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Short communication

DNA identification of *Salvia divinorum* samples☆

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ABSTRACT

Salvia divinorum (diviner's sage) is a plant in the mint family that produces an hallucinogenic compound, salvinorin A. The plant is used, often by chewing or smoking, as a "recreational" drug source and is regulated or banned in several states and countries. We describe a simple DNA technique, polymerase chain reaction of the ribulose bisphosphate carboxylase large subunit (*rbcL*) gene, that can distinguish *S. divinorum* leaf pieces from pieces of tobacco or cannabis. We have also found DNA sequences adjacent to the chloroplast leucine transfer RNA (*trnL*) gene that are specific to *S. divinorum* and distinguish it from other horticulturally popular *Salvia* species. We report some significant differences between the *S. divinorum trnL* sequences we determined and those now published in GenBank.

The mint family is a family of plants known for the variety of their secondary metabolic products. Among species in this family, *Salvia divinorum* Epling & Játiva (*Lamiaceae*) is unusual for its synthesis of a hallucinogenic compound, salvinorin A [1,2]. Salvinorin A is a trans-neoclarodane diterpenoid [2]. It acts through the *kappa*-opioid receptor [3,4] and is similar in efficacy to the receptor's natural peptide ligand, dynorphin A [4].

S. divinorum has been used in religious rites by Mazatec shamans to induce hallucinatory visions [5]. More recently, it has become an experimental drug for teenagers and others seeking new experiences. In a 2008 survey, 2.8% of 55,739 respondents answered "yes" to having "ever, even once, used *Salvia divinorum*" [6]. Many Internet sites describe the effects of smoking or chewing *S. divinorum* leaves as powerful but short-lived. A placebo-controlled study [7] found that the effects of inhaling doses of salvinorin A ranging from 0.375 to 21 μg/kg lasted for about 20 min.

S. divinorum, *S. divinorum* extract, and salvinorin A are not listed in the federal Controlled Substances Act. However, some degree of regulation has been passed by legislatures of 20 US states [http://en.wikipedia.org/wiki/Legal_status_of_Salvia_divinorum]. Canada and California prohibit sales to minors [7]. A number of countries, including Australia, Sweden, Germany, Italy, and Spain have controlled import, sale or use of *S. divinorum* or salvinorin A.

The mint family is very large, including over 7000 recognized species [8]. The genus *Salvia* (sage) has over 900 species [9]. *S. divinorum* is a small to medium-sized shrub with two simple ovate leaves arising from each node in opposite conformation (Fig. 1). Branching at the nodes is frequent. Flowering is rare. However, the plant can easily by propagated by placing a small shoot with an apical or axillary bud in moist soil. *S. divinorum* is an unremarkable plant, easily recognizable as a member of the mint family by its square stem, but without flowers it is more difficult to distinguish from many other members of its genus or of the large mint family. *S. divinorum* leaves are generally large compared to those of other *Salvia* species used in gardening and cooking (Fig. S1). However, the detached leaves are not easy to identify by shape or size, and it is even more difficult to identify *S. divinorum* leaves that have been dried and shredded for smoking (Fig. S2). One direct and relevant

☆ GenBank accession numbers for sequences compared in Figs. S4–S6 and Tables 3 and 4: *S. divinorum*, 5′ segment of trnL-trnF intergenic spacer: voucher LA47663, JQ888119; voucher UC1591867, JQ888120; voucher 89.1680, JQ888121; voucher 92.0391, JQ888122. *S. divinorum*, 3′ segment of trnL-trnF intergenic spacer: voucher LA47663, JQ888123; voucher UC1591867, JQ888124; voucher 89.1680, JQ888125; voucher 92.0391, JQ888126. *Salvia* spp., 5′ segment of trnL-trnF intergenic spacer: *S. apiana*, JQ888127; *S. cedrosensis*, JQ888128; *S. chionophylla*, JQ888129; *S. clevelandii*, JQ888130; *S. dolomitica*, JQ888131; *S. greggii*, JQ888132; *S.* × *jamensis*, JQ888133; *S. lavandulifolia*, JQ888134; *S. microphylla*, JQ888135; *S. officinalis*, JQ888136; *S. recognita*, JQ888137; *S.* × *sylvestris*, JQ888138. *Salvia* spp., 3′ segment of trnL-trnF intergenic spacer: *S. apiana*, JQ888139; *S. cedrosensis*, JQ888140; *S. chionophylla*, JQ888141; *S. clevelandii*, JQ888142; *S. dolomitica*, JQ888143; *S. greggii*, JQ888144; *S.* × *jamensis*, JQ888145; *S. lavandulifolia*, JQ888146; *S. microphylla*, JQ888147; *S. officinalis*, JQ888148; *S. recognita*, JQ888149; *S.* × *sylvestris*, JQ888150.

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Fig. 1. Laboratory-grown *Salvia divinorum* plant. Upper right: inflorescence (from Reisfield, www.sagewisdom.org/reisfield.html).

method is to extract and identify the active principle, salvinorin A [2]. A second method involves the determination of specific DNA sequences to identify tissues of the plant. A recent study used DNA sequences to place *S. divinorum* in the mint family tree [10]. This study indicated that *S. divinorum* is a true species, not a hybrid, and that its closest relative is *Salvia venulosa*, a rare endemic found in Colombia.

The objective of this project was to define DNA procedures that could distinguish *S. divinorum* tissue from that of two other drug plants likely to be prepared for smoking, *Cannabis sativa* and *Nicotiana tabacum*, and from other *Salvia* species.

1. Materials and methods

1.1. Plant samples

Dried, identified samples of *S. divinorum* were obtained from the University and Jepson Herbarium, University of California, Berkeley (accessions UC1591867, dry leaf; LA47663, dry flower). Leaf samples of live plants were obtained from the University of California, Berkeley Botanical Garden (accessions 92.0391 and 89.1680) and from the Botanical Conservatory of the University of California, Davis. A live *S. divinorum* plant was purchased commercially and propagated by cuttings. Dried *S. divinorum* leaves, sold for smoking, were also purchased commercially.

Samples of other *Salvia* species were obtained from the University Arboretum, Davis. Fresh leaves of *N. tabacum* were obtained from a UC Davis laboratory. Samples of *C. sativa* were obtained from the Bakersfield, CA police department.

1.2. Isolation of DNA

DNA was isolated from fresh and dried leaves, following a modification of the CTAB procedure of Murray and Thompson [11]. Samples of live leaves, ca. 0.15 g, were cut into small pieces, frozen in liquid N_2 in a 1.5-mL plastic tube, ground with an acid-treated plastic pestle, and re-ground to homogeneity in the presence of 300 μL of buffer containing 2% cetyltrimethyl ammonium bromide, 1.4 M NaCl, 20 mM ethylenedinitrilotetraacetic acid, and 100 mM Tris buffer, pH 8, plus 2 μL of mercaptoethanol. Dried leaves, ca. 0.05 g, were treated similarly, but not frozen. The mixture was heated at 65 °C for 10 min or more and then cooled and extracted with chloroform. DNA in the aqueous layer was precipitated with isopropanol. After centrifugation, the pellet was washed with 70% ethanol, dried and dissolved in water. The DNA was further purified by adsorption on glass filters, washing the filters with an ethanol–salt solution (Promega Corporation, Madison, WI, USA), and elution of the DNA with 50 μL of water.

1.3. PCR

PCRs were conducted in 20 μL of solution containing 12.1 μL of water, 4 μL of 5× Green Go Taq Buffer (Promega), 1.6 μL dNTPs (2.5 mM of each dNTP), 0.125 μL *Taq* DNA Polymerase (Go Taq, 5 u/μL, Promega), 0.6 μL of each primer (forward and reverse, 20 mM), and 1 μL of template DNA. Normal PCR conditions were 96 °C for 1 min; 35 cycles of 94 °C for 30 s, 52–59 °C (depending on primer) for 30 s, and 72 °C for 1 min; 72 °C for 5 min; 4 °C hold.

1.4. Primer selection

Primers were designed to amplify segments of DNA from the plastid ribulosebisphosphate carboxylase large subunit (*rbcL*) gene and the region around the plastid leucine transfer RNA (*trnL*) gene (Table 1). These sequences are particularly useful in identifying plant materials since the plastid genes that they amplify are missing in genomes of animals and fungi that might contaminate plant samples. The *rbcL* gene has also been recommended for plant barcoding [12]. We chose the *trnL* gene based on the recent sequence work on the genus *Salvia* [10]. Other genes have been recommended for identification of plant species over a wide taxonomic range [12,13].

1.5. Analysis of PCR products

The products of PCR reactions (8 μL) were treated with 1 μL of the indicated restriction enzymes (Promega) plus 1 μL of supplied buffer for 3 h at 37 °C. Restriction products were separated by electrophoresis on 12% acrylamide gels, stained with ethidium bromide, and visualized with ultraviolet light.

DNA sequencing of PCR-amplified DNA was performed by the local College of Biological Science ^{UC}DNA Sequencing Facility. Sequences of *rbcL* and *trnL* genes were also obtained from GenBank.

2. Results

2.1. rbcL: use to distinguish smoking materials

Using PCR to amplify the *rbcL* gene, it was possible to distinguish *S. divinorum* DNA from that of two other materials

Table 1
Primers used in amplification of *rbcL* and *trnL* sequences.

	Primer sequence
rbcLF1	AGTTCCCCCTGAAGAAGCAG
rbcLR1	TTCATTACCCTCACGAGCAAG
trnLF1	AGCTGTTCTAACAAATGGAGTTG
trnLR1	GGACTCTATCTTTGTTCTCGTCC
trnLF3	GAAATTTATAGTAAGAGGAAAATCCGTCG
trnLR2	TTCCTTGCTTCATTTGCAATGTG

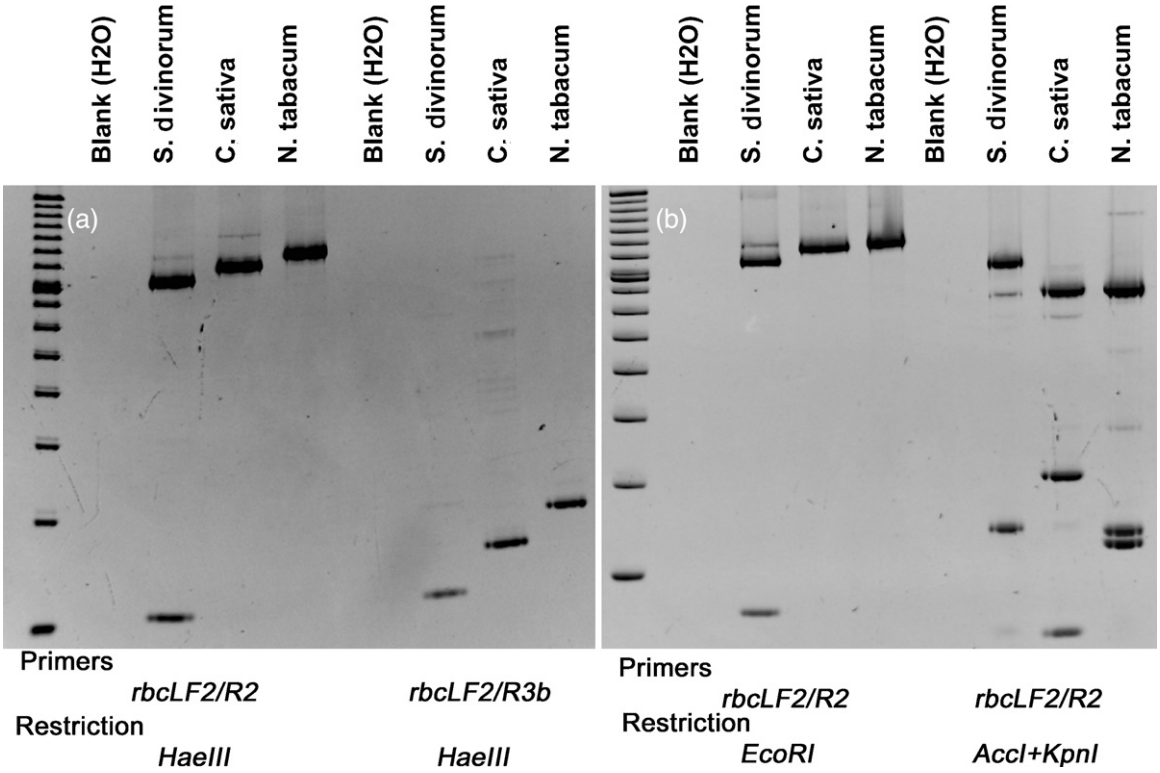

Fig. 2. The patterns of DNA fragments from restriction digests of *rbcL* amplicons produced using template DNA from *S. divinorum*, *C. sativa*, or *N. tabacum*.

commonly used for smoking, *C. sativa* and *N. tabacum*. The amplified DNAs had characteristic sequences (Fig. S3), and products could be easily distinguished by cleaving them with restriction enzymes (Fig. 2a and b). By sequencing amplified *rbcL* DNA, it was also possible to distinguish *S. divinorum* from *Salvia greggii*, *Salvia* × *jamensis*, *Salvia chionophylla*, *Salvia microphylla*, *Salvia dolomitica, Salvia clevelandii*, and *Salvia cedrosensis* (Table 2).

2.2. trnL: use to distinguish Salvias

Although it is possible to distinguish *S. divinorum* from many other *Salvias* using the *rbcL* gene sequence, we expected that better differentiation would result from a comparison of the chloroplast sequences including and adjacent to the *trnL* gene. We chose primers of that region that would amplify DNA of small size (~300 bp), given our experience that DNA in dried and degraded plant material may be fragmented. Of the sequences that would be amplified by primers trnLF1 and trnLR1 from a sample of 156 *trnL* template sequences in GenBank (2 *S. divinorum* accessions plus 154 other *Salvia* species), 132 could be distinguished from *S. divinorum*. Another 22 matched the *S. divinorum* sequences exactly. By using primer sequences trnLF3 and trnLR2 to identify a different segment of the *trnL* gene, all the species, except *S. venulosa*, would be distinguished from *S. divinorum*. *S. divinorum* and *S. venulosa* were indicated as having two inserts of 27 and 32 bases that were not found in any of the other species tested (Fig. 3).

We compared the GenBank data to sequences that we amplified using primer pairs trnLF1/trnLR1 and trnLF3/trnLR2 with DNA templates from four separate accessions of *S. divinorum*, two from the UC Berkeley Herbarium and two from the UC Berkeley Arboretum (Fig. S4a and b). Using trnLF1/trnLR1, our sequences matched GenBank sequences DQ667440.1 and HQ418964.1 exactly, but using trnLF3/trnLR2, our four sequences did not match DQ667440.1 and HQ418964.1—our sequences differed by the two inserts mentioned above, present in the GenBank sequences but not in the ones we determined (Fig. 3 and Fig. S4b).

Using the trnLF1 and trnLR1 primers, we could distinguish the sequence of *S. divinorum* from 11 of the 12 species that we tested, all but *S. cedrosensis* (Table 3, Fig. S5). Using the trnLF3 and trnLR2 primers, there were differences between *S. divinorum* and all 12 of the other species, including *S. cedrosensis* (Table 4, Fig. S6).

Table 2
Sequence differences found between *rbcL* genes of *Salvia* species.

	div	gre	jam	chi	mic	ced	cle	dol
div	–	5	5	5	3	5	9	19
gre	5	–	0	0	2	4	13	18
jam	5	0	–	0	2	4	13	18
chi	5	0	0	–	2	4	13	18
mic	3	2	2	2	–	2	11	18
ced	5	4	4	4	2	–	13	20
cle	9	13	13	13	11	13	–	19
dol	19	18	18	18	18	20	19	–

Sequences were amplified using primers rbcLF1 and rbcLF2. div, *S. divinorum*; gre, *S. greggii*; jam, *S.* × *jamensis*; chi, *S. chionophylla*; mic, *S. microphylla*; dol, *S. dolomitica*; ced, *S. cedrosensis;* cle, *S. clevelandii*.

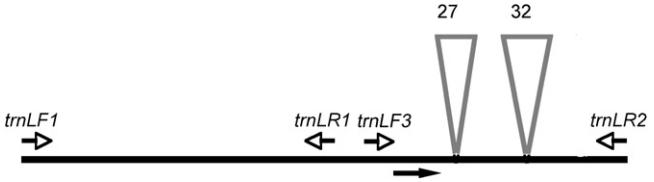

Fig. 3. Graphical description of a section of chloroplast DNA of *Salvia* species containing the *trnL* intron, part of the *trnL* gene and the *trnL-trnF* intergenic spacer, and showing the relative locations of primers used in this work and of two insertions for *S. divinorum* noted in previous GenBank accessions (see text). The total length of the DNA diagrammed here is 598 bp. The solid arrow shows the putative position of the 3′ segment of the *trnL* gene. The 5′ segment of the *trnL* gene starts approximately 200 bp to the left of this section; the trnF gene starts approximately 130 bp to the right.

Table 3
Sequence differences found between *trnL* genes of *Salvia* species.

	div	ced	mic	gre	jam	chi	api	cle	off	lev	rec	dol	syl
div	–	0	1	2	2	2	9	11	12	12	13	17	17
ced	0	–	1	2	2	2	10	11	12	12	13	17	17
mic	1	1	–	3	3	3	11	12	13	13	14	18	18
gre	2	2	3	–	0	3	10	11	10	10	11	15	15
jam	2	2	3	0	–	3	10	11	10	10	11	15	15
chi	2	2	3	3	3	–	11	13	8	13	14	18	17
api	9	10	11	10	10	11	–	1	11	11	12	12	14
cle	11	11	12	11	11	13	1	–	10	10	11	11	13
off	12	12	13	10	10	8	10	11	–	0	1	5	6
lev	12	12	13	10	10	13	10	11	0	–	1	5	6
rec	13	13	14	11	11	14	11	12	1	1	–	6	7
dol	17	17	18	15	15	18	11	12	5	5	6	–	7
syl	17	17	18	15	15	17	13	14	6	6	7	7	–

Sequences were amplified with primers trnLF1 and trnLR1. div, *S. divinorum*; ced, *S. cedrosensis*; mic, *S. microphylla*; gre, *S. greggii*; jam, *S.* × *jamensis*; chi, *S. chionophylla*; off, *S. officinalis*; lev, *S. lavandulifolia*; rec, *S. recognita*; dol, *S. dolomitica*; syl, *S.* × *sylvestris*; cle, *S. clevelandii*; api, *S. apiana*.

Table 4
Sequence differences found between *trnL* genes of *Salvia* species.

	div	mic	jam	chi	cle	api	ced	gre	dol	rec	syl	lev	off
div	–	4	4	4	5	5	7	8	10	11	12	12	12
mic	4	–	0	0	7	7	3	4	8	11	10	12	12
jam	4	0	–	0	7	7	3	4	8	11	10	12	12
chi	4	0	0	–	7	7	3	4	8	11	10	12	12
cle	5	7	7	7	–	0	10	11	11	12	13	13	13
api	5	7	7	7	0	–	10	11	11	12	13	13	13
ced	7	3	3	3	10	10	–	6	10	12	13	14	13
gre	8	4	4	4	11	11	6	–	12	11	10	12	12
dol	10	8	8	8	11	11	10	12	–	5	2	6	6
rec	11	11	11	11	12	12	12	11	5	–	3	1	1
syl	12	10	10	10	13	13	13	10	2	3	–	4	4
lev	12	12	12	12	13	13	14	12	6	1	4	–	0
off	12	12	12	12	13	13	13	12	6	1	4	0	–

Sequences were amplified with primers trnLF3 and trnLR2. *Abbreviations*: see Table 3.

3. Discussion

PCR identification of *S. divinorum* DNA in samples provides a rapid and convenient method of identifying plants, leaves, and shredded samples of the plant. The DNA sequences of the *rbcL* genes include restriction sites that allow a distinction between samples of *S. divinorum*, *C. sativa*, and *N. tabacum* (Fig. 2) and mixtures of these (data not shown). A January, 2012 BLAST search on GenBank, using the *rbcL* amplicon shown in Fig. S3, retrieved the sequence from *S. divinorum* with no ambiguities or differences. However, the next 24 entries, species of *Salvia*—with one exception, another genus of the mint family—showed very high similarities.

A comparison of the *trnL* ampicons also excludes plants of other families and gives better distinction between *Salvia* species. A BLAST search using the amplicon shown in Fig. S5 retrieved sequences, the first 100 of which were solely from *Salvia* species. Using the amplicon shown in Fig. S6, the first 100 sequences retrieved were all from mint family plants; 94 were from species of *Salvia*. Although the numbers of differences between *S. divinorum* and certain other *Salvias* are low (Tables 3 and 4), these numbers relate to a shorter amplified sequence and include some very characteristic multi-base insertion/deletions (Figs. S5 and S6). The sequences that would be amplified by the trnLF1/trnLR1 and trnLF3/trnLR2 primers, accessed from 148 GenBank records (but neglecting the inserts that we could not confirm), were able to distinguish every species from *S. divinorum*, except for *S. venulosa*. *S. venulosa* has already been reported to be a close relative of *S. divinorum* [10] on the basis of gene sequences. *S. venulosa* is a very rare plant that, at least so far, is unlikely to turn up in forensic investigations.

We have no explanation for the differences between *trnL* sequences of *S. divinorum* that we amplified with trnLF3/trnLR2 primers and those reported in GenBank earlier (Fig. 3; Fig. S4). We also found differences in some *trnL* sequences amplified using trnLF1/trnLR1 primers: the sequences from *S. divinorum* and five other species matched the GenBank data, but our sequences of *S. greggii* and *S. sylvestris* differed from the web sequences by lacking inserts of 10 and 6 bases, respectively.

It seems that an investigator using the *trnL* sequence to test an unknown sample might incorrectly reject it as *S. divinorum* if it lacked the insertions noted in Fig. 3. At present, we suggest that an investigator who uses *trnL* sequences to identify a *Salvia* sample consider both web sequences and those presented here.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2012.04.004.

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