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REGULAR PAPER

Evolution and origins of the Mazatec hallucinogenic sage, *Salvia divinorum* (Lamiaceae): a molecular phylogenetic approach

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Abstract *Salvia divinorum* Epl. & Játiva-M. (*Lamiaceae*) is a potent hallucinogenic plant that is classified within *Salvia* subgenus *Calosphace*, section *Dusenostachys*, and hypothesized to be an interspecific hybrid. It is of ethnobotanical significance due to its employment in traditional healing ceremonies by the Mazatecs of Oaxaca, Mexico, and due to its unique pharmacology—a highly selective, non-nitrogenous, κ-opioid receptor agonist. In order to test its phylogenetic position and putative hybridity, we sequenced multiple DNA regions (ITS, *trn*L-*trn*F, and *psb*A-*trn*H) of 52 species—representing the major lineages of subgenus *Calosphace*—and six accessions of *S. divinorum*. Our molecular phylogenetic results suggest that *S. divinorum* should not be classified within *Dusenostachys* and that it is not a hybrid. Additionally, we determine that the closest known relative of this psychoactive Mexican sage is *S. venulosa*, a rare endemic of Colombia.

Keywords Diviner's sage · Hallucinogens · Medicinal plants · Psychoactive plants · *Salvia divinorum* · *Ska Maria Pastora*

Introduction

Salvia divinorum Epling & Játiva-M. [*Labiatae*] is powerful psychoactive plant that together with *teonanácatl,* the so-called "magic mushrooms" (*Psilocybe* spp. [*Strophariaceae*]) (Schultes 1940; Wasson 1961), and the hallucinogenic morning glory, *ololiuqui* (*Turbina corymbosa* (L.) Raf. [*Convolvulaceae*]) (Schultes 1941), plays an important role in psycho-spiritual mythology and healing ceremonies of the Mazatecs of northeastern Oaxaca, Mexico, to whom it is known as *xka pastora* or *ska Maria pastora* ("the leaves of Mary the shepherdess") (Wasson 1962). A diterpene, salvinorin A, has been shown to be pharmacologically responsible for the hallucinogenic activity of *Salvia divinorum* (Siebert 1994), functioning as a highly selective κ-opioid receptor agonist (Roth et al. 2002; Yan and Roth 2004). Medical application in a clinical setting has been documented (Hanes 2001, 2003; Vortherms and Roth 2006).

Wasson (1962) first described the ethnobotany and ceremonial uses of *ska Pastora,* and reported that *Salvia divinorum* only rarely flowers and never sets seed in the Sierra Mazateca. Flowering specimens were sent to Carl Epling who described *Salvia divinorum* as a new species (Epling and Játiva-M. 1962). Epling (1939) had previously revised *Salvia* subgenus *Calosphace* (Benth.) Benth., the largest (~500 spp.) of five *Salvia* subgenera (Bentham 1876; Walker 2006), with species ranging in the North from the United States, through Mexico and Central America, and throughout much of South America. In his

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monograph, Epling (1939) erected 91 sections within the subgenus; *Saliva divinorum* was placed within section *Dusenostachys* (Epl.) Epl. (Epling and Játiva-M 1962), and was hypothesized to be closely related to *S. concolor* Lamb. ex Benth. of that section.

The botany of *Salvia divinorum* was most thoroughly examined by Reisfield (1987, 1993) who not only conducted extensive fieldwork but also implemented a series of greenhouse breeding experiments. In the Sierra Mazateca, Reisfield (1993) located 12 populations, all located within a few square miles, however these populations were reported to be clonal stands and assumed to be the result of human introduction due to their anthropogenic distribution. Chromosome studies indicated that the species was diploid ($2n = 22$) with normal meiotic pairing. Pollen viability was shown to be poor (56% aborted or inviable) when compared with three control species; however, no pollen-tube inhibition was observed, suggesting normal pollination and a lack of self-incompatibility. Hand-pollination experiments resulted in low seed set, attributed to possible post-zygotic embryo abortion or endosperm failure, however it is uncertain how meaningful these results are since no other species were pollinated as a control group. These cumulative results were accounted for by three hypothetical explanations: self-incompatibility (although the absence of pollen-tube inhibition mitigates against this possibility), inbreeding depression, and/or interspecific hybridity. Reisfield (1993) concluded that interspecific hybridity is the most likely explanation for the low pollen viability and low seed set; no putative parental species, however, were suggested. Interspecific hybridization and introgressive speciation are reported to be infrequent within *Calosphace* (Haque and Ghoshal 1981; Ramamoorthy and Elliott 1998), however polyploidy is well-documented and appears to be more frequent among the South American species than the North American (Alberto et al. 2003).

Previous molecular studies (Walker et al. 2004; Walker and Sytsma 2007; Jenks 2009) have strongly supported the monophyly of subgenus *Calosphace*, to which *Salvia divinorum* belongs; however the intra-subgeneric classification has only begun to be elucidated (Walker 2006; Jenks 2009).

In this paper we examine the phylogenetic history of *Salvia divinorum* and test its putative hybrid origin by sequencing multiple regions of nuclear and chloroplast DNA. Specifically, is *S. divinorum* correctly classified and most closely related to the species of section *Dusenostachys*? Is *Dusenostachys* monophyletic? What is the evolutionary history and biogeography of *S. divinorum* and its closest relatives; are they of Mexican origin? Is *S. divinorum* of recent hybrid origin, and if so what are the putative parents?

Materials and methods

Taxon sampling and outgroup selection

Sequence data was obtained for 48 species of *Calosphace* subgenus and 4 outgroups species. *Calosphace* has been strongly supported as monophyletic in previous studies; outgroup taxa were chosen based upon these previous molecular phylogenetic studies (Walker et al. 2004; Walker and Sytsma 2007; Jenks 2009). The outgroup species were *Dorystaechas hastata* Boiss. & Heldr. ex Benth. (a monotypic Asian genus) and three species of *Audibertia, Salvia greatae* Brand., *S. californica* Brand., and *S. mellifera* Greene. Our sampling here includes species from 35 of Epling's sections within *Calosphace*, representing all previously identified major clades (Jenks 2009). Six accessions of *Salvia divinorum*, representing five populations from the Sierra Mazateca, were sequenced to determine whether there was any intra-specific sequence variability. All samples were either collected by the authors or obtained from herbarium specimens. Accession information and vouchers for the taxa analyzed in this study are given (see Electronic Supplementary Materials).

PCR amplification, sequencing, and sequence analyses

Total genomic DNA was extracted via DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Extractions were made from fresh, dried, or frozen leaf material. Polymerase chain reaction (PCR) amplifications were typically prepared in 25 µl reactions; PCR reactions were performed on a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, MA, USA). The PCR profile was as follows: 2 min of initial denaturation at 95°C, 35 cycles with a 1 min denaturation at 94°C, 2 min annealing at 52°C, and 2 min extension at 72°C, with a final extension of 10 min at 72°C. The entire nuclear ribosomal internal transcribed spacer (ITS) region (including ITS1, the 5.8S gene, and ITS2) was amplified with primers ITS5 and ITS4, or alternately in the case of more highly degraded DNA from herbarium specimens, in two parts with primer combinations ITS5-ITS2 and ITS3-ITS4 (White et al. 1990). The noncoding, intergenic spacer region of chloroplast DNA, *psb*A-*trn*H, was amplified using forward and reverse primers as described previously (Kim et al. 1999). In a previous study (Jenks 2009) we were unable to obtain good chloroplast resolution based on this region, so for this study with greatly decreased sample size we sequenced a second chloroplast spacer region, *trn*L-*trn*F, in order to improve resolution, allowing comparison of *Salvia divinorum*'s placement between biparentally-inherited ITS region and maternally-inherited chloroplast DNA while assessing putative hybridity. Forward and reverse primers for

trnL-trnF were used (Taberlet et al. 1991). All PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Sequencing reactions were carried out for the purified PCR products using Big Dye Terminator Cycle Sequencing reagents (Applied Biosystems, Foster City, CA, USA). Sequencing primers used were identical to amplification primers. Sequence fragments were assembled and edited using Sequencer version 4.2.2 (Gene Codes, Ann Arbor, MI, USA). The corrected consensus sequences were aligned manually in MacClade v4.06 (Maddison and Maddison 2000).

Initially all data sets were analyzed independently; however the *psb*A-*trn*H region proved problematic with resolution very poor to none due to extremely low sequence variability (low % of parsimony informative characters) and identical sequences shared by a large number of the taxa. As a result, the two cpDNA datasets were combined for further analysis. The two datasets (ITS and combined cpDNA) were analyzed using an equally weighted, unordered maximum parsimony (MP) approach (Fitch 1971) implemented in PAUP version 4.0 (Swofford 2002). A heuristic search performed with 100 replicates of random addition sequences. Branch-swapping was via stepwise addition with 100 trees held at each step; tree-bisection-recombination (TBR) and MulTrees options were in effect. The maximum number of trees held was 100,000. Branches were collapsed if their score was zero, topological constraints were not enforced, gaps were coded as missing data, and characters were unordered. MP bootstrap analyses (Felsenstein 1985) were performed using 100 replicates, random taxon addition with 10 replicates per replicate and not more than 1,000 trees held at each step.

Results

Phylogenetic analyses

The ITS data matrix had a total aligned sequence length of 642 characters of which 183 were variable and parsimony informative (28.5% of total) (Table 1). The phylogenetic analysis yielded 547 equally most parsimonious trees with a tree length of 909 (CI = 0.4653, HI = 0.5347, RI = 0.5525). The combined *psb*A-*trn*H/*trn*L-F matrix had a aligned sequence length of 1,510 characters of which only 84 were variable and parsimony informative (5.6% of total) (Table 1). Phylogenetic analysis resulted in more than 100,000 equally parsimonious trees, however, the heuristic MP search was unable to run to completion and was truncated after 4 days. These most parsimonious trees had a tree length of 250 (CI = 0.8200, HI = 0.1800, RI = 0.8732). The 50% majority-rule consensus tree of each data set is presented (Figs. 1, 2; ITS and combined cpDNA, respectively).

Table 1 Numbers of taxa and characters with tree statistics for the maximum parsimony (MP) analyses of both ITS and combined cpDNA datasets

	ITS	Combined cpDNA
Number of taxa		
Ingroup	48	48
Total	52	52
Sequence characters		
Aligned sequence length (bp)	642	1,510
Constant sites	363 (56.5%)	1,321 (87.5%)
Total variable sites	277	189
Parsimony informative sites	183 (28.5%)	84 (5.6%)
Tree statistics		
Number of MP trees	547	>100,000
Tree length	909	250
Consistency index	0.4653	0.8200
Retention index	0.5525	0.8732

In the ITS phylogeny (Fig. 1), subgenus *Calosphace* is moderately-supported as monophyletic [72% bootstrap probability (BP)], with *S. axillaris* sister to the rest of the subgenus. Section *Dusenostachys* is strongly supported as monophyletic (100% BP). Section *Erythrostachys* is also supported as monophyletic (77% BP). Additional clades with support and previously identified (Jenks 2009) are "*Hastatae* clade" (98% BP), "*Uliginosae* clade" (60% BP), and "core *Calosphace*" (52% BP), which includes the "*Fulgentes* clade" (93% BP). Since it is not the purpose of this paper to discuss the phylogeny of the subgenus at large, these clades will not be discussed further herein. However, it should be noted that these clades are congruent with those previously identified and described (Jenks 2009). *Salvia divinorum* and *S. venulosa* are strongly supported as sister species (98% BP).

Calosphace is strongly supported as monophyletic (100% BP) in the cpDNA phylogeny as well (Fig. 2). The monophyly of "*Hastatae* clade" (100% BP), "*Uliginosae* clade" (100% BP), and the "core *Calosphace*" clade (100% BP) is supported by our results as is section *Dusenostachys* (100% BP). *Salvia divinorum* and *S. venulosa* are strongly supported as sister to each other (100% BP) in the cpDNA phylogeny (Fig. 2) as they were in the ITS phylogeny (Fig. 1).

Sequence analysis

The ITS sequence data of all six sampled accessions of *Salvia divinorum* were searched, base-pair by base-pair, for

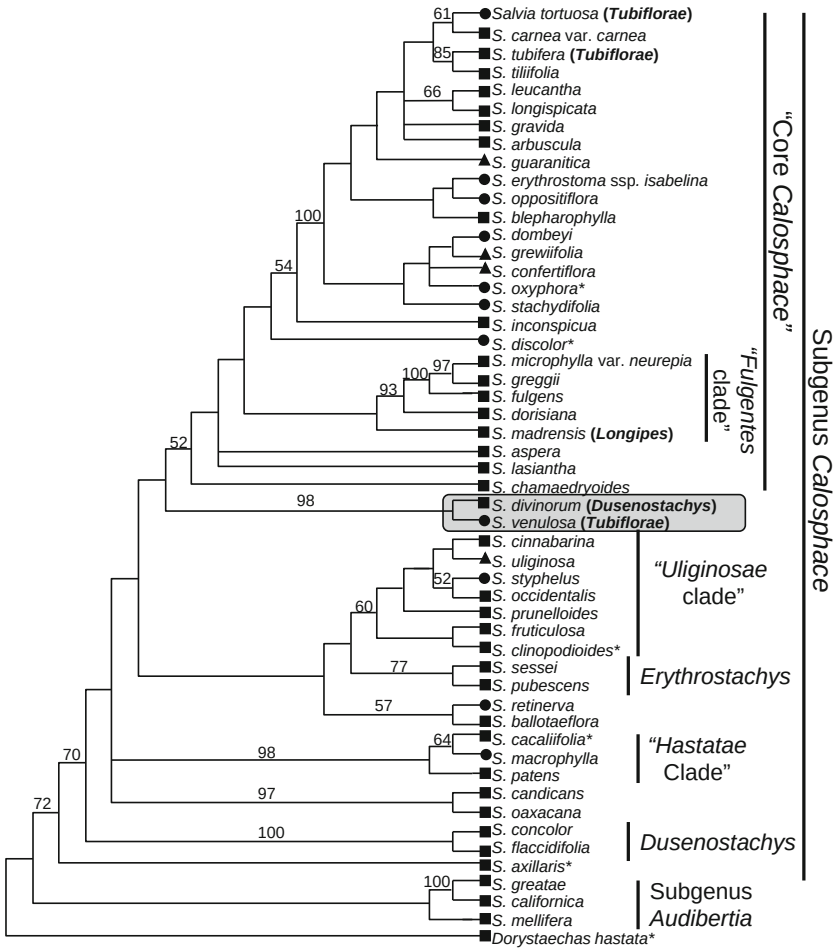

Fig. 1 Majority-rule (50%) consensus tree of 547 based on MP analysis of ITS dataset. The MP bootstrap support values above 50% are shown above the branches. Species distribution are represented by shape: *square* Mexico/Central America, *circle* Andes, and *triangle* eastern Brazil. An *asterisk* following a species name indicates a monotypic section or genus. *Bold* names in parentheses following species names are their respective taxonomic sections as discussed in the text

sequence additivity. Since ITS is inherited bi-parentally, a recent interspecific hybrid would exhibit DNA regions with a high percentage of polymorphisms, i.e. two different nucleotides at each site, one from each parent donor. Sequence additivity in the six sampled accessions of *S. divinorum* was not identified. Additionally, the two sequenced accessions of *S. venulosa*, representing two populations, shared an identical ITS sequence containing no polymorphic loci.

Discussion

Phylogenetic position and closely related species of *Salvia divinorum*

Epling's original placement of *Salvia divinorum* within section *Dusenostachys* and closely related to *S. concolor* (Epling and Játiva-M. 1962) were not supported by our results; the other sampled *Dusenostachys* species, *S. concolor* and *S. flaccidifolia* Epl., formed a strongly supported monophyletic clade which does not include *S. divinorum,* in either the ITS (Fig. 1) or cpDNA phylogenies (Fig. 2). In a previous study (Jenks 2009), a third *Dusenostachys* species, *S. recurva* Benth., was also well resolved within this section—strongly supported (100% BP) as sister to *S. concolor* and *S. flaccidifolia*. Morphologically, *Dusenostachys* is characterized by large, ventricose violet-blue corollas, calyces with upper lips with 5 or 7 major veins, typically hollow fragile stems, and a generally hydrophytic habit as exhibited by large clonal stands in seasonal or perennially-flowing waterways. Although *S. divinorum* is certainly hydrophytic, its floral morphology is significantly different from other species in *Dusenostachys*. *Salvia divinorum* possesses white corollas of moderate size and slightly sigmoidal rather than ventricose conformation, and it has violet calyces with upper lips distinctly three-veined. Aside from the single Brazilian *Dusenostachys* species, *S. oligantha* Dusen. (synonym, *S. lepida* Epl.), *Dusenostachys* is comprised of entirely endemics of central and southern Mexico. While our results do not support the inclusion of *S. divinorum* within *Dusenostachys, S. divinorum* is strongly supported as sister to the Colombian endemic, *S. venulosa,* in both the ITS and cpDNA phylogenies (Figs. 1, 2).

There are a number of morphological similarities between *Salvia divinorum* and *S. venulosa*, strikingly

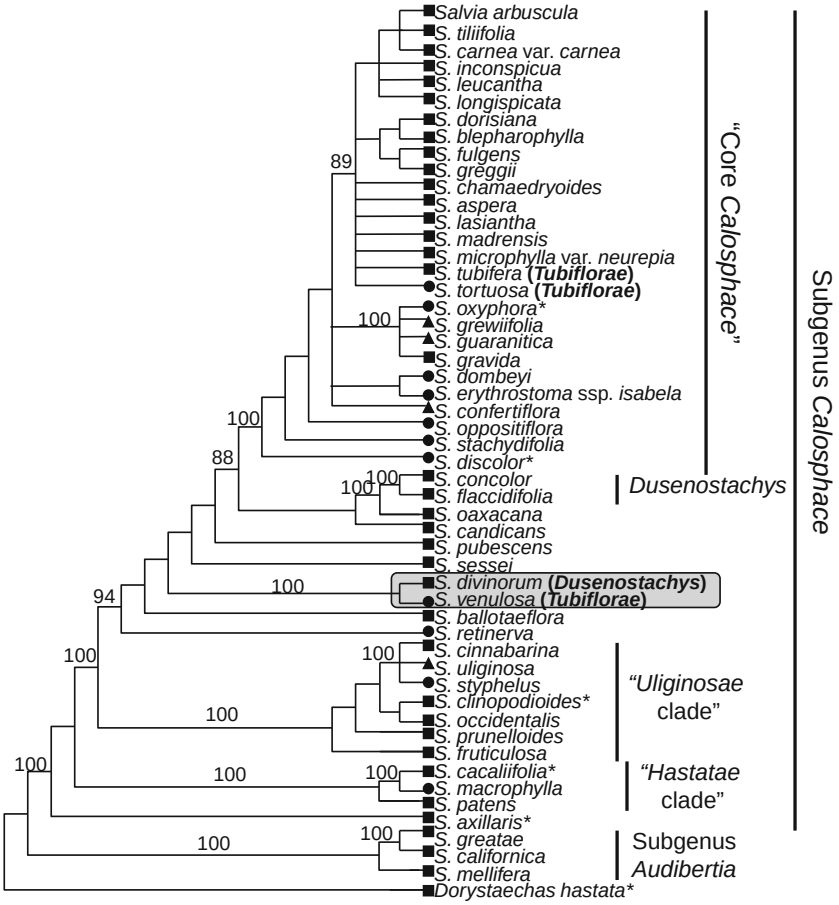

Fig. 2 Majority-rule (50%) consensus tree of 100,000 based on MP analysis of combined cpDNA dataset (*trn*L-F and *psb*A-*trn*H). The MP bootstrap support values above 50% are shown above the branches. Species distribution are represented by shape: *square* Mexico/Central America, *circle* Andes, and *triangle* eastern Brazil. An *asterisk* following a species name indicates a monotypic section or genus. *Bold* names in parentheses following species names are their respective taxonomic sections as discussed in the text

resembling each other morphologically, and most significantly in floral reproductive structures. Both species have racemes of well-spaced verticillasters of three to six flowers subtended by deciduous violet bracts. The calyces of both species are violet and pedicelate, while the interiorly epapillate corollas are densely villous externally and have upper and lower labia of nearly equal length. The pubescent styles of each are slightly exserted while the anthers are included within the galea. Both species have "Type 4" stamens (Jenks 2009), the most derived and common of four stamen types within *Calosphace*: defined as having an elongated, lever-forming connective, the posterior branches of which are sterile, fused, and possessed a small retrorse tooth. The floral differences between the two species are corollas of different colors (*S. divinorum* = white to pink; *S. venulosa* = a light red) and calyces with occasionally different numbers of veins on their upper lips (*S. divinorum* = 3; *S. venulosa* = 3 or 5). Vegetatively, these two species are quite similar to one another with narrowly ovate, typically glabrous leaves, acuminate at the apex and attenuate at the base (the blade decurrent onto the petiole and the petiole scarcely differentiated if at al from the blade). The hollow stems of both species are decumbent and when in contact with moist soil or water rooting vigorously at the nodes and characteristically, even along the internodes. They share a similar habit of growing in shaded, wet ravines or ditches.

Salvia venulosa is a very local endemic, known from only three locations found in deeply shaded, moist ravines in the westernmost cordillera of the Colombian Andes in the district of Risaralda from 1,500 to 2,000 m elevation. It is of interest that no other Colombian natives are reported to grow in this north-westernmost extension of the Colombian Andes (Wood and Harley 1988). We are unable to find any documented use of medicinal or other uses of *S. venulosa*, which was placed in section *Tubiflorae* (Epl.) Epl. (Epling, 1939), a primarily Colombian section. Two other species of the section are sampled in our study, *S. tubifera* Cav. of Mexico and *S. tortuosa* Kunth of Colombia, however, *S. venulosa* is not closely related to either species (Figs. 1, 2), *Tubiflorae* in our results proving to be highly polyphyletic. Morphologically *S. tortuosa* does not appear to be closely related to the rest of the section (Wood and Harley 1988), therefore further sampling of Colombian *Tubiflorae* (specifically, *S. camaraefolia* Benth., *S. secundiflora* Rusby and *S. falcata* Wood & Harley) is needed to determine whether any of these species are closely related to *S. divinorum* and *S. venulosa*.

Biogeography

Previous work has concluded that Mexico is the center of diversity (Neisess 1983; Reisfield 1987; Ramamoorthy et al. 1988; Harley and Heywood 1992; Walker 2006; Jenks 2009) for the monophyletic (Walker et al. 2004; Jenks 2009) subgenus *Calosphace* and that the South American species and lineages arose from within the Mexican taxa (Walker 2006; Jenks 2009). Previous results (Walker 2006; Jenks 2009) show that there have been multiple long-distance dispersal events from Mexico to South America. Further sampling of the Colombian *Tubiflorae* is required to determine whether *S. divinorum* and *S. venulosa* are part of a larger Colombian lineage. At present, the most parsimonious explanation is that the common ancestor of both species arose in the Mexican center of diversity with subsequent dispersal of the *S. venulosa* ancestor to the northern Andes.

Regardless, the reported lack (Wasson 1962; Reisfield 1987) of sexual reproduction of *Salvia divinorum* in the Sierra Mezateca (the only region from which it is known) is somewhat problematic if that is indeed the native range of the species and introduces again the question of whether *S. divinorum* is the Aztec plant *pipilzintzintli* as has been hypothesized (Wasson 1962; Ott 1995). *Pipilzintzintli* was consumed orally by the Aztecs (as *S. divinorum* is by the Mazatecs) to psychoactive effect (De Vetancurt 1698) and is depicted in the Dresden Codex as having bilabiate corollas (Emboden 1983) providing some slight evidence for this identification. If *pipilzintzintli* is correctly identified as *S. divinorum*, it would be reasonable to assume that this species was once used and potentially found growing over a much larger geographic area than its current, extremely limited distribution in the Sierra Mazateca. Reports of other indigenous groups besides the Mazatecs, i.e. the Cuicatec, Chinantec, and Otomí, using leaves in the same manner for divination and visions (Reko 1945; Weitlaner 1952) also supports the hypothesis of a once-larger range and distribution. Ott (1995) makes a case for *S. divinorum* being non-native and a post-conquest introduction to the Sierra Mazateca based most strongly upon linguistic evidence: there is no indigenous name for *S. divinorum* in the Mazatec language, *Ska Maria Pastora* ("Leaves of Mary the Shepherdess") being a Spanish modernism referencing two post-Spanish introductions: the Virgin Mary and sheep. Seeming to support this are the lack of any documented wild populations in the Sierra Mazateca and Reisfield's (1993) observations that all of the known populations there are clonal and all were at least potentially the result of human introduction. Nahua trade arteries passed through the Papaloapan drainage basin (Reisfield 1987), in the eastern Mazatec country, indicating at least a potential anthropogenic vector for presence of *S. divinorum* in the Sierra Mazateca. Regardless of whether or not *S. divinorum* is native to the Sierra Mazateca, the conditions there do not appear to be adequate for sexual reproduction, suggesting that the origins of this plant are elsewhere.

Putative hybrid origin/reproductive biology

In the most thorough experimental study of *Salvia divinorum,* Reisfield (1993) concluded that this species was probably of recent interspecific hybrid origin and possibly self-incompatible or suffering from inbreeding depression. Our results suggest that *S. divinorum* is not a recent hybrid. Nuclear additivity, showing biparental inheritance, as evidenced by a high degree of polymorphisms, was not observed in the ITS sequence of *S. divinorum* (6 accessions sampled). Additionally, our results do not show incongruence between the ITS (Fig. 1) or chloroplast (Fig. 2) phylogenies, i.e. *S. divinorum* is strongly supported as sister to *S. venulosa* in both phylogenies, and these two species form a clade that is positioned similarly in both trees. Non-additivity and congruence between the ITS and cpDNA phylogenetic placement of *S. divinorum* together strongly suggest that this species is not an interspecific hybrid. Therefore, if *S. divinorum* is not a hybrid as our results suggest, how can we account for Reisfield's (1993) findings that led him to conclude that it was an interspecific hybrid?

While Reisfield (1993) observed no irregular pairing during meiosis in *Salvia divinorum*, some stage of gametogenesis was irregular since half the pollen grains aborted. While our results indicate that this is not due to interspecific inbreeding depression (or depression due to hybridity) as Reisfield speculated, a lack of genetic diversity within the Sierra Mazateca due to repeated clonal propagation in an already limited gene-pool could account for this depression. In his pollination experiment, Reisfield (1993) reported extremely low percentages of *S. divinorum* nutlet maturation. Thus, he concluded that the low seed set was most likely the results of hybridity (and accompanying disharmonious genic or chromosomal interactions) or perhaps self-incompatibility or inbreeding depression. Self-incompatibility had been speculated previously to be the cause of low or lack of seed set (Valdés et al. 1987), but Reisfield (1993) observed no pollen-tube inhibitions during pollination as would be expected in a self-incompatible species. Further pollination experiments should be commenced to determine whether *S. divinorum* truly has a lower percentage of nutlet maturation than other species of subgenus *Calosphace*.

Conclusions

Salvia divinorum is not closely related to the other sampled species of the Mexican section *Dusenostachys* as previously

described (Epling and Játiva-M. 1962) and should no longer be classified within that section. In both the ITS and cpDNA phylogenetic trees, *S. divinorum* is strongly supported as sister species to *S. venulosa,* and not resolved within "core *Calosphace*". *Salvia divinorum* and *S. venulosa* are not closely related to the other sampled species of the largely Colombian section *Tubiflorae* (in which *S. venulosa* is classified) which is highly polyphyletic in our results.

Salvia divinorum does not appear to be an interspecific hybrid as previously hypothesized (Reisfield 1993) since it shows neither additivity in the biparentally-inherited, nuclear ITS sequence nor incongruence between the ITS and chloroplast DNA phylogenetic trees. *Salvia divinorum* appears to be a reproductively capable species, however because of its extensive anthropogenically-mediated, clonal propagation in the Sierra Mazateca, its ability to reproduce sexually seems to be somewhat limited as evidenced by the decreased pollen viability previously reported (Reisfield 1993). While it is uncertain whether *Salvia divinorum* once prospered over a larger geographic range or whether it is correctly identified as the Aztec etheogen, *Pipilzintzintli* (although both speculations are certainly plausible based on the scant available evidence), there has doubtless been an ancient alliance between *Salvia divinorum* and humans who have used it to visionary effect. However, this close association does not necessarily mean that this species is a cultigen, i.e. a species requiring human intervention to reproduce. Further research is needed to determine what barriers to sexual reproduction might be responsible for the seemingly low sexual viability of the species and to determine the genetic diversity within the species. And, while *S. venulosa* does not appear to biosynthesize the psychoactive clerodane diterpenoid, salvinorin A (D. Siebert, personal communication), further studies are underway to determine the chemical constituents of this species.

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