Phylogenetic Analysis of *Cestrum* Section *Habrothamnus* (Solanaceae) Based on Plastid and Nuclear DNA Sequences

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**ABSTRACT.** Phylogenetic analysis of DNA sequences from chloroplast regions and nuclear ribosomal ITS was conducted to test the monophyly of *Cestrum* sect. *Habrothamnus* and investigate its relationships with other sections of *Cestrum*. Molecular divergence was very low among the sampled species, suggesting a rapid diversification in *Cestrum*. Individual and combined analyses of these molecular data sets using maximum parsimony and Bayesian inference reject the monophyly of the traditionally recognized sections of *Cestrum*, including sect. *Habrothamnus*. Infrageneric classifications will require significant revision. Nevertheless, the resolved monophyletic clades in this molecular analysis are geographically structured.


The genus *Cestrum* ranges from southern Florida and northern Mexico to Chile, including the Antilles. However, a few species (e.g., *C. nocturnum*, *C. elegans*, and *C. parqui*) have become naturalized in several regions of the world. Species of *Cestrum* range from trees, shrubs, to vines with sympodial, polyaxial, and monochasial branching (Bell and Dines 1995). Species of *Cestrum* are well-known for their ornamental (Beckett 1987), chemical, and pharmacological potential (e.g., Prema and Raghuramulu 1994; Backhouse et al. 1996; Haraguchi-Mitsue et al. 2000). Attention has recently been brought to *Cestrum* because of its chromosomal structural novelties that imply systems of chromosome maintenance unknown in the eudicots (Sykorova et al. 2003). *Cestrum* is easily confused with the Andean genus *Sessea*. *Cestrum* has berries with angular seeds, whereas *Sessea* has capsular fruits with winged seeds (Benítez and D’Arcy 1998; Benítez and D’Arcy 1999; Benítez and Nee 2001). Nevertheless, Macbride (1962) and Carvalho and Schnnoor (1997) have united the two genera. Also *Cestrum* and *Sessea* share distinctive chromosomes (Sykorova et al. 2003), and in a phylogenetic analysis of the Antillean subfamily Goetzeoideae (Santiago-Valentin and Olmstead 2003) *Cestrum* and *Sessea* were resolved as well-supported sister taxa.

The genus *Habrothamnus* (Schlechtendal 1833; Endlicher 1839) was reduced to a section of *Cestrum* (Schlechtendal 1847), which has since been followed by other botanists (e.g., Dunal 1852; Francey 1935, 1936; D’Arcy 1973). Urban (1903) proposed sect. *Pseudocestrum* to include the Hispaniolan endemic, *C. inclusum*. According to Nee’s nomenclatural synopsis (Nee, in prep.) three sections are recognized in *Cestrum*: sections *Cestrum*, *Habrothamnus*, and *Pseudocestrum*. Section *Habrothamnus* includes eight species, which contrasts with Francey’s classification (1935, 1936) where 28 species and eight varieties are included. All species of section *Habrothamnus* are confined to the mountains of Central Mexico, Chiapas-Guatemala, Costa Rica-Panama, and Hispaniola, and are morphologically distinguished by inflorescences mostly terminal, club-shaped, bright-red or yellow corolla tube, diurnal flowering, and hummingbird pollination (D’Arcy 1999; Nee 2001; Nee, in prep.). Although species of *Cestrum* have been included in phylogenetic studies of the family Solanaceae (Marshall 1999; Olmstead and Palmer 1992; Olmstead et al. 1999; Chase et al. 2003; Santiago-Valentin and Olmstead 2003; Clarkson et al. 2004) the monophyly of *Cestrum* or the inter-relationships of its constituent species have never been addressed. The aim of this paper is to address these issues, with emphasis in the monophyly of *Habrothamnus*, using nuclear ribosomal 5.8S and ITS DNA sequences and chloroplast DNA sequences from the regions trnT-trnL, trnL-trnF, and matK-trnK. Likewise, we explore the utility of these sequence data for resolving a species-level phylogeny for subsequent studies that will include all species of *Cestrum*.

**MATERIALS AND METHODS**

**Taxon Sampling.** Species considered part of sect. *Habrothamnus* (18 accessions, Appendix 1) primarily were targeted for analysis. *Cestrum dasyanthum*, the recently described *C. milciomejiae* (Zanoni 1995), and *Cestrum* sp. nov. were also included because they have the diagnostic
characteristics of sect. *Habrothamnus*. Representative species of the other two *Cestrum* sections were sampled using morphology as guide to capture maximum diversity, including eight species of sect. *Cestrum* and five species of sect. *Pseudocestrum* (sensu Nee, in prep.). Thus, a total of 32 *Cestrum* species sampled across the three sections of *Cestrum* (Appendix 1) was analyzed. Twenty five species from Mexico, Guatemala, Dominican Republic, Costa Rica, and Panama were freshly collected and preserved in silica gel (Chase and Hills 1991). Voucher specimens are deposited at the National Herbarium of Mexico (MEXU) unless noted otherwise in Appendix 1. Collections of the following herbaria were consulted: A, BIGU, C, CGE, CR, F, G, GH, HAL, INB, JBDS, M, MEXU, MICH, MO, NY, PMA, S, UC, US, USJ, XAL, Z. Species of *Sessea*, *Salpiglossis*, and *Vestia* are known to be closely related to *Cestrum* (Olmstead et al. 1999) and thus serve as the outgroups. Samples of *Sessea* were donated by Michael Nee (New York Botanical Garden) and Lynn Bohs (University of Utah). Some sequences of *C. elegans*, *C. nocturnum*, *Vestia foetida*, and *Salpiglossis sinuata* were obtained from the GenBank (Appendix 1).

### DNA Extraction, Amplification, and Sequencing.

Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Inc.). The ITS region was amplified with the oligos 18 and 26 used by Wäss and Wink (1997). The trnL-trnF region was amplified with primers designed by Taberlet et al. (1991) and new internal primers designed for this study: A3 (5′-TCATTTTCAATTTGTAATGC-3′) and B4 (5′-GAATCTTTGACCATGTCG-3′). For the *matK-trnK* region we used the oligo r2 developed by Steele and Vilgalys (1994) and the oligo 1309F designed by Paul Kores (unpublished, Jodrell Laboratory, Royal Botanic Gardens, Kew, UK). Amplification reactions were performed according to the manufacturer's instructions (Qiagen Inc.). PCR programs differ for each region; for the ITS region the thermocycler was programmed using the Wäss and Wink (1997) profile, but annealing at 48°C. For the trnL-trnF region, a PCR cycling profile from Taberlet’s protocol (1997) was carried out. In the case of the trnT-trnL region, the previous routine was modified given the annealing temperature to 48°C. PCR cycling conditions for *matK-trnK* region were used following the protocol of Salazar et al. (2003). All PCR reactions were performed in a 9700 (PE Applied Biosystems) thermal cycler. PCR products were cleaned with the QIAquick PCR Purification Kit (Qiagen Inc.). DNA sequencing was performed with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit, v. 3.0, polymerase (Applied Biosystems). Both forward and reverse sequences were detected in an ABI Prism 3100 (Applied Biosystems Inc.) automated sequencer. All DNA sequences obtained in this study have been deposited in GenBank. Sequences alignments were obtained initially with ClustalW (Thompson et al. 1994), and subsequently refined manually following the guidelines of Kelchner (2000) using the on-line program BIOEDIT (Hall 1997–2001). Aligned data sets are available on TreeBASE (study number S1560).

### Phylogenetic Analyses.

Each of the ITS and plastid regions was analyzed separately and in combination, and all phylogenetic analyses were performed with PAUP 4.0b (Swofford 1998). All characters and character-state changes were weighted equally. All character transformati ons were treated as unordered (Fitch 1971). Phylogenetically informative indels were coded as separate presence-absence characters according to the simple indel coding scheme of Simmons and Ochoterena (2000). Maximum parsimony analysis was carried out with three thousand random addition replicates using the heuristic search option with TBR branch-swapping, and 50 trees were held at each step. Clade stability was assessed by bootstrap (BP; Felsenstein 1985), with 500 replicates using the heuristic search option with TBR branch swapping. Partition homogeneity tests (ILD Test; Farris et al. 1995) were carried out with 500 replicates using heuristic settings as in the bootstrap analysis. PAUP and ModelTest version 3.7 (Posada and Crandall 1998) were employed to select among alternative models of evolution using Akaike Information Criterion (AIC, Akaike 1974) outlined by Posada and Crandall (1998). Bayesian phylogenetic analysis was performed with MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). Monte Carlo Markov chains starting with a random tree were run for seven million generations. The majority consensus tree was computed after “burn in” and sampling every 5,000 generations.

### Results

Individual data sets show extremely low sequence divergence among *Cestrum* taxa and only some phylogenetic information (Table 1). The molecular divergence found in *Cestrum* species is below that found in other Solanaceae genera (Table 2). The best fit models were GTR+G for ITS data, F81 for trnT-trnL data, and TVM+G for trnL-trnF and *matK-trnK* data. The ILD test reveals no conflict between the ITS and chloroplast data sets (p = 0.1914). The combined data set included 2,944 sites and 19 indel characters. The parsimony analysis of the combined data set produced 129,250 most-parsimonious trees. Despite this large number, the strict consensus resolved most nodes (Fig. 1).

All *Cestrum* species were resolved as a monophyletic group sister to *Sessea vestita* (Fig. 1). In contrast, *Sessea* was resolved as paraphyletic with *S. stipulata* placed as sister to *Vestia foetida*. *Habrothamnus sensu lato* was not resolved as monophyletic and neither were the other two sections of *Cestrum* (Fig. 1). Although some clades had low bootstrap support, they showed geographical structure (Fig. 1). The Central-Mex clade contains species distributed in mountains of Central Mexico, and includes six species of sect. *Habrothamnus* and two of sect. *Cestrum*. The Talamancan clade contains species distributed throughout Costa Rica and Panama, and comprises two species of sect. *Habrothamnus* and one of sect. *Cestrum*. The Guatemala clade includes species inhabiting the mountains of Chiapas and adjacent Guatemala, and comprises two species of sect. *Cestrum* as basal grade and a subclade with six taxa of sect. *Habrothamnus*. The Central Mex, Tala manca, and Guatemala clad es are part of a polytomy that also contains the unresolved *C. poasanum*, *C. fulvescens*, and *Cestrum* sp. nov. Although this large clade has no bootstrap support, all 22 constituent species are continental. The Hispaniola clade is resolved with some support and comprises species restricted to Haiti and Dominican Republic.

**Discussion**

The molecular divergence in Solanaceae is low in comparison to other lineages of plants (Shaw et al. 2005). The nuclear ITS region was the most informative in this study, having more clades with support and more parsimony informative characters than any of the chloroplast partitions (Table 1). Even though *Cestrum* is well resolved as monophyletic, the molecular divergence among the *Cestrum* subgroups is the lowest reported for any of the Solanaceae genera (Table 2), even as low as *Ceratocamia* (Gonzáles and Vovides 2002). These low levels of molecular divergence among the sampled species of *Cestrum* resulted in clades with low bootstrap support. In contrast to the low molecular divergence, there is a remarkable morphological diversity in the genus, which suggests a recent and rapid diversification in *Cestrum*. Regardless, the limited sampling in this analysis suggests that *Sessea* is distinct from *Cestrum* (Fig. 1), thus supporting the traditional circumscription of *Cestrum*. The paraphyly of *Sessea* needs to be validated with additional sampling of this and related genera.

In all of the analyses, *Habrothamnus* (sensu Schlechtendal 1847; Dunal 1852; Francey 1935, 1936; Nee, in prep.) was never resolved as monophyletic. The infrageneric classifications of *Cestrum* have relied primarily on floral structures related to pollination syndromes, which according to the evidence presented here, have evolved convergently. In *Habrothamnus*, Schlechtendal (1847) included *Cestrum elegans*, *C. endlicheri*, *C. fasciculatum*, and other species.

**Table 1.** Nucleotide sequence statistics for ITS and chloroplast regions *trnL-trnF*, *trnT-trnL*, and *matK-trnK*. Information is given for separate and combined analyses. The consistency index (CI) was computed excluding uninformative characters. Simple indel indicates gaps that have similar 5' and 3' termini. Compound indel indicates gaps that have different 5' or 3' termini.

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<th><em>trnL-trnF</em> spacer</th>
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<td>49800 / 42</td>
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**Table 2.** Sequence statistics of ITS and chloroplast regions *trnL-trnF*, *trnT-trnL*, and *matK-trnK* for various genera of Solanaceae. The number of species analyzed in each study is reported in parentheses after the genus. Values derived from Miller (2002) and Whitson and Manos (2005) involved species of *Grabowskia* and *Margaranthus* because these genera were resolved as nested within *Lycium* and *Physalis*, respectively.

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<td>910</td>
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and *C. roseum*. These species grouped within the Central-Mex clade, as did others such as *C. laxum* and *C. miradorense*. These newly resolved clades are inconsistent with the diagnosis of Schlechtendal.

Similarly, all other circumscriptions of *Habrothamnus* (Dunal 1852; Francey 1935; Nee, in prep.) are inconsistent with Schlechtendal’s classification and also with the clades resolved in this study.
With regard to the validity of sect. Pseudocestrum, our results show that the Hispaniola clade includes species of sections Habrothamnus and Pseudocestrum, all occurring in Haiti and Dominican Republic. Included among these is C. inclusum, the type species of sect. Pseudocestrum (Urban 1903; Nee, in prep.). This assemblage may be monophyletic given its geographic distinction, and thus probably should be referred to as sect. Pseudocestrum. More Antillean species need to be sampled to validate this.

The combined analysis resolved four clades, each of which has a moderately narrow geographical distribution. The Central-Mex clade is confined almost entirely to Mexico and comprises three subclades, each with their own somewhat distinctive geographic localization. One of these includes two narrow endemics, C. miradorensis and C. endlicheri, along with C. fasciculatum and C. elegans that all occur mainly along the Sierra Madre Oriental (SMO). Another subclade includes C. roseum from the Transmexican Volcanic Belt (TVB) and C. oblongifolium distributed along SMO, from Tamaulipas to Hidalgo. Both of these species overlap in Hidalgo. The third clade includes C. laxum and C. thyrsoidenum, both distributed along TVB. Cestrum laxum is also found in Guatemala.

The Talamanca clade includes three species that all occur in the Talamanca Cordillera. The narrow endemic C. irazuense is localized in the northwest portion of this mountain range, and C. chiriquianum is restricted to Panama in the southeast portion of this Cordillera. Cestrum fragile occurs throughout this range.

The species of the Guatemala clade converge in the Chiapan-Guatemalan Highlands. The narrow endemic C. dasyanthum is restricted to Cuchumatanes Mountains, where it co-occurs with C. regelii, which also can be found in other Chiapas localities. The geographically restricted C. pacayense occurs on the volcanoes near Guatemala City. With wider ranges, C. guatemalense and C. lutteovirens occur their distributions from Chiapas to Honduras and El Salvador. Cestrum aurantiacum occurs in the mountains from western Mexico to Costa Rica, but C. aurantiacum var. macrocalyx is distributed in Guatemala and in the northwest portion of Talamanca Cordillera. The species with the broadest distribution in this clade is C. nocturnum, which has been collected from northern South America to Mexico and several islands of Greater and Lesser Antilles. However, Nee (2001) considers that this species has escaped from cultivation and its original distribution is more restricted in the region of Veracruz, Mexico, south to Nicaragua. The detection of the Guatemala clade somewhat supports this idea.

The species of the Hispaniola clade are also a combination of widely distributed and narrow endemics. Cestrum acutifolium, C. milciomejae, and C. tuerckheimii are distributed in different locations along the Central Cordillera. Cestrum violaceum is localized in the southern Sierra de Bahoruco-Massif de la Selle, whereas C. virgareum is known only from the Sierra de Neiba-Chaînes des Mathews. Cestrum inclusum and C. sphareocarpum are distributed throughout all of these same mountain ranges. The sister group of the Hispaniola clade is weakly supported as monophyletic (Fig. 1), and includes three continental species. One of these is a Guatemalan endemic, C. mortonianum, and two are more broadly distributed, C. tomentosum and C. glanduliferum. The Hispaniola clade and its sister clade are separated by an oceanic barrier, thus forming a transcontinental crown clade that could be of recent origin via dispersal given the lack of nucleotide sequence variation among these island endemics and their mainland relatives (cf. Lavin et al. 2004).

Each geographically confined clade includes both narrow endemics and more broadly distributed species. Geographical proximity predicts phylogenetic relationships in Cestrum. Although this could be explained by vicariance events, long distance dispersal is thought to be as equally explanatory if not more (Lavin et al. 2004). Similar patterns have been observed in Annonaceae, Rhamnaceae (Richardson et al. 2004), Melastomataceae (Renner 2004), and Leguminosae (Lavin et al. 2004; Schrire et al. 2004). The limited molecular divergence among the sampled species of Cestrum together with the geographical phylogenetic structure suggests that this pattern could be recently evolved, thus implicating dispersal over vicariance. But to test this hypothesis will require an evolutionary rates analysis (e.g., Lavin et al. 2004), for which fossils of Cestrum (or closely related genera) will be needed as time constraints. Such analysis will also require a broader sampling of South American and Antillian species to validate the geographical integrity of the clades resolved in this study.

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Biología Bosque Nuboso Monteverde), Instituto Nacional de Biodiversidad and Museo Nacional de Costa Rica; Universidad de San Carlos, Guatemala; Universidad de Panamá and Smithsonian Tropical Research Institute; Jardín Botánico Nacional, Dr. Rafael Ma. Moscosa, Dominican Republic.

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MARBH, J. A. 1999. Molecular systematics of Solanum section Lycopersicun: nuclear ITS rDNA sequences. Thesis of Ph. D. Department of School of Biosciences, Nottingham University, University Park, Nottingham, U.K.


APPENDIX 1. Voucher specimens associated with GenBank accession numbers of all sequences analyzed in this study. The infrageneric classification of Cestrum was modified from Nee (in prep.). All vouchers are deposited at MEXU unless otherwise noted. The first author identified all specimens except Sessea stipulata, which was identified by Michael Nee.


1. Voucher specimens associated with GenBank

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