = 95%; H2 = 68%; H3 = 63%) than *D. barbeyi* specific STS markers (H1 = 7%; H2 = 50%; H3 = 20%) (Table 4). These results suggest that the three hybrid populations are not composed of F₁ hybrid plants of the parental species, *D. occidentale* and *D. barbeyi*, but segregating offsprings of different generations from original hybrids. All of the results for STS and CAPS were in agreement with earlier RAPD results (Li et al. 2002).

This final set of STS and CAPS markers (Table 4; Figs. 2 and 3) for larkspur species can be used to identify single-

plant larkspur samples using routine PCR assay methods with optimized programmed conditions (Tables 2 and 3). A *D. occidentale* plant would have at least three positive results when assayed with the four STS primer sets. More than two STS markers should be amplified by the three *D. barbeyi* specific primer sets if the plant is *D. barbeyi*. Identification of *D. glaucum* is the least difficult. None of *D. occidentale*, *D. barbeyi*, or their hybrids should have amplification products using the *D. glaucum* specific STS primer sets.

The selected CAPS and STS markers have not been tested on other *Delphinium* species, thus it is not possible to assess their usefulness in the identification of plants belonging to other species of this genus. Nevertheless, in conjunction with morphological characterization, molecular analysis using this set of STS and CAPS primer pairs will be an additional reliable tool for identifying field samples of *Delphinium* plants belonging to the three species common in the western United States.

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