



Perspectives on the systematics and phylogenetics of *Guaiacum* (Zygophyllaceae): complexities in conservation of endangered hardwoods due to fragmentation, introgression, and intermittent gene flow

Perspectiva en la sistemática y filogenia de *Guaiacum* (Zygophyllaceae): dificultad en la conservación de maderas duras económicamente importantes debido a fragmentación, introgresión y flujo intermitente de genes

Joseph R. Dertien¹✉ and Melvin R. Duvall²

¹Department of Biological Sciences, 3700 West 103rd Street, Saint Xavier University, Chicago, IL 60655, USA.

²Department of Biological Sciences, 1425 W. Lincoln Hwy, Northern Illinois University, DeKalb, IL 60115, USA.

✉ dertien@sxu.edu

Abstract. The systematics of the economically important, endangered hardwoods in *Guaiacum* are unclear with regard to taxonomic ranks, and the relationships among taxa. This is partially due to a lack of diagnostic characters and minimal geographic sampling in previous studies. Nevertheless, systematic relationships are important to inform trade regulations and management practices for these species. This is especially true for Mexico, which is both the primary exporter and the center of diversity for *Guaiacum*. Systematic and biogeographic issues in *Guaiacum* were investigated by analyses of nuclear and chloroplast DNA markers from specimens sampled throughout the range. Phylogenetic and statistical parsimony analyses showed well-supported divergences within the group, including a deep divergence between *G. officinale* and other taxa with additional biogeographically correlated subdivisions. There is also an indication that accessions from Chiapas, Mexico are genetically intermediate between *G. sanctum* and *G. coulteri*, while minor segregates of *Guaiacum* (*G. palmeri*, *G. guatemalense*) were not well distinguished by either morphological or molecular characters. The genetic structuring among the major groups of *Guaiacum* shows evidence of isolation induced by fragmentation of the range, with the structure largely maintained with only occasional long distance gene flow between remote populations.

Key words: dry tropical forest, haplotypes, hardwoods, Larreoideae.

Resumen. La sistemática de las especies maderables, económicamente importantes y en peligro de extinción en *Guaiacum* aún no aclara con respecto a la clasificación taxonómica y las relaciones entre los taxa. Esto se debe parcialmente a la falta de rasgos diagnósticos y a reducidas muestras geográficas en estudios previos. Sin embargo, los aspectos en la sistemática y las relaciones filogenéticas de las especies son importantes a considerar en la regulación comercial y en las prácticas de manejo de las especies. Esto es especialmente importante para México ya que es tanto el exportador más importante, como el centro de diversidad para *Guaiacum*. En este trabajo se investigaron aspectos sistemáticos y biogeográficos en *Guaiacum* analizando marcadores nucleares y de cloroplastos de ADN de especímenes muestreados de su área de distribución. Los análisis filogenéticos y de parsimonia estadística mostraron divergencia bien sustentada dentro del grupo, incluyendo una divergencia profunda entre *G. officinale* y los otros taxa con subdivisiones adicionales asociadas a la biogeografía. También hay evidencia de accesiones de Chiapas, México, como genéticamente intermedias entre *G. sanctum* y *G. coulteri*, mientras que otras especies de *Guaiacum* (*G. palmeri*, *G. guatemalense*) no se distinguen bien sea por rasgos morfológicos o moleculares. La organización genética entre los mayores grupos de *Guaiacum* muestra evidencia de un aislamiento provocado por fragmentación en su distribución, con la estructura en gran parte mantenida por un flujo ocasional de genes a larga distancia entre poblaciones lejanas.

Palabras clave: bosques tropicales secos, haplotipos, árboles maderables, Larreoideae.

Introduction

Guaiacum L. (Zygophyllaceae R. Br.) is a New World genus comprised of a group of 4 to 8 commonly recognized taxa of tropical and subtropical hardwoods. These trees and shrubs are distributed primarily within the arid and semi-arid regions of the Caribbean basin and Mexico, and represent economically important species characterized by extremely dense and resinous wood.

Guaiacum sanctum L. is the most abundant and economically important species, and has therefore been the species of greatest conservation focus. Studies of population structure, density, and regeneration in the chief exporting country of Mexico have been conducted (López-Toledo et al., 2010, 2011b), and population level genetic studies have been conducted for Caribbean, Mexican, Floridian (Dertien and Duvall, 2009), and Costa Rican populations (Fuchs and Hamrick, 2010a, b). Furthermore, *Guaiacum sanctum* could act as an umbrella species in terms of conservation, as protection of populations could also benefit other endangered flora and fauna (López-Toledo et al., 2011a). *Guaiacum coulteri* A. Gray has been studied less extensively, primarily because it has lower economic importance and does not occur in protected areas (López-Toledo et al., 2010). The population genetics of *G. unijugum* Brandegees have also been studied for conservation purposes as it is a relatively rare endemic of the Cape region of Baja California, Mexico (McCauley et al., 2010).

Despite various conservation and genetic studies, the number of species within the genus remains unresolved, with as few as 4 and as many as 8 species commonly recognized. (Grow and Schwartzman, 2001a, b; Axelrod, 2002). The absence of clear species delimitation within the genus is particularly problematic for trade regulation by the Convention on International Trade in Endangered Species or Wild Fauna and Flora (UNEP-WCMC, 2007), developing management practices of *Guaiacum* species by exporting countries, and assessing extinction risk for species or local populations (IUCN, 2007). In practice, taxonomic distinctions are frequently based on geographic origin rather than morphological, reproductive, or genetic criteria. Currently, the entire genus is listed on CITES Appendix II because of the inability to distinguish timber of the different species and a taxonomy that is complicated by numerous synonyms, unsettled taxonomic ranks, and invalidly published names. The listing effectively renders all species of *Guaiacum* endangered from a regulation standpoint (López-Toledo et al., 2010) regardless of what is known about the status of individual populations.

Previous phylogenetic studies of *Guaiacum* have only included a single species as an exemplar, and were

therefore not informative at the species level (Sheahan and Chase, 2000; Lia et al., 2001). Other studies attempting to delimit species using morphological characters were not inclusive of all species, and failed to identify characters suitable for consistent unambiguous identification (Grow and Schwartzman, 2001b). Specifically, suites of morphological character combinations yield conflicting results in taxonomic identifications for specimens found in southern Mexico and Central America (Grow and Schwartzman, 2001b), an area coincidentally of great importance to the harvesting and trade of *Guaiacum*. A more robust analysis of evolutionary relationships within *Guaiacum* could provide useful insight for a future taxonomic revision, and a clearer understanding of these phylogenetic relationships could be directly applicable to conservation practices.

This study is an attempt to resolve evolutionary relationships, identify consistent genetic patterns, and discover novel genetic patterns among *Guaiacum* species by using a combination of analyses appropriate for several taxonomic levels. Maximum parsimony methods can resolve deeper nodes but leave shallower relationships unresolved where variation is lacking among closely related accessions. Statistical parsimony analyses, however, can resolve relationships down to single mutations separating closely related individuals in a manner similar to DNA barcoding (Schindel and Miller, 2005; Collins and Cruickshank, 2013). Any contrasting patterns from nuclear and chloroplast DNA markers can indicate potential hybridization events (Soltis and Kuzoff, 1995), and geographic correlations with genetic patterns may indicate populations of greater taxonomic or conservation interest.

In addition to broadly resolving evolutionary relationships, this study is designed to address the following particular phylogenetic and taxonomic issues associated with the genus:

- 1) *Guaiacum guatemalense* Planch. ex Vail and Rydberg while often identified as a distinct species in herbarium collections, is most commonly accepted as a synonym of *G. sanctum* (<http://www.theplantlist.org>). Individual specimens identified as *G. guatemalense* possess intermediate and shared morphological characteristics of *G. sanctum* and *G. coulteri*, suggesting a hybrid origin (Porter, 1972). This putative species is found in a region near the intersection/overlap of the ranges *G. coulteri* and *G. sanctum*;
- 2) *Guaiacum angustifolium* Engelm. and its synonym *Porlieria angustifolia* (Engelm.) A. Gray, may be more accurately described as a subspecies or variety of *G. coulteri*;
- 3) *Guaiacum unijugum* is a species endemic to the Cape region of the Baja peninsula. The species status is questionable, as its unique morphology may be

an environmental response of *G. coulteri* to the extreme desert conditions of the area, and 4) *Guaiacum coulteri* var. *palmeri*, while sometimes identified as the species *G. palmeri* Vail, may not have consistent diagnostic morphological characteristics or a unique genetic signature to warrant a distinction from *Guaiacum coulteri* var. *coulteri*.

Materials and methods

Taxon sampling. A total of 98 accessions of *Guaiacum* species were obtained from herbarium collections, living collections, or field-collected from populations in Mexico, Puerto Rico, and the Florida Keys (Table 1). Exemplars representing all currently recognized species were collected, as well as accessions of putative species and varieties. Specimens were also collected from major geographic regions covering the extent of the generic distribution, as well as specific regions of potential biogeographic (i. e., Curaçao) or other evolutionary interest. Multiple accessions of *Guaiacum* were collected from regions where species identification is difficult or inconsistent, particularly southern Mexico (states of Oaxaca, Chiapas, and Campeche) and Guatemala.

Porlieria chilensis was chosen as an outgroup based on previous phylogenetic studies that included *Guaiacum* and related genera (Sheahan and Chase, 2000; Lia et al., 2001).

DNA Extraction, PCR, and purification and sequencing. DNA was extracted from leaflets stored in silica gel or from herbarium specimens using either a modified CTAB method (Doyle and Doyle, 1987) or the DNeasy Plant Mini Kit (Qiagen, Valencia, California). The manufacturer's protocol was followed for the DNeasy Plant Mini Kit; however modifications were applied to the extraction protocol to increase DNA yield from degraded herbarium material. Modifications included an extended elution time of 10-20 minutes (Drábková et al., 2002) and in some cases, an overnight alcohol precipitation prior to RNase digestion. A PEG/NaCl (polyethylene glycol/salt) precipitation following RNase digestion was used to remove potentially PCR inhibiting soluble polysaccharides and secondary metabolites (Li et al., 1994).

Genetic markers were amplified via polymerase-chain reaction (PCR) using the FailSafe PCR system (Epicentre Technologies, Madison, Wisconsin) with buffer premixes "I" or "H".

The *trnL-F* chloroplast marker was amplified using primer pairs "C" and "F" and by following the PCR protocol described in Taberlet et al. (1991). The *trnS-G* marker, containing both the *trnS-trnG* intergenic spacer and the *trnG* intron, was amplified using primers "trnS"

and "3'trnG" and following the PCR protocol described in Shaw et al. (2005). Two additional internal primers, "trnG2G" and "trnG2S" (Shaw et al., 2005) were occasionally used in sequencing reactions in cases where short reads were obtained from external primers or where large poly-T regions created downstream sequencing artifacts. The complete nuclear ITS region was amplified (including ITS1, 5.8s, and ITS2) using the primer pair ITS4 and ITS5 (White et al., 1990). Additional accessions were sampled for the ITS2 region alone using the primer pair P3K (Kim and Jansen, 1994) and P4 (White et al., 1990; Simpson et al., 2004).

PCR products were prepared for sequencing using either the Wizard SV PCR clean-up system (Promega Corp., Madison, Wisconsin) or the Microcon YM100 system (Millipore Corp., Billerica, Massachusetts). Automated capillary sequencing was performed with ABI3730xl DNA analyzers (Macrogen, Seoul, South Korea) or Beckman Coulter CEQ-8000 DNA analyzers at the Northern Illinois University Core Sequencing Facility (NIU, DeKalb, Illinois).

Sequence alignment and analyses. Sequences were machine-aligned using the "Geneious" align function within the Geneious Pro V. 4.5 software package (Drummond et al., 2008). Parameters were set for a global alignment with free end gaps of 70% similarity (IUB 5.0/-4.5) with a gap opening penalty setting of 12 and gap extension penalty setting of 3. Minor manual adjustments were made to alignments to match patterns of tandem repeats within regions of insertion/deletion mutations (indels) within the sequence matrix. Variable positions were confirmed against original chromatograms using Geneious Pro V. 4.5 (Drummond et al., 2008) to confirm the nucleotide identity and signal strength at the variable position. Positions of variable nucleotides that could not be confidently confirmed due to weak or conflicting signal in chromatograms were treated as sequencing artifacts and excluded from analyses. Regions of high variability and ambiguous alignment were removed prior to analysis, as were regions of poly-nucleotide runs in which proper homology could not be assessed. Additionally, portions of the *trnS-G* marker were excluded as a result of poor quality or little overlap between forward and reverse primers in some accessions. Autapomorphic indel mutations in the outgroup taxon *Porlieria chilensis*, were also removed, including a 153 base pair (bp) insertion in the *trnS-G* region that was absent in all of the ingroup taxa. Indel mutations were coded as multi-state (0, 1, 2, 3) (González et al., 2006) and added to the sequence data matrix prior to phylogenetic analysis.

Phylogenetic analysis. Phylogenetic relationships were inferred using PAUP* (Swofford, 2002). The Maximum

Table 1. Collection information and GenBank accessions for all taxa used in this study. Specimens with shared accession numbers indicate identical DNA sequences for that marker, represented by a single GenBank submission

<i>Species</i>	<i>Extract ID</i>	<i>Voucher</i>	<i>Location</i>	<i>trnLF</i>	<i>trnSG</i>	<i>ITS</i>
<i>Guaiacum coulteri</i>	GuCou03	DEK: <i>L. López and Martínez M. 195</i>	Oaxaca, Mexico	JX682625	JX682626	JX486716
<i>G. coulteri</i>	GuCou04	DEK: <i>L. López and Martínez M., 197</i>	Oaxaca, Mexico	JX682629	JX682630	JX486722
<i>G. coulteri</i>	GuCou05	DEK: <i>L. López and Martínez M., 199</i>	Oaxaca, Mexico	JX682629	JX682630	-
<i>G. coulteri</i>	GuCou06	MICH: <i>W. R. Anderson and Anderson C., 5592</i>	Oaxaca, Mexico	JX682629	JX682630	-
<i>G. coulteri</i>	MEX02	MEXU: <i>R. Marroquín, s.n.</i>	Guatemala	EU253464	JX888937	JX486717
<i>G. coulteri</i>	MEX03	MEXU: <i>C.G. Hernández, 1553</i>	Oaxaca, Mexico	JX682631	JX682632	JX486722
<i>G. coulteri</i>	MEX04	MEXU: <i>M. Véliz and R. Luarca, 11265</i>	Guatemala	EU253464	JX888937	JX486717
<i>G. coulteri</i>	GuCou08	MEXU: <i>R. Marroquín, s.n.</i>	Guatemala	EU253464	EU258927	JX486717
<i>G. coulteri</i>	GuCou02	DEK: <i>L. López and Martínez M. 196</i>	Oaxaca, Mexico	EU253466	EU253475	JX486716
<i>G. coulteri</i> var. <i>palmeri</i>	GH06	GH: <i>C. Ritchie Bell, 17693</i>	Sonora, Mexico	-	-	JX486715
<i>G. coulteri</i> var. <i>palmeri</i>	GuCoPa01	MICH: <i>R.W. Cruden 1038</i>	Sonora, Mexico	JX888921	JX888920	JX486715
<i>G. coulteri</i> var. <i>palmeri</i>	TEX03	LL: <i>A.L. Reina G., 99-112A</i>	Sonora, Mexico	JX888921	JX888922	JX486715
<i>G. guatemalense</i>	GuGua01	DEK: <i>J.R. Dertien, 514</i>	Guatemala	EU253464	EU258927	JX486717
<i>G. officinale</i>	F06	F: <i>J. A. Steyermark, 62908</i>	Venezuela	EU253467	EU253476	JX901015
<i>G. officinale</i>	F07	F: <i>S. J. Record, 55, series 16,454</i>	Colombia	EU253467	EU253476	JX901016
<i>G. officinale</i>	GuOff04	DEK: <i>J.R. Dertien, 407</i>	Puerto Rico	EU253467	EU253476	JX901017
<i>G. officinale</i>	GuOff06	DEK: <i>J.R. Dertien, 424</i>	US Virgin Islands	EU253467	EU253476	JX901018
<i>G. officinale</i>	GuOff10	DEK: <i>J.R. Dertien, 423</i>	US Virgin Islands	EU253467	EU253476	JX901019
<i>G. officinale</i>	GuOff11	DEK: <i>J.R. Dertien, 501</i>	Puerto Rico	EU253467	EU253476	JX901020
<i>G. officinale</i>	GuOff18	DEK: <i>J.R. Dertien 505</i>	Puerto Rico	EU253467	JX888916	JX901021
<i>G. officinale</i>	GuOff22	Y: <i>E. Edwards, 93</i>	Dominican Rep.	EU253467	JX888923	JX901022
<i>G. officinale</i>	GuOff23	DEK: <i>J.R. Dertien 425</i>	Puerto Rico	EU253467	JX888924	JX901023
<i>G. officinale</i>	GuOff24	DEK: <i>J.R. Dertien 517</i>	Jamaica	EU253467	JX888917	JX901024
<i>G. officinale</i>	GuOff27	FTG: <i>accession 961380a</i>	Haiti	EU253467	JX888915	JX901025
<i>G. sanctum</i>	F04	F: <i>R. Espinosa and R. Aguilar 1035</i>	Costa Rica	JX888919	JX888918	JX486714
<i>G. sanctum</i>	F08	F: <i>Eduardo Lépiz, 206</i>	Costa Rica	JX888926	JX888925	JX669509
<i>G. sanctum</i>	GH02	GH: <i>ASJ van Proosdij, 575</i>	Curacao	EU253457	EU253473	JX486718
<i>G. sanctum</i>	GH03	GH: <i>ASJ van Proosdij, 989</i>	Bonaire	EU253457	EU253473	JX486718
<i>G. sanctum</i>	GuSan01	DEK: <i>J.R. Dertien, 508</i>	cultivated	EU253457	EU253473	-
<i>G. sanctum</i>	GuSan03	DEK: <i>J.R. Dertien 404</i>	Puerto Rico	EU253457	EU253473	JX486718
<i>G. sanctum</i>	GuSan04	DEK: <i>J.R. Dertien 413</i>	Puerto Rico	EU253457	JX888927	JX486718
<i>G. sanctum</i>	GuSan06	DEK: <i>J.R. Dertien 429</i>	Puerto Rico	EU253457	EU253473	JX669510
<i>G. sanctum</i>	GuSan07	DEK: <i>J.R. Dertien 428</i>	Puerto Rico	EU253457	EU253473	JX669510
<i>G. sanctum</i>	GuSan08	NIU Greenhouse	cultivated	EU253457	EU253473	-
<i>G. sanctum</i>	GuSan09	DEK: <i>J.R. Dertien 403</i>	Puerto Rico	EU253457	EU253473	-
<i>G. sanctum</i>	GuSan10	DEK: <i>J.R. Dertien 415</i>	Puerto Rico	EU253457	EU253473	-
<i>G. sanctum</i>	GuSan11	DEK: <i>J.R. Dertien 510</i>	Bahamas	EU253457	EU253473	-
<i>G. sanctum</i>	GuSan12	DEK: <i>J.R. Dertien 511</i>	Bahamas	EU253457	JX888928	-
<i>G. sanctum</i>	GuSan13	DEK: <i>L. López and Martínez M., 204</i>	Campeche, Mexico	EU253458	JX888929	-

Table 1. Continues

<i>Species</i>	<i>Extract ID</i>	<i>Voucher</i>	<i>Location</i>	<i>trnLF</i>	<i>trnSG</i>	<i>ITS</i>
<i>G. sanctum</i>	GuSan14	DEK: <i>L. López and Martínez M., 210</i>	Campeche, Mexico	EU253459	JX888930	JX486718
<i>G. sanctum</i>	GuSan15	DEK: <i>L. López and Martínez M., 200</i>	Campeche, Mexico	EU253464	EU258927	-
<i>G. sanctum</i>	GuSan16	DEK: <i>L. López and Martínez M., 203</i>	Campeche, Mexico	EU253461	JX888931	JX486718
<i>G. sanctum</i>	GuSan18	DEK: <i>J.R. Dertien 512</i>	Bahamas	EU253457	JX888932	-
<i>G. sanctum</i>	GuSan19	DEK: <i>J.R. Dertien 513</i>	Bahamas	JX888935	JX888933	-
<i>G. sanctum</i>	GuSan20	DEK: <i>L. López and Martínez M., 194</i>	Yucatan, Mexico	EU253464	EU258927	-
<i>G. sanctum</i>	GuSan21	DEK: <i>L. López and Martínez M., 202</i>	Campeche, Mexico	EU253462	JX888934	JX486718
<i>G. sanctum</i>	GuSan22	DEK: <i>L. López and Martínez M., 201</i>	Campeche, Mexico	JX888935	EU253469	JX486718
<i>G. sanctum</i>	GuSan23	DEK: <i>L. López and Martínez M., 209</i>	Campeche, Mexico	EU253464	EU258927	JX486718
<i>G. sanctum</i>	GuSan24	DEK: <i>L. López and Martínez M., 211</i>	Campeche, Mexico	EU253464	EU258927	JX486718
<i>G. sanctum</i>	GuSan25	DEK: <i>L. López and Martínez M., 205</i>	Campeche, Mexico	EU253463	EU253470	JX486718
<i>G. sanctum</i>	GuSan26	DEK: <i>L. López and Martínez M., 206</i>	Campeche, Mexico	EU253464	EU258927	JX486718
<i>G. sanctum</i>	GuSan27	DEK: <i>J.R. Dertien 518</i>	Florida, USA	EU253464	EU258927	JX486718
<i>G. sanctum</i>	GuSan28	DEK: <i>J.R. Dertien 519</i>	Florida, USA	EU253457	EU253473	JX486718
<i>G. sanctum</i>	GuSan29	DEK: <i>J.R. Dertien 524</i>	Florida, USA	EU253457	EU253473	JX486718
<i>G. sanctum</i>	GuSan30	DEK: <i>J.R. Dertien 523</i>	Florida, USA	EU253457	EU253473	JX486718
<i>G. sanctum</i>	GuSan31	DEK: <i>J.R. Dertien 527</i>	Florida, USA	EU253464	EU258927	-
<i>G. sanctum</i>	GuSan32	DEK: <i>J.R. Dertien 528</i>	Florida, USA	EU253464	EU258927	-
<i>G. sanctum</i>	GuSan35	DEK: <i>L. López and Martínez M., 207</i>	Campeche, Mexico	EU253464	EU258927	JX486719
<i>G. sanctum</i>	GuSan36	DEK: <i>J.R. Dertien 521</i>	Florida, USA	EU253457	EU253473	-
<i>G. sanctum</i>	GuSan37	DEK: <i>J.R. Dertien 533</i>	Florida, USA	EU253464	EU258927	-
<i>G. sanctum</i>	GuSan38	DEK: <i>J.R. Dertien 526</i>	Florida, USA	EU253464	EU258927	-
<i>G. sanctum</i>	GuSan39	DEK: <i>J.R. Dertien, 531</i>	Florida, USA	EU253457	EU253473	-
<i>G. sanctum</i>	GuSan40	DEK: <i>J.R. Dertien, 532</i>	Florida, USA	EU253457	EU253473	-
<i>G. sanctum</i>	GuSan41	DEK: <i>J.R. Dertien, 525</i>	Florida, USA	EU253457	EU253473	JX486718
<i>G. sanctum</i>	GuSan42	DEK: <i>J.R. Dertien, 522</i>	Florida, USA	EU253457	EU253473	-
<i>G. sanctum</i>	GuSan43	DEK: <i>J.R. Dertien, 520</i>	Florida, USA	EU253464	EU258927	JX486718
<i>G. sanctum</i>	GuSan44	DEK: <i>J.R. Dertien, 529</i>	Florida, USA	EU253457	EU253473	-
<i>G. sanctum</i>	GuSan45	DEK: <i>J.R. Dertien, 530</i>	Florida, USA	EU253457	JX888936	-
<i>G. sanctum</i>	GuSan47	FTG: <i>accession X 3-4c</i>	cultivated	EU253464	EU258927	-
<i>G. sanctum</i>	GuSan48	FTG: <i>accession 61478A</i>	unknown	EU253464	EU258927	JX669511
<i>G. sanctum</i>	GuSan50	MICH: <i>N. H. Nickerson, 4216</i>	Bahamas	EU253457	EU253473	JX486718
<i>G. sanctum</i>	MEX01	MEXU: <i>C. Salazar et al., 08</i>	Guatemala	EU253464	JX888937	JX486717
<i>G. sanctum</i>	GH01	GH: <i>J.J. Castillo, 1594</i>	Guatemala	EU253464	EU258927	JX486717
<i>G. unijugum</i>	GuUni01	DEK: <i>J.R. Dertien 509</i>	Baja California Sur, Mexico	JX682627	JX682628	JX486720
<i>G. unijugum</i>	GuUni02	DEK: <i>R. McCauley s.n.</i>	Baja California Sur, Mexico	JX682629	JX682630	JX486721
<i>G. angustifolium</i>	GuAng01	TEX: <i>B. Turner 12-I041</i>	Texas, USA	JX669512	JX669513	JX468346
<i>G. angustifolium</i>	GuAng02	DEK: <i>J.R. Dertien 534</i>	cultivated	EU253465	EU253474	JX486127

Table 1. Continues

<i>Species</i>	<i>Extract ID</i>	<i>Voucher</i>	<i>Location</i>	<i>trnLF</i>	<i>trnSG</i>	<i>ITS</i>
<i>G.angustifolium</i>	-	-	-	-	-	AY260974
<i>Guaiacum sp.</i>	CHIP01	CHIP: R. Gutiérrez 29	Chiapas, Mexico	JX669514	JX669515	JX486713
<i>Guaiacum sp.</i>	CHIP02	CHIP: unknown collector s.n.	Chiapas, Mexico	JX682623	JX682624	JX486713
<i>Guaiacum sp.</i>	LL289	CHIP: L. López and M. Martínez 289	Chiapas, Mexico	JX669514	JX669515	JX486713
<i>Porlieria chilensis</i>	PoChi01	ULS: M.A. Previtali, 01	Chile	JX888914	JX888913	JX901026

Parsimony (MP) method was implemented to make use of parsimony informative indels. Heuristic searches with 100 random addition sequence replicates and tree-bisection reconnection branch swapping were performed to find the set of most parsimonious trees. A MP bootstrap analysis (Felsenstein, 1985) was performed with 1 000 bootstrap pseudoreplicates. Accessions with identical cpDNA sequences were removed from the data matrices prior to analysis, leaving exemplars of each unique haplotype. Phylogenetic analyses were conducted using a data matrix of combined cpDNA and nrDNA sequences (*trnL-F*, *trnS-G*, ITS1, 5.8s, ITS2) and separate data matrices of cpDNA (combined *trnL-F*, *trnS-G*) and nuclear (ITS1, 5.8s, ITS2) data.

Incongruence length difference tests. Wilcoxon signed-ranks tests (Templeton, 1983) and Kishino-Hasegawa (KH) tests (Kishino and Hasegawa, 1989) were conducted using PAUP* (Swofford, 2002) to test incongruence between chloroplast and nuclear trees.

Statistical Parsimony Analysis. Phylogenetic networks of interconnected haplotypes were inferred through the statistical parsimony method of estimating gene genealogies, as implemented by the TCS v 1.21 software package (Clement et al., 2000). Unlike the maximum parsimony method of phylogenetic analyses, these phylogenetic reconstructions make use of multiple accessions with identical haplotypes (Table 2). Furthermore, the individual mutational steps separating unique haplotypes are mapped in the network. As such, biogeographic and similar data can be overlaid on these networks, making them useful for studies in which biogeographical elements, hybridization, and speciation are prominent components (Shaw and Small, 2005; Ran et al., 2006). Autapomorphies are similarly mapped, providing further insight into levels of genetic diversity.

Separate aligned sequence data matrices were generated for nrDNA (ITS1, 5.8s, ITS2), the ITS2 marker, and cpDNA (*trnL-F* + *trnS-G*). Sites of ambiguous base calls or unconfirmed mutations were removed prior to analysis. Statistical parsimony networks were constructed using the

95% connection limit criterion, and reticulating patterns in networks were resolved following guidelines outlined by Crandall (1994).

Results

The aligned data matrix for the combined analysis of cpDNA and nrDNA (*trnL-F* + *trnS-G* + ITS1, 5.8s, ITS2) consisted of 3 055 bp for 41 ingroup taxa and 1 outgroup taxon. Maximum parsimony analysis of the combined sequence data included a total of 2 324 characters, with 119 being parsimony informative. The result was 24 equally parsimonious trees with a tree length of 351 steps, with a consistency index (CI)= 0.8433 and a retention index (RI)= 0.9417. The strict consensus tree included 18 nodes resolved with a bootstrap support value exceeding 70%, and ingroup taxa divided into 4 strongly supported clades (Fig. 1, clades A-D).

The basal clade is a monophyletic group inclusive of all accessions identified as *Guaiacum officinale* (100% bootstrap support) that is sister to all other *Guaiacum* species. These data also indicate significant intraspecific genetic structuring within this clade with 2 strongly supported subclades (100% bootstrap support each) separating accessions from Jamaica (GuOff24), Haiti (GuOff27), and the Dominican Republic (GuOff22) from accessions collected in Colombia, Venezuela, Puerto Rico, and the Virgin Islands. A second well-supported clade (98% bootstrap support) includes accessions confidently identified as *G. sanctum*, as well as accessions collected from Guatemala dubiously identified as *G. sanctum*, *G. coulteri*, or *G. guatemalense* (Fig. 1, clade B). A third clade with 99% bootstrap support includes accessions identified as *G. angustifolium*, *G. unijugum*, *G. coulteri* var. *palmeri*, and *G. coulteri* (var. *coulteri*) of non-Guatemalan origin (Fig. 1, clade C). This clade has a sister relationship (100% bootstrap support) to all accessions identified as *G. sanctum* based on morphology. The fourth clade (Fig. 1, clade D) contains 3 accessions (CHIP01, CHIP02, LL289) collected from Chiapas, Mexico lacking clear morphological

Table 2. Haplotype designations for all specimens used in this study

<i>Species</i>	<i>Extract ID</i>	<i>Location</i>	<i>cpDNA haplotype</i>	<i>nrDNA haplotype</i>	<i>ITS2 haplotype</i>
<i>Guaiacum coulteri</i>	GuCou02	Oaxaca, Mexico	LFSG_NMX_04	ITS_OAX_02	ITS2_OAX_02
<i>G. coulteri</i>	GuCou03	Oaxaca, Mexico	LFSG_NMX_05	ITS_OAX_02	ITS2_OAX_02
<i>G. coulteri</i>	GuCou04	Oaxaca, Mexico	LFSG_NMX_01	ITS_OAX_01	ITS2_OAX_01
<i>G. coulteri</i>	GuCou05	Oaxaca, Mexico	LFSG_NMX_01	-	-
<i>G. coulteri</i>	GuCou06	Oaxaca, Mexico	LFSG_NMX_01	-	-
<i>G. coulteri</i>	MEX02	Guatemala	LFSG_SMX_02	ITS_CEN_01	ITS2_GUA_01
<i>G. coulteri</i>	MEX03	Oaxaca, Mexico	LFSG_NMX_03	ITS_OAX_01	ITS2_OAX_01
<i>G. coulteri</i>	MEX04	Guatemala	LFSG_SMX_02	ITS_CEN_01	ITS2_GUA_01
<i>G. coulteri</i>	GuCou08	Guatemala	LFSG_SMX_01	ITS_CEN_01	ITS2_GUA_01
<i>G. coulteri</i> var. <i>palmeri</i>	GH06	Sonora, Mexico	-	ITS_SON_01	ITS2_SON_01
<i>G. coulteri</i> var. <i>palmeri</i>	GuCoPa01	Sonora, Mexico	-	ITS_SON_01	ITS2_SON_01
<i>G. coulteri</i> var. <i>palmeri</i>	TEX03	Sonora, Mexico	-	ITS_SON_01	ITS2_SON_01
<i>G. guatemalense</i>	GuGua01	Guatemala	LFSG_SMX_01	ITS_CEN_01	ITS2_GUA_01
<i>G. sanctum</i>	F04	Costa Rica	LFSG_SMX_01	ITS_CEN_03	ITS2_GUA_02
<i>G. sanctum</i>	F08	Costa Rica	LFSG_SMX_03	-	ITS2_GUA_02
<i>G. sanctum</i>	GH02	Curacao	LFSG_CAF_01	ITS_CAR_01	ITS2_MCF_01
<i>G. sanctum</i>	GH03	Bonaire	LFSG_CAF_01	ITS_CAR_01	ITS2_MCF_01
<i>G. sanctum</i>	GuSan01	cultivated	LFSG_CAF_01	-	-
<i>G. sanctum</i>	GuSan03	Puerto Rico	LFSG_CAF_01	ITS_CAR_01	ITS2_MCF_01
<i>G. sanctum</i>	GuSan04	Puerto Rico	LFSG_CAF_02	ITS_CAR_01	ITS2_MCF_01
<i>G. sanctum</i>	GuSan06	Puerto Rico	LFSG_CAF_01	-	ITS2_MCF_02
<i>G. sanctum</i>	GuSan07	Puerto Rico	LFSG_CAF_01	-	ITS2_MCF_02
<i>G. sanctum</i>	GuSan08	cultivated	LFSG_CAF_01	-	-
<i>G. sanctum</i>	GuSan09	Puerto Rico	LFSG_CAF_01	-	-
<i>G. sanctum</i>	GuSan10	Puerto Rico	LFSG_CAF_01	-	-
<i>G. sanctum</i>	GuSan11	Bahamas	LFSG_CAF_01	-	-
<i>G. sanctum</i>	GuSan12	Bahamas	LFSG_CAF_03	-	-
<i>G. sanctum</i>	GuSan13	Campeche, Mexico	LFSG_SMX_05	-	-
<i>G. sanctum</i>	GuSan14	Campeche, Mexico	LFSG_SMX_08	-	ITS2_MCF_01
<i>G. sanctum</i>	GuSan15	Campeche, Mexico	LFSG_SMX_01	-	-
<i>G. sanctum</i>	GuSan16	Campeche, Mexico	LFSG_SMX_07	-	ITS2_MCF_01
<i>G. sanctum</i>	GuSan18	Bahamas	LFSG_CAF_04	-	-
<i>G. sanctum</i>	GuSan19	Bahamas	LFSG_SMX_09	-	-
<i>G. sanctum</i>	GuSan20	Yucatan, Mexico	LFSG_SMX_01	-	-
<i>G. sanctum</i>	GuSan21	Campeche, Mexico	LFSG_SMX_06	-	ITS2_MCF_01
<i>G. sanctum</i>	GuSan22	Campeche, Mexico	LFSG_SMX_04	ITS_CAR_01	ITS2_MCF_01
<i>G. sanctum</i>	GuSan23	Campeche, Mexico	LFSG_SMX_01	-	ITS2_MCF_01
<i>G. sanctum</i>	GuSan24	Campeche, Mexico	LFSG_SMX_01	-	ITS2_MCF_01
<i>G. sanctum</i>	GuSan25	Campeche, Mexico	LFSG_SMX_10	-	ITS2_MCF_01
<i>G. sanctum</i>	GuSan26	Campeche, Mexico	LFSG_SMX_01	-	ITS2_MCF_01
<i>G. sanctum</i>	GuSan27	Florida, USA	LFSG_SMX_01	ITS_CAR_01	ITS2_MCF_01
<i>G. sanctum</i>	GuSan28	Florida, USA	LFSG_CAF_01	ITS_CAR_01	ITS2_MCF_01
<i>G. sanctum</i>	GuSan29	Florida, USA	LFSG_CAF_01	-	ITS2_MCF_01
<i>G. sanctum</i>	GuSan30	Florida, USA	LFSG_CAF_01	-	ITS2_MCF_01
<i>G. sanctum</i>	GuSan31	Florida, USA	LFSG_SMX_01	-	-
<i>G. sanctum</i>	GuSan32	Florida, USA	LFSG_SMX_01	-	-
<i>G. sanctum</i>	GuSan35	Campeche, Mexico	LFSG_SMX_01	ITS_CAR_02	ITS2_MCF_01
<i>G. sanctum</i>	GuSan36	Florida, USA	LFSG_CAF_01	-	-
<i>G. sanctum</i>	GuSan37	Florida, USA	LFSG_SMX_01	-	-

Table 2. Continues

<i>Species</i>	<i>Extract ID</i>	<i>Location</i>	<i>cpDNA haplotype</i>	<i>nrDNA haplotype</i>	<i>ITS2 haplotype</i>
<i>G. sanctum</i>	GuSan38	Florida, USA	LFSG_SMX_01	-	-
<i>G. sanctum</i>	GuSan39	Florida, USA	LFSG_CAF_01	-	-
<i>G. sanctum</i>	GuSan40	Florida, USA	LFSG_CAF_01	-	-
<i>G. sanctum</i>	GuSan41	Florida, USA	LFSG_CAF_01	-	ITS2_MCF_01
<i>G. sanctum</i>	GuSan42	Florida, USA	LFSG_CAF_01	-	-
<i>G. sanctum</i>	GuSan43	Florida, USA	LFSG_SMX_01	-	ITS2_MCF_01
<i>G. sanctum</i>	GuSan44	Florida, USA	LFSG_CAF_01	-	-
<i>G. sanctum</i>	GuSan45	Florida, USA	LFSG_CAF_05	-	-
<i>G. sanctum</i>	GuSan47	cultivated	LFSG_SMX_01	-	-
<i>G. sanctum</i>	GuSan48	unknown	LFSG_SMX_01	-	ITS2_MCF_03
<i>G. sanctum</i>	GuSan50	Bahamas	LFSG_CAF_01	-	ITS2_MCF_01
<i>G. sanctum</i>	MEX01	Guatemala	LFSG_SMX_02	ITS_CEN_01	ITS2_GUA_01
<i>G. sanctum</i>	GH01	Guatemala	LFSG_SMX_01	ITS_CEN_01	ITS2_GUA_01
<i>G. unijugum</i>	GuUni01	Baja California Sur, Mexico	LFSG_NMX_02	ITS_UNI_01	ITS2_UNI_01
<i>G. unijugum</i>	GuUni02	Baja California Sur, Mexico	LFSG_NMX_01	ITS_UNI_02	ITS2_UNI_01
<i>G. angustifolium</i>	GuAng01	Texas, USA	LFSG_TEX_01	ITS_ANG_03	ITS2_ANG_01
<i>G. angustifolium</i>	GuAng02	cultivated	LFSG_TEX_02	ITS_ANG_01	ITS2_ANG_01
<i>G. angustifolium</i>	-	-	-	ITS_ANG_02	-
<i>Guaiacum sp.</i>	CHIP01	Chiapas, Mexico	LFSG_CHI_01	ITS_CEN_02	ITS2_GUA_03
<i>Guaiacum sp.</i>	CHIP02	Chiapas, Mexico	LFSG_CHI_02	ITS_CEN_02	ITS2_GUA_03
<i>Guaiacum sp.</i>	LL289	Chiapas, Mexico	LFSG_CHI_01	ITS_CEN_02	ITS2_GUA_03

character states that readily distinguish them as either *G. coulteri*, *G. sanctum*, or *G. guatemalense*. This clade shows only moderate support (73% bootstrap) for a sister relationship to the *G. sanctum* clade.

Sequence data were divided into separate groupings of cpDNA (*trnL-F* + *trnS-G*) and nrDNA (ITS1, 5.8s, ITS2). Maximum parsimony analysis of cpDNA included 1 782 bp, with 48 parsimony informative characters (~2.7%). Three equally parsimonious trees with a length of 135 steps (CI= 0.9185, RI= 0.8935) were produced (Fig. 2).

The strict consensus tree includes 8 nodes resolved with a bootstrap support value exceeding 70%. Analysis of nrDNA included 542 characters, with a total of 71 characters being parsimony informative. (~13%). Maximum Parsimony produced 743 equally parsimonious trees with tree lengths of 208 steps; CI= 0.8269, RI= 0.9332. (Fig. 2) The strict consensus tree includes 13 nodes resolved with a bootstrap support value exceeding 70%. The nrDNA contains more parsimony informative characters than the cpDNA data (71 vs. 48, respectively). Furthermore, the nrDNA contains 8 parsimony informative indel mutations, compared to the 5 indels scored in the cpDNA data set. The indels in the nrDNA are small indels of 1 to 2 bp, whereas the cpDNA indels range from 1 to 38 bp.

Topologies for strict consensus trees generated from cpDNA and nrDNA are largely similar; however, both KH

and Templeton tests of incongruence conducted between cpDNA and nrDNA indicate strongly incongruent tree topologies between the 2 data subsets ($p < 0.001$).

A sister relationship between *G. officinale* and other *Guaiacum* species is strongly supported in both trees (Fig. 2).

The cpDNA tree unites *G. angustifolium*, *G. unijugum*, and non-Guatemalan *G. coulteri* with strong support (B', 100% bootstrap support). A second strongly-supported clade unites accessions of *G. sanctum*, *G. coulteri* of Guatemalan origin, and *G. guatemalense* (C', 96% bootstrap support) with moderate support (79% bootstrap) uniting accessions from Guatemala, regardless of identity based on morphology. A third clade unites accessions collected from Chiapas Mexico (D', 99% bootstrap support) and is positioned as sister to clade containing *G. angustifolium*, non-Guatemalan *G. coulteri*, and *G. unijugum* and the clade containing *G. sanctum* and Guatemalan accessions.

Incongruence in topology is notable for the accessions from Chiapas (D, D'). These accessions are united with accessions of *G. sanctum* in the nrDNA tree, although support for this clade (C) is weak (53% bootstrap support). The cpDNA reconstruction places these accessions in a sister relationship to other (non-*officinale*) *Guaiacum* species.

The nrDNA data failed to resolve a single clade containing all of the accessions the *G. sanctum*. These data

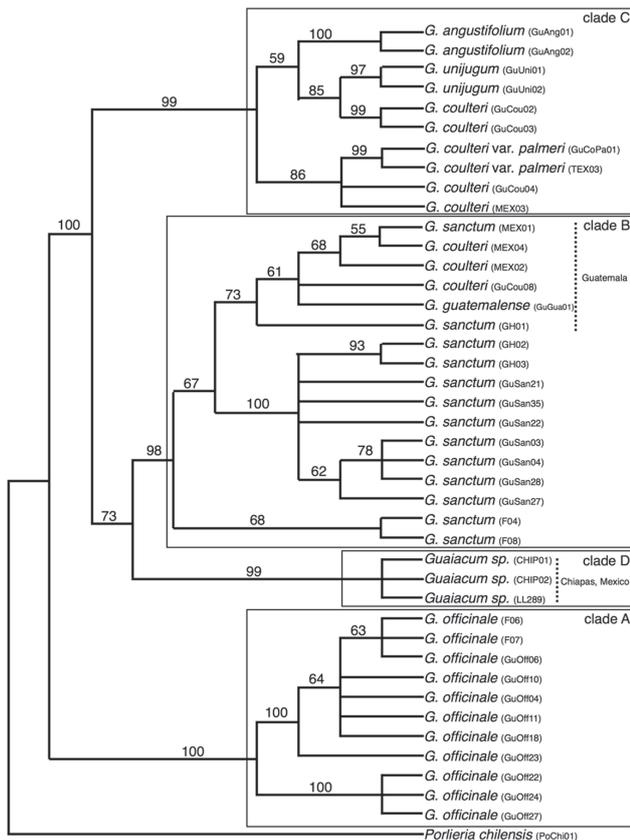


Figure 1. Maximum parsimony bootstrap analysis of combined cpDNA and nrDNA sequence data. Numbers along the branches represent bootstrap support values. Accession numbers are indicated for each terminal. Four clades A-D are identified (see text).

also failed to support a clade containing the accessions of non-Guatemalan *G. couleri*, *G. unijugum*, and *G. angustifolium*.

Conversely, the cpDNA phylogenetic reconstruction shows strong support for a clade uniting *G. sanctum* (96% bootstrap support) and another clade in which accessions of *G. unijugum*, and *G. angustifolium* were united with non-Guatemalan accessions of *G. couleri* (100% bootstrap support). This reconstruction fails to resolve sister relationships among the 3 well-supported clades containing non-*officinale* accessions.

Phylogenetic network estimation using statistical parsimony. The phylogenetic network constructed from combined nrDNA data (Fig. 3) connects 30 taxa at a 95% confidence interval from a data matrix consisting of 517 bp. *Guaicum sanctum* accessions represent a majority of haplotypes that are separated from Central American and Chiapas accessions by 3 mutational steps. Accessions of *G. couleri* from Oaxaca, Mexico are divided into 2 haplotypes

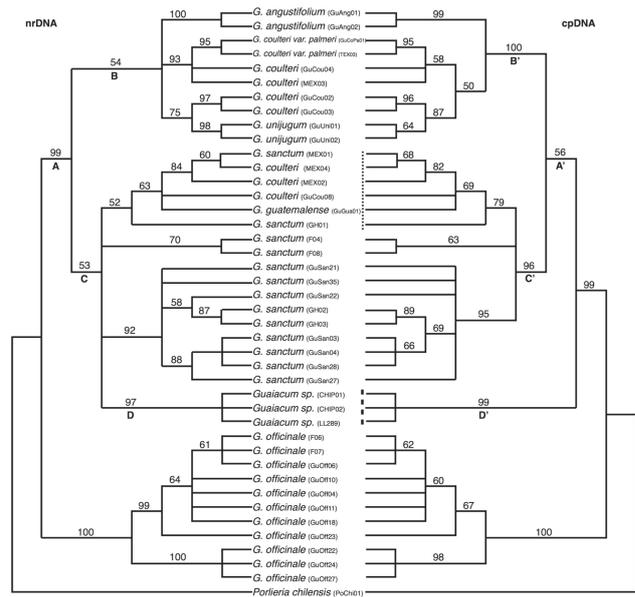


Figure 2. Maximum parsimony bootstrap analysis of (a) cpDNA sequence data and (b) nrDNA sequence data. Numbers along the branches are bootstrap values.

separated by 6 mutational steps. Accessions identified as *G. couleri* var. *palmeri* are derived from one of these *G. couleri* haplotypes with a single additional mutational step. Accessions from Baja California Sur identified as *G. unijugum* have the closest connection in the network to *G. couleri*, with 8 mutational steps separating the nearest accession of *G. unijugum* from either sampled haplotype of *G. couleri*.

The phylogenetic network constructed from the ITS2 marker data (Fig. 4) connects 45 taxa at the 95% confidence interval. The ITS2 network is constructed with fewer mutational steps separating sampled haplotypes; however the overall network patterns are visually congruent with the combined nrDNA network.

The phylogenetic network constructed from cpDNA (Fig. 5) connects 66 taxa at a 95% confidence interval. This network segregates *G. sanctum* into 2 major clades, consistent with the patterns outlined in Dertien and Duvall (2009). Accessions identified as *G. couleri*, and *G. unijugum* form a complex containing shared haplotypes, and *G. angustifolium* is connected to this complex by a single mutational step. Accessions from Chiapas, Mexico, show intermediacy, being connected to the nearest sampled haplotypes of the *G. sanctum* and *G. couleri* complexes by 10 and 14 mutational steps, respectively.

Two equally parsimonious networks are generated from cpDNA, representing a differential placement of Chiapas accessions in the network. Although equally parsimonious,

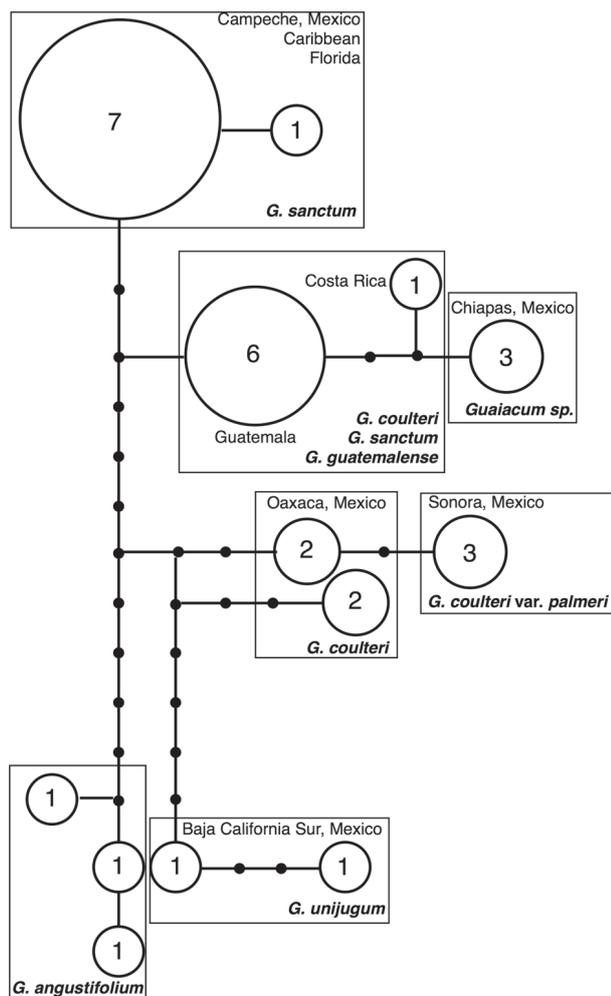


Figure 3. Haplotype network of *Guaiacum* from nrDNA (ITS1, 5.8s, ITS2) for all taxa excluding *G. officinale*. Large circles represent sampled haplotypes, with numbers and relative size indicating frequency of observation. Dots represent unobserved haplotypes with inferred mutational steps. Lines connecting haplotypes represent mutational steps.

one network depicts the reversal of an ordered state indel mutation. Given the mutational mechanism of slipped-strand mispairing generating the derived states of either an inserted or deleted copy of a tandem repeat found in an ancestral condition, this network can be ruled out because it requires an insertion from a condition in which the tandem repeat has already been removed through deletion. The alternate network (Fig. 5) does not require such a reversal, but rather a parallel insertion from the ancestral condition, and is therefore considered the more probable network. Specific details about the ancestral and derived states of this mutation are explained in Dertien and Duvall (2009).

Discussion

Guaiacum officinale. The phylogenetic relationship of *G. officinale* as sister to the remaining taxa is unambiguous. This result based on the genetic evidence is expected given the high levels of morphological divergence between *G. officinale* and remaining *Guaiacum* taxa. Nevertheless, misidentification between *G. officinale* and *G. sanctum* occasionally occurs among specimens collected in areas of the Caribbean where both species occur sympatrically. A screening of 1 694 specimens from 11 herbaria showed misidentification to occur at a rate of approximately 10%. Misidentified specimens between these species did not demonstrate ambiguous or intermediate character states, and it is therefore likely that such misidentification is attributable to error.

Genetic divergence and intraspecific structuring for *G. officinale* was evident in all trees (Figs. 1, 2). These data show strong support for a genetic divergence between accessions from Jamaica/Hispaniola (GuOff22, GuOff24, GuOff27) from those collected in the rest of the Caribbean. The monophyly of the sister group to the Jamaica/Hispaniola clade was resolved more strongly

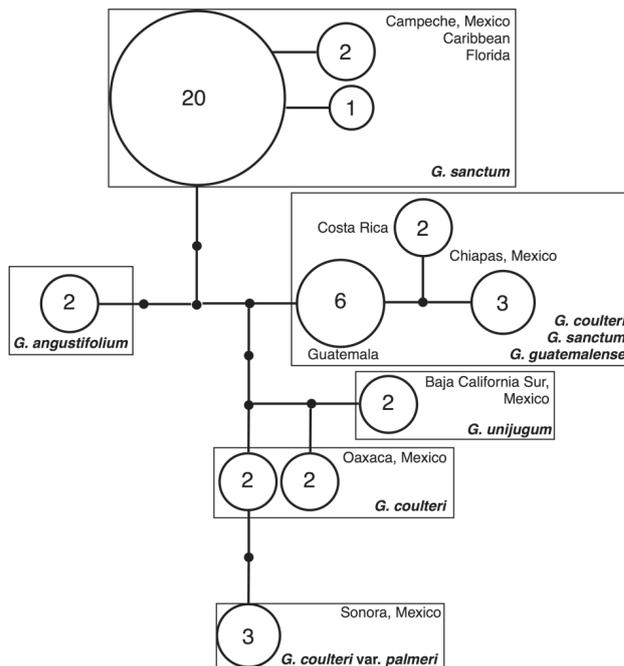


Figure 4. Haplotype network of *Guaiacum* from ITS2 region of nrDNA for all taxa excluding *G. officinale*. Large circles represent sampled haplotypes, with numbers and relative size indicating frequency of observation. Dots represent unobserved haplotypes with inferred mutational steps. Lines connecting haplotypes represent mutational steps.

with the nrDNA data, which could be explained by a stronger phylogenetic signal from the more variable nrDNA, or indicative of chloroplast capture from a past hybridization event (Soltis and Kuzoff, 1995) confounding the phylogenetic signal. It is anticipated that additional sampling of *G. officinale* from Caribbean populations could reveal a complex evolutionary pattern coinciding with the region's rich geologic history.

Guaiaicum sanctum, *G. guatemalense*, *G. coulteri*. *Guaiaicum* along the Pacific coast of Mexico are generally identified as *G. coulteri* based on having linear to linear oblong leaflets whereas *Guaiaicum* growing in Yucatán and Campeche are distinguished as *G. sanctum* based on their oblong to obovate leaflets. However, these morphological character states converge in parts of southern Mexico and Central America (Grow and Schwartzman, 2001b) making identification difficult. It has been hypothesized that populations of *G. coulteri* and *G. sanctum* could be occurring sympatrically or possibly hybridizing in these regions, where species distributions presumably overlap (Grow and Schwartzman, 2001b) Furthermore, *Guaiaicum* found in Guatemala and parts of Central America have been treated as *G. guatemalense*, a novel species that possesses some intermediate characteristics of *G. sanctum* and *G. coulteri*. However, this distinction is often indiscernible or otherwise ill-defined because of the absence of discrete character states (e. g., leaf and petal shape, and hairiness of abaxial leaf surfaces).

The difficulty in distinguishing and defining species in these regions is not only interesting in the context of systematic botany and taxonomy, but also has potential impact on resource management and regulation of trade of *Guaiaicum*, as Mexico is the largest global exporter (CITES, 2002).

The molecular data of this study show similar challenges as morphological data in efforts to clearly distinguish between *G. coulteri* and *G. sanctum*. Most accessions are readily distinguished by mutation patterns in both cpDNA and nrDNA. However, some accessions from southern Mexico show intermediate patterns in cpDNA. Specifically, 3 accessions collected from Chiapas, Mexico represent a genetic intermediate between *G. sanctum* and *G. coulteri* in the statistical parsimony analyses of cpDNA. The cpDNA network places these accessions in a position that is nearly equidistant from the nearest accession of *G. sanctum* (15 mutational steps) or of *G. coulteri* (14 mutational steps). Furthermore, statistical parsimony networks generated from the cpDNA data matrix with these accessions removed failed to place remaining accessions in a single network at the 95% confidence limit (networks not shown). Networks generated from the nrDNA dataset demonstrated a different pattern in which the Chiapas accessions are

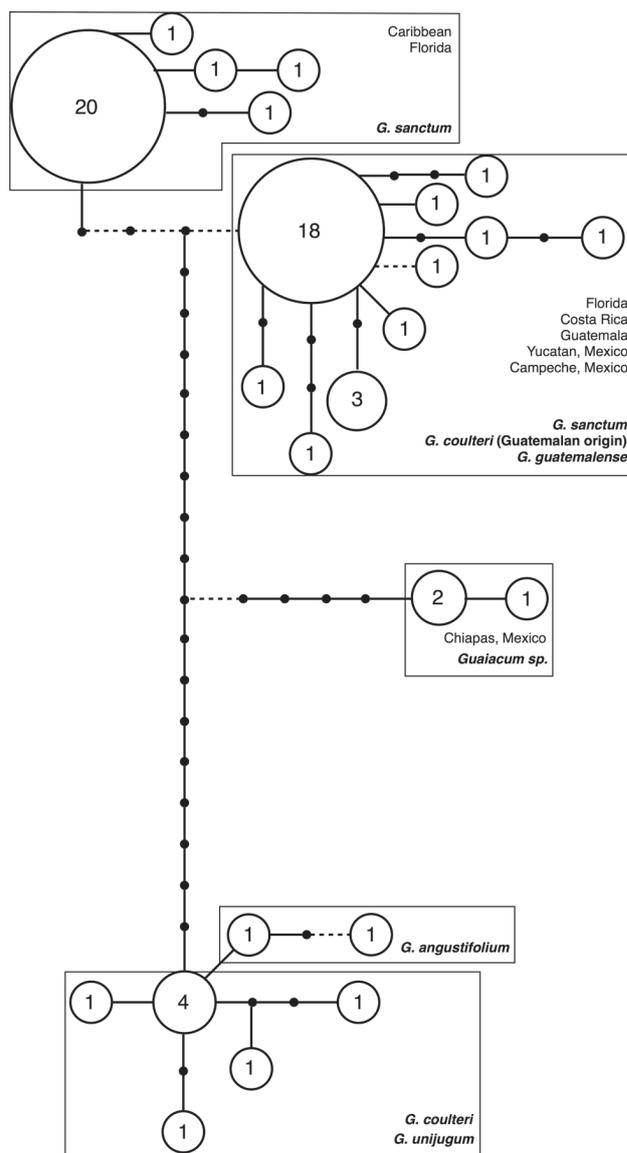


Figure 5. Haplotype network of *Guaiaicum* from cpDNA for all taxa excluding *G. officinale*. Large circles represent sampled haplotypes, with numbers and relative size indicating frequency of observation. Dots represent unobserved haplotypes with inferred mutational steps. Solid lines connecting haplotypes represent single nucleotide polymorphisms and dashed lines represent indel mutations.

closely connected to the subgroup of *Guaiaicum* collected from Guatemala and Costa Rica. The intermediate cpDNA pattern and different pattern in nrDNA is the most likely explanation for the difference in bootstrap support and topology in the maximum parsimony analyses. However, removal of these accessions failed to induce congruence between the 2 trees, most likely due to further differences

in topology involving the *G. coulteri*, *G. angustifolium*, *G. unijugum* accessions.

These data show no evidence of contemporary hybridization between *G. sanctum* and *G. coulteri*. However, the intermediate position of the Chiapas accessions in the cpDNA network can be explained by a past event of hybridization and introgression of ancestral populations that subsequently diverged further into what are now distinct populations of *G. coulteri* and *G. sanctum*. Specifically, we hypothesize that the Chiapas region of Mexico could have served as an ancient refuge, thereby becoming a center of diversity during range expansion of what are now populations of *G. sanctum* in the Yucatan peninsula, Caribbean and Central America, and *G. coulteri* along the western coast of Mexico. Under this scenario, Chiapas populations later hybridized with Central American populations, retaining the intermediate cpDNA molecular signature while gaining an nrDNA pattern that is most closely related to the Central American populations. Interestingly, this scenario aligns with the suggestion by Porter (1972) that *G. guatemalense* is the result of hybridization and introgression between *G. sanctum* and *G. coulteri*. However, the data from this study show that such a scenario can only be applied to the Chiapas population(s), rather than to all accessions dubiously identified as *G. guatemalense*.

The species status of *Guaiaacum guatemalense* is supported by these data inasmuch as accessions from Guatemala are monophyletic in the phylogenetic trees and gene networks for nrDNA. However, accessions from Costa Rica and Chiapas appear to be derived from Guatemalan nrDNA haplotypes (Figs. 3, 4), and there is strong support uniting most accessions collected from the same geographic region. Therefore, it is suggested that these phylogenetic patterns are best interpreted as significant intraspecific structuring within *G. sanctum* rather than recognizing *G. guatemalense* as a separate species, especially in the absence of distinguishing morphological characters. Additional sequence data would most likely raise bootstrap support values for subclades within *G. sanctum*, and raising *G. guatemalense* to a full species based on a molecular signature would likely warrant the splitting of other regional variants (e. g., Aruba and Curaçao, Costa Rica, Chiapas) to species status. Conversely, sampling of additional populations (e. g., Honduras, Nicaragua) may reveal additional intermediate haplotypes that could reduce support for these subclades. This interpretation is congruent with the hypothesis that gene flow is limited and populations remain relatively isolated from one another due to natural and anthropologic habitat fragmentation. The haplotype networks and tree topologies for cpDNA and nrDNA data indicate that *Guaiaacum* from Guatemala

is not likely *G. coulteri*, and species identifications should be treated with care.

Guaiaacum unijugum, *G. coulteri* and *G. angustifolium*. Maximum parsimony and statistical parsimony methods of phylogenetic reconstruction fail to clearly resolve sister relationships among accessions of *G. unijugum*, *G. coulteri* and *G. angustifolium*. This result is somewhat unexpected given that these taxa are readily distinguished based on morphology. Floral characters such as carpel number and basal filament appendages are generally distinct and unambiguous for species identification and sterile specimens can be readily distinguished based on leaf morphology, with leaflet number and width to length ratio being particularly useful characters.

The lack of resolution in the maximum parsimony analyses and patterns in the statistical parsimony networks are indicative of either recent or incomplete speciation, as well as possible hybridization among taxa. One reason the molecular data fail to resolve these relationships is the lack of cpDNA divergence and presence of shared haplotypes among taxa in this group, which can be explained by the slower mutational rate of the chloroplast genome (Wolf et al., 1987). Accessions of *G. coulteri*, *G. coulteri* var. *coulteri*, and *G. unijugum* share cpDNA haplotypes, and *G. angustifolium* is derived from this complex by a single mutation. Conversely, the nrDNA haplotypes do not show *G. angustifolium* as derived from the *G. coulteri* complex. Accessions of *G. coulteri* have multiple nrDNA haplotypes, with *G. unijugum* and *G. coulteri* var. *palmeri* being derived from different *G. coulteri* ancestral haplotypes.

This species complex may have arisen from a polyploidization event. Evidence of polyploidy in both *G. coulteri* and *G. angustifolium* (McCauley et al., 2008) has been identified; however karyotyping has not been conducted to resolve the full extent of these events. As such, the paraphyly in *G. coulteri* may be the result of differential amplification and sequencing of paralogous ITS loci.

Guaiaacum angustifolium appears to have evolved from a shared ancestor with the *G. coulteri* complex, as evidenced by the nrDNA network. However, a subsequent hybridization in which *G. coulteri* cpDNA was introgressed back into the population may have occurred, which could explain the close, derived condition seen in the cpDNA network. Nevertheless, this taxon displays significant differences in morphology from other members in this clade, and the designation as a species is likely valid despite low phylogenetic resolution.

Taxonomically, we recommend that *G. unijugum* retain species status based on morphological, geographic, and molecular criteria. Morphologically, *G. unijugum* possess

a suite of characters, both floral and vegetative that make it readily distinguishable from other taxa. The distribution of this taxon is also highly limited to a small region of the Baja peninsula that does not overlap with other taxa (Porter, 1963). Finally, nrDNA data show that *G. unijugum* is as divergent from *G. coulteri* as *G. coulteri* is from *G. sanctum* and *G. angustifolium* (8 mutational steps, Fig. 3). This is consistent with conclusions of McCauley et al. (2010) that *G. unijugum* and *G. coulteri* have evolved independently from a common ancestor, with microsatellite data also suggesting that remnant populations of *G. unijugum* are maintained primarily by selfing with little evidence of outcrossing or hybridization with outside populations.

Accessions identified as *G. coulteri* var. *palmeri* share a nrDNA haplotype that is derived from 1 of the 2 haplotypes found in other *G. coulteri* accessions, which is indicative of isolation and a separate evolutionary trajectory from other *G. coulteri* populations. However, this haplotype is not significantly divergent from other *G. coulteri* (var. *coulteri*) in cpDNA and nrDNA phylogenetic networks where it is separated by a single mutational step. Furthermore, the 2 nrDNA haplotypes found in *G. coulteri* var. *coulteri* accessions are more divergent from one another than the *G. coulteri* var. *palmeri* haplotype (Fig. 3), indicating that *G. coulteri* may have intraspecific genetic structuring similar to that seen in *G. sanctum*.

Accessions of *G. coulteri* var. *palmeri* are distinguished by a single morphological character, i. e., the presence of a pubescent ovary. This character is not discrete, and intermediate or partially pubescent ovaries were observed in several accessions. The pubescent ovary is also shared with *G. unijugum* and *G. angustifolium*, and it has been suggested that the pubescent character state is an environmental response shared by specimens in the northern distributional range of *Guaiaicum*. An herbarium specimen identified as *G. coulteri* var. *palmeri* retained the pubescent ovary when planted outside of its natural range as an ornamental specimen (*R. Grether 2513*, 14-Aug-1989, MEXU), thereby providing some evidence of a genetic condition and not a strict environmental response. Ovary pubescence is retained in the developing and immature fruits, but the character becomes less discernible as the fruit develops and ripens. Sterile specimens of *G. coulteri* var. *palmeri* are indistinguishable from *G. coulteri* var. *coulteri*.

The lack of clear morphological differences and minimal genetic divergence lead to the conclusion that the distinction of varieties is likely unwarranted in *G. coulteri*.

Taxonomic conclusions. The phylogenetic relationships resolved in this study do not suggest major taxonomic changes such as splitting of species are necessary in

Guaiaicum, although many nomenclatural issues remain to be addressed. While accessions from Guatemala form a moderately supported clade, these data do not show strong support of *G. guatemalense* as a distinct species based on genetic divergence. The majority of publications involving *Guaiaicum* already treat *G. guatemalense* as a synonym of *G. sanctum* and from an application standpoint the absence of diagnostic morphological characters would make species identification difficult.

Varietal distinctions in *G. coulteri* are not supported by monophyly or unique haplotypes. The formal dissolution of these taxa would not have a large impact as the varietal distinction of var. *palmeri* is rarely referenced in the literature. Taxonomic distinction of *G. coulteri* var. *coulteri* was not observed in any of the 1 800+ herbarium specimens reviewed for this study, and distinction of *G. coulteri* var. *coulteri* from *G. coulteri* var. *palmeri* was not included among the 31 *Guaiaicum* taxa listed in the International Plant Names Index (2009), nor listed in Tropicos, the leading database for tropical plant taxa (2009). The distinction between varieties does not follow the guidelines established by the International Code of Botanical Nomenclature (2009), and it is therefore assumed that a formal taxonomic revision is not necessary or practical for taxa that are so rarely recognized. The intermediate genetic fingerprint of the accessions from Chiapas provide evidence that past hybridization may have occurred, and contemporary hybridizations could be possible. These accessions also show that the taxonomic separation of *G. sanctum* and *G. coulteri* is not distinct at the molecular level. While it can be argued that speciation has not fully occurred between these taxa, to combine them as one species would not be prudent, as there is significant genetic divergence between the 2, and the range of overlap is relatively small.

Conservation application. The most significant finding of this study that is directly applicable to conservation is the biogeographic genetic structuring found within the various taxa. The *Guaiaicum* taxa that were sampled in multiple locations display intraspecific genetic structuring, with *G. officinale*, *G. sanctum*, and *G. coulteri* all possessing unshared haplotypes among sampled populations. Given the resolution of the molecular methods utilized in this study, it can be concluded that this structuring is most likely a result of historical isolation and fragmentation, which is maintained by minimal long distance gene flow among distant populations. So while negative genetic effects caused by anthropogenic fragmentation are a conservation concern, this study indicates that much of the genetic variability among populations is more likely indicative of pre-fragmentation genetic patterns. This is likely attributable to the longevity of these trees, effective

dispersal of seeds and efficient pollen transfer in what are generally naturally fragmented populations (Dertien and Duvall, 2009; Fuchs and Hamrick 2010a). This historical genetic structure is also consistent with the conclusions drawn from population level allozyme and microsatellite studies in *G. sanctum* (Fuchs and Hamrick, 2010a) and *G. unijugum* (MacCauley et al., 2010).

The historical genetic patterns demonstrate that individual populations are likely following different evolutionary trajectories than distant neighbors, with the implication that populations could be highly adapted to local environmental conditions or microclimates (Lopez-Toledo, 2011b). This could also explain the intermediate phylogenetic position between *G. sanctum* and *G. coulteri* of the Chiapas accessions. Genetic diversity levels of populations are not likely to be maintained through long distance gene flow, and even selective logging could have a high impact on a population that is genetically distinct because of its biogeographical history.

The current phylogenetic signature created from the dynamic biogeographical and evolutionary history of *Guaiacum* makes species delimitation difficult in a context useful for regulating harvesting and trade. As such, it may be more pragmatic to protect and regulate trade on the generic taxonomic level rather than the species level to provide proper levels of protection for remaining *Guaiacum* populations. However, the consistent correlation between distinct haplotypes and geography show promise that DNA barcoding methods could be developed as a tool for specimen identification and determining provenance.

Acknowledgements

This work was supported in part by the Plant Molecular Biology Center and the Department of Biological Sciences at Northern Illinois University. The authors would like to thank Frank Axelrod (University of Puerto Rico at Río Piedras), Leonel López-Toledo (San Diego Zoo's Institute for Conservation Research), and Ross A. McCauley (Fort Lewis College) for their insight and assistance in collecting specimens. Mr. Cosme Becerra (Northern Illinois University) graciously provided Spanish language translation.

Literature cited

- Axelrod, F. 2002. Proposal to conserve the name *Guaiacum* (Zygophyllaceae) with that spelling. *Taxon* 51:203-204.
- CITES. 2002. Inclusion of *Guaiacum* spp. in Appendix II in accordance with Article II. Annotation: designates all parts and derivative, including wood, bark and extract.
- Clement, M., D. Posada and K. Crandall. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9:1657-1660.
- Crandall, K. 1994. Intraspecific cladogram estimation: accuracy at higher levels of divergence. *Systematic Biology* 43:222-235.
- Collins, R. A. and R. H. Cruickshank. 2013. The seven deadly sins of DNA barcoding. *Molecular Ecology Resources* 13:969-975.
- Dertien, J. R. and M. Duvall. 2009. Biogeography and divergence in *Guaiacum sanctum* (Zygophyllaceae) revealed in chloroplast DNA: Implications for conservation in the Florida Keys. *Biotropica* 41:120-127.
- Drábková, L., J. Kirschner and C. Vleck. 2002. Comparison of seven DNA extraction and amplification protocols in historical herbarium specimens of Juncaceae. *Plant Molecular Biology Reporter* 20:161-175.
- Doyle, J. J. and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemistry Bulletin* 19:11-15.
- Drummond, A., B. Ashton, M. Cheung, J. Heled, M. Kearse, R. Moir, S. Stones-Havas and T. Thierer. 2008. Geneious v4.5. <http://www.geneious.com/>; last access: 08.IV.2011.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783-791.
- Fuchs, E. and J. Hamrick. 2010a. Genetic diversity in the endangered tropical tree, *Guaiacum sanctum* (Zygophyllaceae). *Journal of Heredity* 101:284-291.
- Fuchs, E. and J. Hamrick. 2010b. Spatial genetic structure within size classes of the endangered tropical tree, *Guaiacum sanctum* (Zygophyllaceae). *American Journal of Botany* 97:1200-1207.
- González, D., M. Cubeta and R. Vilgalys. 2006. Phylogenetic utility of indels within ribosomal DNA and β -tubulin sequences from fungi in the *Rhizoctonia solani* species complex. *Molecular Phylogenetics and Evolution* 40:459-470.
- Grow, S. and E. Schwartzman. 2001a. The status of *Guaiacum* species in trade. *Medicinal Plant Conservation* 7:19-20.
- Grow, S. and E. Schwartzman. 2001b. A review of the taxonomy and distribution of the genus *Guaiacum* in Mexico. *In* Report to the Eleventh Meeting of the Plants Committee, CITES PC11 Doc. 8.2., CITES, Langkawi, Malaysia. p. 1-22.
- International Code of Botanical Nomenclature. 2009 <http://www.bgbm.org/IAPT/Nomenclature/Code/SaintLouis/0002Preface.htm>, last access: 01.VI.2009.
- International Plant Names Index. 2009. <http://www.ipni.org>, last access: 01.VI.2009.
- IUCN 2007. 2007 IUCN Red List of Threatened Species. www.iucnredlist.org; last access: 12.XI.2007.
- Kim, K. and R. Jansen. 1994. Comparison of phylogenetic hypotheses among different data sets in dwarf dandelions (*Krigia*: Asteraceae): additional information from internal transcribed spacer sequences of nuclear ribosomal DNA. *Plant Systematics and Evolution* 190:157-185.
- Kishino H. and M. Hasegawa. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidae.

- Journal of Molecular Evolution 29:170-179.
- Li, Q. B., Q. Cai and C. Guy. 1994. A DNA extraction method for RAPD analysis from plants rich in soluble polysachharides. *Plant Molecular Biology Reporter* 12:215-220.
- Lia, V., V. Confalonieri, C. Comas and J. Hunziker. 2001. Molecular phylogeny of *Larrea* and its allies (Zygophyllaceae): reticulate evolution and the probable time of creosote bush arrival to North America. *Molecular Phylogenetics and Evolution* 21:309-320.
- Lopez-Toledo, L., C. Gonzalez-Salazar, D. Burslem and M. Martínez-Ramos. 2010. Conservation assessment of *Guaiaicum sanctum* and *Guaiaicum coulteri*: historic distribution and future trends in Mexico. *Biotropica* 43:246-255.
- Lopez-Toledo, L., G. Ibarra-Manríquez, D. Burslem, E. Martínez-Salas, F. Pineda-García and M. Martínez-Ramos. 2011a. Protecting a single endangered species and meeting multiple conservation goals: an approach with *Guaiaicum sanctum* in Yucatan Peninsula, Mexico. *Diversity and Distributions* 18:575-587.
- Lopez-Toledo, L., A. Murillo-García, M. Martínez-Ramos and C. Perez-Salicrup. 2011b. Demographic effects of legal timber harvesting on *Guaiaicum sanctum* L., an endangered neotropical tree: implications for conservation. *Interciencia* 36:650-656.
- McCauley, R., A. Cortés-Palomec and K. Oyama. 2008. Isolation, characterization, and cross-amplification of polymorphic microsatellite loci in *Guaiaicum coulteri* (Zygophyllaceae). *Molecular Ecology Resources* 8:671-674.
- McCauley, R., A. Cortés-Palomec and K. Oyama. 2010. Distribution, genetic structure, and conservation status of the rare microendemic species, *Guaiaicum unijugum* (Zygophyllaceae) in the Cape Region of Baja California, Mexico. *Revista Mexicana de Biodiversidad* 81:745-758.
- Porter, D. 1963. The taxonomy and distribution of the Zygophyllaceae of Baja California, Mexico. *Contributions from the Gray Herbarium of Harvard University* vol. CXCII:99-110.
- Porter, D. M. 1972. The genera of the Zygophyllaceae in the southeastern United States. *Journal of the Arnold Arboretum* 53:531-532.
- Ran, J., X. Wei and X. Wang. 2006. Molecular phylogeny and biogeography of *Picea* (Pinaceae): implications for phylogeographical studies using cytoplasmic haplotypes. *Molecular Phylogenetics and Evolution* 41:405-419.
- Schindel, D. E. and S. E. Miller. 2005. DNA barcoding a useful tool for taxonomists. *Nature* 435:17.
- Shaw, J., E. Lickey, J. Beck, S. Farmer, W. Liu, J. Miller, K. Siripun, C. Winder, E. Schilling and R. Small. 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* 92:142-166.
- Shaw, J. and R. Small. 2005. Chloroplast DNA phylogeny and phylogeography of the North American plums (*Prunus* subgenus *Prunus* section *Prunocerasus*, Rosaceae). *American Journal of Botany* 92:2011-2030.
- Sheahan, M. and M. Chase. 2000. Phylogenetic relationships within Zygophyllaceae based on DNA sequences of three plastid regions, with special emphasis on Zygophylloideae. *Systematic Botany* 25:371-384.
- Simpson, B., A. Weeks, D. Helfgott and L. Larkin. 2004. Species relationships in *Krameria* (Krameriaceae) based on ITS sequences and morphology: implications for character utility and biogeography. *Systematic Botany* 29:97-108.
- Soltis, D. E. and R. K. Kuzoff. 1995. Discordance between nuclear and chloroplast phylogenies in the *Heuchera* group (Saxifragaceae). *Evolution* 49:727-742.
- Swofford, D. 2002. *PAUP* 4.0b10: Phylogenetic analysis using parsimony (and other methods). Sinauer Associates, Sunderland, Massachusetts.
- Taberlet, P., L. Gielly, G. Pautou and J. Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17:1105-1109.
- Tropicos. 2009. www.tropicos.org; last accessed: 06.VIII.2009.
- Templeton, A. 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. *Evolution* 37: 221-244.
- UNEP-WCMC. 2007. UNEP-ECMC Species Database: CITES Listed Species. [http:// sea.unep-wcmc.org/](http://sea.unep-wcmc.org/); last accessed: 12 .XI.2007.
- White, T., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* PCR protocols: a guide to methods and applications, M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White (eds.). Academic Press, Inc., New York. p. 315-322.
- Wolf, K., W. H. Lee and P. Sharp. 1987. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proceeding of the National Academy of Sciences of the United State of America* 84:9054-9058.