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PHYLOGENETIC RELATIONSHIPS OF *CAPSICUM* (SOLANACEAE) USING DNA SEQUENCES FROM TWO NONCODING REGIONS: THE CHLOROPLAST *atpB-rbcL* SPACER REGION AND NUCLEAR *waxy* INTRONS

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This study focuses on three phylogenetic problems related to *Capsicum* (Solanaceae): (1) the monophyly of the genus, (2) species delimitation within the genus, and (3) phylogenetic relationships of species within *Capsicum*. The chloroplast *atpB-rbcL* noncoding spacer region was used to derive a phylogeny for seven outgroup genera and 11 species of *Capsicum*. Data derived from five introns within the nuclear gene *waxy* were used, both separately and in combination with the *atpB-rbcL* spacer data, to resolve further questions of species delimitation and phylogenetic relationships within *Capsicum*. *Capsicum* is monophyletic, with moderate support. *Capsicum ciliatum*, which is both molecularly and morphologically distinctive, is sister to a highly supported clade consisting of all other *Capsicum* species studied. *Capsicum cardenasii* and *C. eximium* are sister species and are, in turn, sisters to a moderately supported clade consisting of *C. tovarii*, *C. pubescens*, *C. chacoense*, *C. baccatum*, *C. galapagoense*, *C. chinense*, *C. frutescens*, and *C. annuum*. *Capsicum galapagoense*, whose taxonomic affinities have been largely unstudied, is included in a weakly supported clade consisting of *C. annuum*, *C. chinensis*, and *C. frutescens*. Many species of *Capsicum* have sufficient molecular markers in the *waxy* data set (both nucleotide substitutions and insertions/deletions) to be useful in species delimitation. An informal classification of the genus is proposed.

Keywords: *Capsicum*, *atpB-rbcL* spacer, *waxy*, chilies, peppers, Solanaceae, phylogeny, species delimitation.

Introduction

Capsicum (chilies and other peppers) consists of annual or perennial herbs or shrubs native to South and Central America and the Galápagos. Because humans have been affecting dispersal since prehistoric times, the original geographic distribution of *Capsicum* is difficult to determine. Of the 20–27 currently recognized species within the genus that appear to be native to Central and South America, ca. 17 have ranges overlapping in Bolivia. In the past 50 yr, several *Capsicum* species have been identified that were previously unknown to botanists, including *C. tovarii* (Eshbaugh et al. 1983), *C. cardenasii*, *C. praetermissum*, and *C. galapagoense* (Heiser and Smith 1958). Because of limited study, the affinities of *C. galapagoense* (Galápagos Islands) is of particular biogeographic and morphological interest.

Capsicum exhibits considerable morphological variation, especially in fruit shape, color, and size. Pubescence of leaves and stems range from glabrous to very pubescent. Inflorescences vary from solitary to seven flowers at one node. The calyx may range from long, green sepals to truncate sepals to spinelike projections. The corolla is rotate or infrequently campanulate, with highly variable coloration between and among species. Seeds are cream colored, except for *C. pubescens*, which has black seeds. *Capsicum* species, with few exceptions, are diploid ($2n = 24$, infrequently $2n = 26$) and have similar karyotypes (Lippert et al. 1966; Moscone et al. 1993). Many species have overlapping morphological character states, potentially leading to unresolved or erroneous species identification. A combination of diagnostic characters is usually required to identify and differentiate *Capsicum* species.

Archaeological evidence from Mexico indicates that humans have been using wild chili peppers as a food source possibly as early as 7200 B.C. (Pickersgill 1966; Heiser 1969). The oldest evidence of domesticated chilies was found in a cave in Tehuacan Valley (south-central Mexico) and dates to 5000–6500 B.C. (Davenport 1970), which establishes chilies as one of the earliest domesticated plants in the New World. There are four ancient agricultural centers in the New World, three of which are believed to have domesticated the chili pepper independently (Pickersgill 1969, 1977). After several thousand years of domestication, the varieties of chilies, along with other crops and technologies, were traded between the agricultural centers and dispersed over half of North and South America. Trading and migration rapidly expanded the ranges of many *Capsicum* species into small, fragmented populations scattered over vast regions, increasing the potential for interbreeding between domesticated and wild populations. While interbreeding is quite common in laboratory situations, it does not appear to occur frequently in the wild, possibly due to a strong tendency toward self-pollination in domesticates (Eshbaugh 1970, 1976).

The first known Europeans to come in contact with chilies were the crew of Columbus's initial transatlantic voyage to the New World. Peter Martyr, a historian who accompanied Columbus on his voyages, wrote in 1493 that the New World

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has a pepper more pungent than the black and white pepper from Asia (*Piper nigrum*; Lippert et al. 1966). The association between the spicy bite of black pepper and chilies is how the name "pepper" was inappropriately linked with *Capsicum*. Columbus returned to the Old World with several pungent forms of *Capsicum*, most of which were members of the species *C. annuum*. In Europe, the chili was enthusiastically and rapidly incorporated into many cultures. Within 50 yr, chilies spread from Spain to England (Lippert et al. 1966) and as far west as India (Deb 1979).

From the time chilies arrived in Europe up to the mid-1900s, taxonomists have disagreed on the criteria delimiting *Capsicum* species and varieties. Often the characters used in these studies were the same morphological features manipulated by domestication for 3500–7000 yr. In these studies, workers compared morphological differences between *Capsicum* varieties and deduced common ancestry based on such shared features as fruit shape, color, position, and pungency. These studies served only to obscure evolutionary relationships. Some early botanists recognized up to 100 species of *Capsicum*, while others recognized only a few (Eshbaugh 1980).

The morphological differences between wild and cultivated chilies are easily discerned. All wild forms of chilies have small, red, berry-like fruits with colors and sizes attractive to birds. Wild chilies have deciduous fruits, which, if not eaten by birds, fall to the ground while the seeds are still at peak viability. Domesticated forms exhibit variable fruit and flower coloration (designed to appeal to the human eye); gigantism of the fruits, seeds, flowers, and leaves (Cochran 1940; Eshbaugh 1976); and retention of the fruit on the peduncle at maturity (Pickersgill 1969; Eshbaugh 1976). When early taxonomists compared various *Capsicum* taxa, they noted that chilies sorted into two distinct groups: one typified by small, red fruits and the other by large fruits. This classification effectively separated the wild and domesticated forms of *Capsicum* but bore no relevance to evolutionary relationships.

Capsaicin, a volatile phenolic amine, is a very stable molecule and is responsible for the pungency commonly associated with chili peppers (Heiser 1969). When some chilies, such as the habañero, are ground into a powder, capsaicin can be detected by taste at dilutions up to 1 ppm. Presence of capsaicin was once thought to be an identifying characteristic found in all species within the genus (excluding only nonpungent, domesticated varieties). However, *C. ciliatum* is never pungent (Eshbaugh 1980), and several wild nonpungent forms of *C. chacoense* have been found. However, *C. anomalum*, despite its pungent fruit (determined by taste test by B. M. Walsh), has been removed from *Capsicum* to the monotypic genus *Tubocapsicum*, which is relatively distantly related to *Capsicum* (Olmstead et al. 1999; this study).

Enzymatic studies of *Capsicum* (Jensen et al. 1979; McLeod et al. 1979*a*, 1979*b*, 1982, 1983) have demonstrated that species could be grouped into taxonomic categories that somewhat agreed with groupings based on flower color (fig. 1). This system of classification is useful for separating some *Capsicum* species into subgeneric categories. However, less than half of the commonly recognized species of *Capsicum* were included in these studies. In addition, some of the excluded species do not fit into this categorization, such as the yellow flowers of *C. ciliatum* and *C. scolnikianum* and the white flowers of *C. chacoense*, which seem to be more closely related to the purple-flowered group than to the white-flowered group (McLeod et al. 1982).

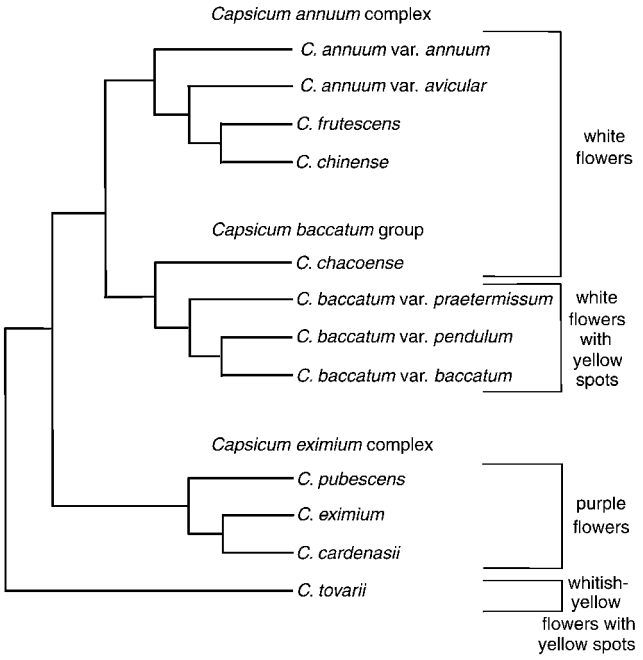

Fig. 1 *Capsicum* dendrogram constructed from standard genetic distance estimates based on isozyme data compared with a classification based on flower color (from McLeod et al. 1982).

During the past 40 yr, hybrid analyses have been used extensively to resolve species relationships in *Capsicum* (Heiser and Smith 1948, 1953, 1958; Smith and Heiser 1951, 1957; Emboden 1961; Lippert et al. 1966; Eshbaugh 1970, 1976; Pickersgill 1971; Eshbaugh et al. 1983). To determine the viability of hybrids between various species of *Capsicum*, pollen staining and F1 seed germination studies were used. The results of these hybrid analyses are helpful in grouping closely related species into subgeneric categories but have limited usefulness in determining evolutionary relationships (fig. 2).

Numerical comparisons of morphological traits (Cochran 1940; Eshbaugh 1970; Jensen et al. 1979; Pickersgill et al. 1979) and cytogenetic analyses (Shopova 1966; Ballard et al. 1970; McLeod et al. 1979*a*, 1979*b*, 1982; Moscone et al. 1993) have been used to resolve relationships. The numerical analyses typically included a limited number of species and focused primarily on the relationships of cultivated varieties to their wild progenitors. Cytogenetic analyses allowed greater resolution of the relationships between species and varieties but have achieved limited taxonomic resolution between closely related species, such as the *C. annuum/frutescens/chinense* and the *C. cardenasii/eximium* complexes. All of these studies correlate well with the hybrid analyses.

Species delimitation within two *Capsicum* species complexes remain problematic: (1) the *C. annuum* complex, consisting of *C. annuum*, *C. frutescens*, and *C. chinense*, and (2) the *C. eximium* complex, consisting of *C. eximium* and *C. cardenasii*. Species of the *C. annuum* complex contain both domesticated

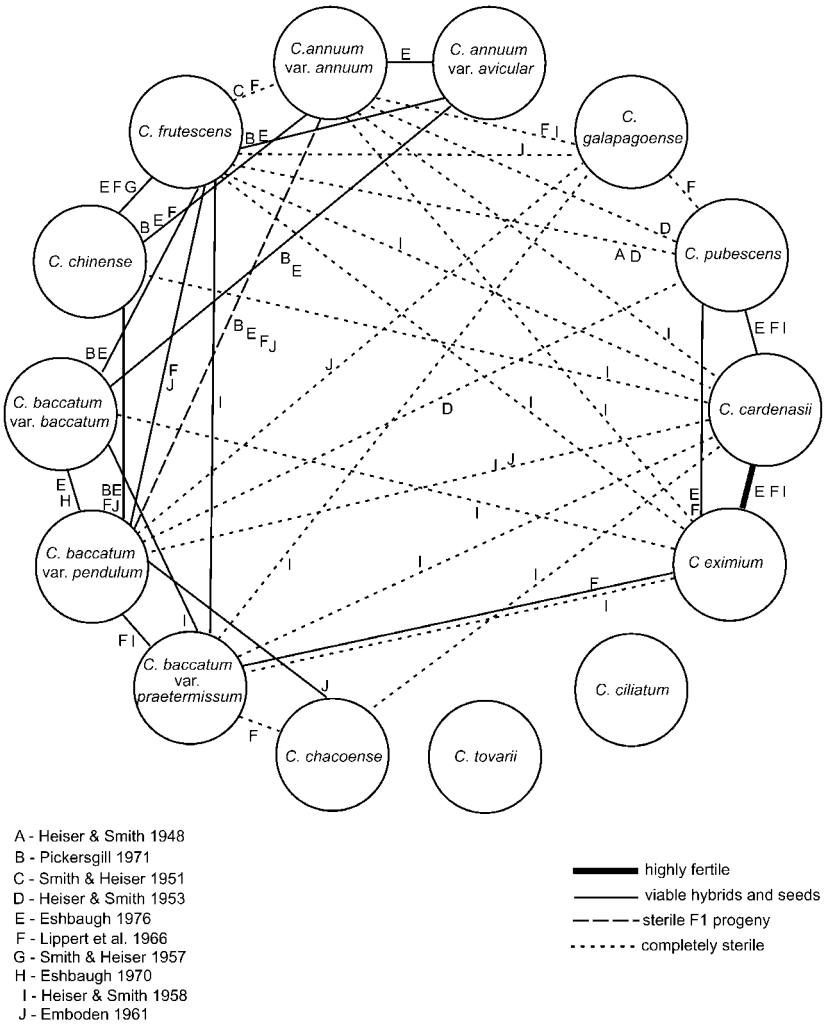

Fig. 2 Summary of *Capsicum*–hybrid crossing studies with associated citations indicated by the letters below

and wild varieties, as well as a wide range of intermediates, which are all similar morphologically and indistinguishable based on enzyme profiles (Jensen et al. 1979). Some researchers have argued that *C. frutescens* and *C. chinense* should be combined into one species (Pickersgill 1966, 1971; McLeod et al. 1979*b*) because they interbreed fairly readily (Smith and Heiser 1957; Lippert et al. 1966; Pickersgill 1966) and intergraded into a morphological continuum. *Capsicum frutescens* displays features considered typical of a wild species and is not cultivated on a large scale, except relatively recently on the Tabasco farms of Louisiana (Pickersgill 1971). *Capsicum chinense* does not have any true wild form and is cultivated extensively in South America. Several characteristics, such as nondehiscent fruit, fruit shape, and gigantism of leaves, fruit, and flower structure, suggest it has been cultivated for a long time (Pickersgill 1966, 1971).

The *C. eximium* complex consists of *C. eximium* and *C. cardenasii*, which are morphologically quite distinct. *Capsicum eximium* produces the rotate flowers typical of *Capsicum*, while *C. cardenasii* produces vaselike, campanulate flowers. Furthermore, *C. cardenasii* is the only species in the genus that obligately outbreeds (McLeod et al. 1979*a*). However, the ranges of these species overlap, and they appear to form natural hybrids (McLeod et al. 1979*a*). Hybrid studies indicate a high level of fertility, with 90%–100% pollen stainability (fig. 2; Lippert et al. 1966; Eshbaugh 1976). Hybrids of *C. eximium* and *C. cardenasii* are more fertile than some crosses between varieties within a species (Eshbaugh 1976). In addition, based on allozyme data, these two species are indistinguishable from each other (Jensen et al. 1979; McLeod et al. 1979*a*). It has been suggested that *C. eximium* and *C. cardenasii* be consolidated into a single, morphologically variable species (Ballard et al. 1970; Eshbaugh 1976; Jensen et al. 1979; McLeod et al. 1979*a*).

This study focused on three phylogenetic problems associated with *Capsicum*: (1) monophyly of the genus *Capsicum*, (2) species delimitation, and (3) phylogenetic relationships of the species within *Capsicum*. Recent work by Olmstead and Palmer (1997), Bohs and Olmstead (1997), and Olmstead et al. (1999) using both chloroplast sequences and restriction site data indicates that the genus *Capsicum* is derived from *Lycianthes*, making *Lycianthes* paraphyletic. Because of this close

Table 1

List of Species, Source of Plant Material or DNA, Voucher Information, and GenBank Numbers (*atpB-rbcL* spacer/*waxy*)

Species	Source/type of material	Voucher information	GenBank number
Aureliana fasciculata (Sendt.) Barb. & A. Hunz.	R. Olmstead/DNA	K. Brown s.n., UEC	AF397083
Capsicum annuum:			
var. *annuum** L.	USDA-ARS/seeds	B. Walsh 1, UWM	AF397108/AF397129
var. *annuum**	USDA-ARS/seeds	B. Walsh 24, UWM	AF397110/AF397131
var. *annuum* cv. Early CalWonder*	Green Valley Seed/seeds	B. Walsh 14, UWM	AF397109/AF397130
var. *aviculare* (Dierb) D'Arcy & Eshbaugh	USDA-ARS/seeds	B. Walsh 5, UWM	AF397106/AF397127
var. *aviculare*	CATIE 8191	B. Walsh 12, UWM	AF397107/AF397128
C. baccatum:			
var. *baccatum* L.	USDA-ARS/seeds	B. Walsh 6, UWM	AF397100/AF397120
var. *pendulum** (Willd.) Eshbaugh	USDA-ARS/seeds	B. Walsh 9, UWM	AF397101/AF397121
C. cardenasii Heiser & Smith	USDA-ARS/seeds	B. Walsh 26, UWM	AF397095/AF397116
C. chacoense Hunz.	USDA-ARS/seeds	B. Walsh 7, UWM	AF397099/AF397122
C. chinense Jacq.	USDA-ARS/seeds	B. Walsh 3, UWM	AF397102/AF397123
C. ciliatum (H.B.K.) O. Kuntze	R. Olmstead/DNA	C. Heiser 7518, IND	AF397094/AF397115
C. eximium Hunz.	B. Pickersgill/seeds	B. Walsh 35, UWM	AF397096/AF397117
C. frutescens L.	USDA-ARS/seeds	B. Walsh 20, UWM	AF397104/AF397124
cv. Tabasco*	Shepherds Garden Seeds/seeds	B. Walsh 15, UWM	AF397105/AF397125
C. galapagoense Hunz.	USDA-ARS/seeds	B. Walsh 18, UWM	AF397103/AF397126
*C. pubescens** Ruiz. & Pav.	USDA-ARS/seeds	B. Walsh 17, UWM	AF397098/AF397119
C. tovarii Eshbaugh, Smith & Nickrent	B. Pickersgill/seeds	B. Walsh 34, UWM	AF397097/AF397118
Datura stramonium L.	S. Hoot/leaves	B. Walsh 29, UWM	AF397076
Jaltomata auriculata (Miers) Mione	R. Olmstead/DNA	BIRM S1596/76	AF397081
Lycianthes ciliolata (Mart. & Gal.) Bitter	R. Olmstead/DNA	BIRM S0607/70	AF397085
L. cuchumatanensis J. L. Gentry	R. Olmstead/DNA	R. Olmstead 94-06, WTU	AF397092
L. glandulosa Bitter	R. Olmstead/DNA	BIRM S1616/75	AF397089/AF397114
L. heteroclita (Sendtn.) Bitter	L. Bohs/DNA	Bohs 2376, UT	AF397091/AF397113
L. lenta Bitter	R. Olmstead/DNA	R. Olmstead 96-92, WTU	AF397093
L. lycioides Hassl.	R. Olmstead/DNA	R. Olmstead S-87, WTU	AF397087/AF397111
L. magdalenae Bitter	R. Olmstead/DNA	Det. D. Symon s.n.	AF397090
L. rantonnei (Carrière) & Bitter	R. Olmstead/DNA	R. Olmstead S-96, WTU	AF397086/AF397112
Solanum aviculare Forst. f.	USDA-ARS/seeds	B. Walsh 33, UWM	AF397077
S. lycopersicum L.	R. Olmstead/DNA	No voucher[a]	AF397080
S. pimpinellifolium (L.) P. Miller	S. Hoot/leaves	B. Walsh 13, UWM	AF397079
S. pseudocapsicum L.	USDA-ARS/seeds	B. Walsh 32, UWM	AF397078
S. shanesii F. Muell. (=*Lycianthes* sp.)[b]	R. Olmstead/DNA	Clarkson 6674, AD	AF397088
Tubocapsicum anomalum (Franchet & Savat.) Makino	USDA-ARS/seeds	B. Walsh 27, UWM	AF397082
Withania coagulans (Stocks) Dun.	R. Olmstead/DNA	BIRM S0678/69	AF397084

Note. An asterisk denotes species and varieties of *Capsicum* commonly found in cultivation. BIRM = University of Birmingham Solanaceae seed collection; CATIE = Centro Agronómico de Investigación y Enseñanza, Costa Rica.

[a] Same DNA accession used in Olmstead and Palmer (1992, 1997) and Bohs and Olmstead (1997).

[b] *Solanum shanesii* is now considered a *Lycianthes* species, but a formal recombination has not yet been made (L. Bohs, personal communication).

relationship between *Capsicum* and *Lycianthes* and the limited sampling of both genera in the above studies, a goal of this research was to verify the monophyly of *Capsicum* and, with broader sampling (18 species and eight genera), determine the placement of *Capsicum* within Solanaceae. To accomplish this goal, the noncoding chloroplast DNA region between *atpB* and *rbcL* was sequenced. This spacer region is ca. 800 bp long in *Capsicum* and is suitable for taxonomic studies at the generic and family level (Golenberg et al. 1993; Savolainen et al. 1994; Manen and Natali 1995; Natali et al. 1995; Hoot and Douglas 1998).

To test species delimitations and phylogenetic relationships within *Capsicum*, sequence data from both the chloroplast *atpB-rbcL* spacer region and a 1200-bp segment from the nuclear gene *waxy*, encoding an essential enzyme in granule-bound starch synthesis (GBSS), were used. The *waxy* gene contains 12 introns and is ca. 3 kb long in *Solanum tuberosum* (van der Leij et al. 1991). The *waxy* region used in this study includes introns 2–6 and is ca. 900 bases long. The *waxy* gene has had limited use for phylogenetic work to date but is becoming increasingly popular. Unlike the ribosomal internal transcribed spacer (ITS) regions, which contain at least two, nonidentical paralogues in *Capsicum*, *waxy* appears to be single copy (van der Leij et al. 1991; Miller et al. 1999) and is most useful at the generic level (Mason-Gamer et al. 1998; Miller et al. 1999).

Material and Methods

Eleven of the 27 most commonly recognized species of *Capsicum* (Eshbaugh 1980), several varieties of some species, and seven outgroup genera were sampled in this study (table 1).

Included in the sampling are the five domesticated and some of the wild progenitor species (table 1). Because many species are difficult to attain and little systematic work has been done on the genus as a whole, it is difficult to know how representative this sampling is of the overall variation found in *Capsicum*. All taxa in this study were positively identified (see tables 2 and 3 in Walsh 1999 for a list of diagnostic characters used in species verification and botanical descriptions of *Capsicum* species). Sequencing, accession, voucher, and GenBank information are included in table 1. All seeds were germinated and grown in the greenhouses of the Department of Biological Sciences, University of Wisconsin—Milwaukee.

Total cellular DNA was isolated from fresh leaf material according to the miniprep method of Doyle and Doyle (1987). In most cases, DNA was further purified and concentrated after extraction using Wizard Column PCR Preps (Promega) or ethanol precipitation (Sambrook et al. 1989).

For amplification (PCR) of the *atpB-rbcL* spacer, four different 25-mer amplification primers were used (Hoot et al. 1995). The primer rbcL1 was used in all reactions. This primer complements the 5′ end of the *rbcL* gene but with opposite orientation, allowing amplification through the spacer region toward the *atpB* gene. The primer S385R within *atpB* was used in conjunction with rbcL1 for all samples, except *Lycianthes cuchumatanensis* and *L. lenta*, which were amplified with the primers S2R and S766R. The protocol for the amplification of the spacer region used a reaction mixture containing the final concentrations: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.5 μM of each amplification primer, 2.5 U Taq polymerase, and 0.3–2.0 μL template DNA per 100 μL reaction (depending on concentration). The sample was then amplified using the cycling parameters described in Hoot et al. (1995).

The regions of *waxy* were amplified using primers waxy 5′ and waxy 3′ based on the van der Leij et al. (1991) *Solanum tuberosum* sequence (kindly provided by D. Spooner) or two 20-mer primers located ca. 100 bases in from the two previous primers (primer sequences available by request from S. Hoot). Samples were amplified using ready-to-go PCR Beads (Pharmacia Biotech), adding 0.3 mM of each primer, 0.5 μL of template DNA, and 30 μL of mineral oil for a 25-μL reaction. The thermocycler was programmed with the following parameters: premelt at 94°C for 3 min, 40 cycles each consisting of a denaturation step at 94°C for 30 s, annealing step at 45°C for 30 s, and an extension step at 72°C for 2 min, followed by a final extension step of 72°C for 5 min. In some cases (13 of the 21 samples), 2% low-melting agarose gel plugs of the PCR product were diluted with 75–125 μL of ddH_2O, depending on the size of the plug, and used as a template for further PCR.

One sample, *Capsicum annuum* var. *annuum* cv. Early CalWonder, which did not amplify for *waxy* in large enough quantities for sequencing with the protocol described above, was cloned from the PCR product using a T-overhang vector kit (T-Easy, Promega). The vector was used to transform Promega ultracompetent cells. DNA-Pure Plasmid Mini-Prep Kit (CPG) was used to isolate, purify, and concentrate the vector DNA from cloudy cultures for use in automated sequencing.

PCR products were purified for automated sequencing by electrophoresis on a 2% low-melting agarose gel (Fisher Biotech) with 1% TAE buffer. Ethidium-bromide-stained bands were visualized over UV illumination and then removed as gel plugs. Either Wizard PCR Preps (Promega) or QIAquick PCR Purification columns (Qiagen) were used to remove agarose and concentrate the PCR product.

Both 5′ and 3′ strands of DNA were sequenced for the *atpB-rbcL* spacer and *waxy* with 100% overlap for the spacer region and at least 80% for *waxy*. In the case of *waxy*, the sequences amplified using waxy 5′ and waxy 3′ were pruned to the same length as those using the more internal amplification primers. In spite of the numerous indels, alignments could be accomplished to a rough approximation using Sequencher 3.0 (Gene Codes Corporation) with subsequent manual corrections.

The following alignment criteria and methodology were used: (1) Alignments maximized two elements—matching nucleotides at a sequence position and consideration of gap or indel type. Recognition of two types of indels (type Ia indels, runs of the same nucleotide of any length, and type Ib indels, regions of two or more base pairs with more complex repeated nucleotide motifs) are often helpful in assessing gap homology and reliability (Golenberg et al. 1993; Hoot and Douglas 1998). (2) Gaps were scored using simple indel coding (Simmons and Ochoterena 2000). (3) Phylogenetically informative indels (variable in two or more taxa) were scored as one event at the end of the data set. (4) Regions of the alignment that consisted of gaps in at least 50% of the taxa were removed from the data set before analysis (these regions were almost universally phylogenetically uninformative).

Phylogenetic analyses were performed using PAUP* 4.0b4a (Swofford 1998) with the branch-and-bound search option. PAUP* was also used to perform bootstrap analyses with 2000 replications using the branch-and-bound search option (Felsenstein 1985). Before combining the data sets, several methods of assessing congruence among the two data sets were implemented: visual comparison of the various clades found in the minimal trees, their bootstrap support, and implementation of the incongruence length difference test (Farris et al. 1995; implemented in PAUP*), which tests whether the predefined partitions in the data differ significantly from random partitions of the combined data set. The analysis was conducted with 1000 replications, heuristic search with simple addition, TBR (tree bisection/reconnection) branch swapping, and saving up to 2000 trees for each replicate.

Outgroup taxa for both analyses were selected based on the results of several previous phylogenetic analyses of the Solanaceae (Olmstead and Palmer 1992, 1997; Bohs and Olmstead 1997; Olmstead et al. 1999). The studies cited above indicate that, of the outgroup taxa sampled in this study, *Datura* occupies the most basal position within the Solanaceae. For this reason, *Datura* was selected as the outgroup in the analysis at the family level. We also rooted the family-level analysis with the immediate sister group (*Tubocapsicum*/*Areliana*/*Witheringia*) to *Capsicum* to test for changes in tree topology. Several studies have shown *Lycianthes* to be paraphyletic (Olmstead and Palmer 1992, 1997; Bohs and Olmstead 1997; Olmstead et al. 1999) or as a monophyletic group sister to *Capsicum* (this study). To be consistent with the results from our family-level analysis, we assumed monophyly of both *Lycianthes* and *Capsicum* and rooted the generic-level tree accordingly.

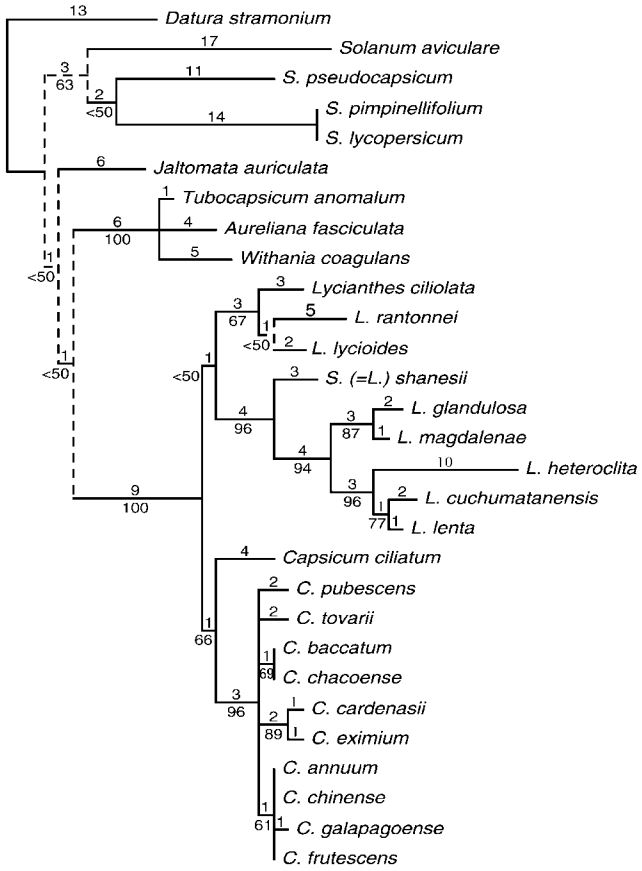

Fig. 3 One of the 18 shortest trees based on *atpB-rbcL* spacer data for *Capsicum* and outgroups. Numerals above lines are number of substitutions supporting branches; numerals below lines are bootstrap values. Dotted lines indicate where branches collapse in strict consensus tree.

Results

The family-level analysis of the *atpB-rbcL* spacer data resulted in 18 equally parsimonious trees based on 67 variable sites and a consistency index excluding autapomorphies (CI) = 0.77 and a retention index (RI) = 0.90. One of the 18 shortest trees is presented in figure 3. Tree topology within *Capsicum* and *Lycianthes* remained the same whether rooted with *Datura* or the more immediate sister clade of *Tubocapsicum*, *Aureliana*, and *Withania*. While the tree lacks support for many of the more basal nodes, several more derived clades are well supported. The genera *Tubocapsicum*, *Aureliana*, and *Withania* form a well-supported (bootstrap = 100%) trichotomy. *Lycianthes* and *Capsicum* together form a highly supported clade (bootstrap = 100%; fig. 3). Within *Lycianthes*, two clades are recognized: a strongly supported clade (bootstrap = 96%), consisting of *Solanum* (=*Lycianthes*) *shanesii*, *L. glandulosa*, *L. magdalenae*, *L. heteroclita*, *L. cuchumatanensis*, and *L. lenta*, and a smaller, weakly supported clade (bootstrap = 67%), consisting of *L. rantonnei*, *L. lycioides*, and *L. ciliolata*. All species of *Capsicum* form a largely unresolved monophyletic group weakly supported with one base substitution (bootstrap = 66%) with *C. ciliatum* as sister to all remaining *Capsicum*. The remaining *Capsicum* species (excluding *C. ciliatum*) form a strongly supported monophyletic group (bootstrap = 96%). Several clades are formed within this core *Capsicum* group, which correspond to known species complexes: the *C. annuum/chinense/frutescens* complex, now also including *C. galapagoense* (bootstrap = 61%); the well-supported *C. eximium/cardenasii* complex (bootstrap = 89%); and the *C. baccatum/chacoense* group (bootstrap = 69%).

The generic-level analysis of four species of *Lycianthes* and 17 *Capsicum* taxa using *waxy* data consisted of 200 variable characters (including 20 gaps) and 113 parsimony informative characters. These data resulted in one most parsimonious tree (CI = 0.87, RI = 0.94). The *atpB-rbcL* spacer data (60 variable characters including gaps, 20 informative characters) for the same taxa also resulted in one shortest tree (CI = 0.87, RI = 0.93). The tree resulting from the *atpB-rbcL* spacer data was similar to the *waxy* tree but with considerably less resolution. Both visual inspection of the tree topologies and the partition homogeneity test indicated that the two data sets were highly congruent (*P* value = 1.00). For this reason, only the tree resulting from the combined *atpB-rbcL* spacer and *waxy* data is presented here (fig. 4).

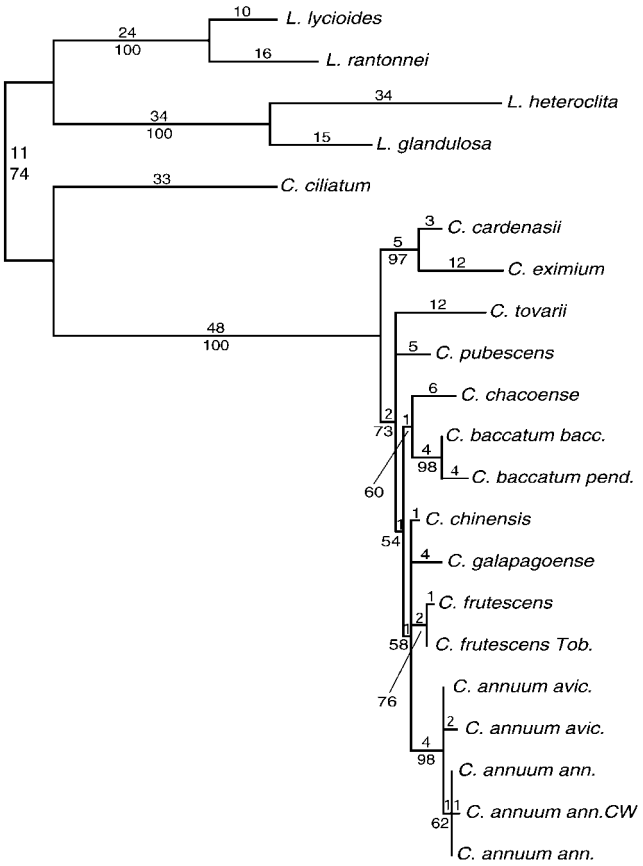

Fig. 4 The shortest tree resulting from the combined *atpB-rbcL* spacer and *waxy* data. Numerals are as in fig. 3; *bacc.* = var. *baccatum*, *pend.* = var. *pendulum*, *Tob.* = cv. Tobasco, *avic.* = var. *aviculare*, *ann.* = var. *annuum*, and *CW* = cv. Early CalWonder.

The combination of the *atpB-rbcL* spacer and *waxy* data (including gaps) resulted in one most parsimonious tree (fig. 4) derived from 133 informative characters (CI = 0.87, RI = 0.94). Reanalysis excluding gap data resulted in two trees with identical topology except for the collapse of the monophyletic *C. frutescens* clade (trees not presented). The division of *Capsicum* and *Lycianthes* is moderately well supported, with 11 nucleotide changes and 74% bootstrap. The monophyly of the core *Capsicum* group (excluding *C. ciliatum*) is extremely well supported, with 48 characters and 100% bootstrap. Within the core *Capsicum*, the following received moderate to strong bootstrap support: a clade consisting of all *Capsicum*, excluding *C. cilatum*, *C. cardenasii*, and *C. eximium* (73%); a *C. cardenasii* and *C. eximium* clade (97%); and clades consisting of two varieties of *C. baccatum* (98%), two taxa of *C. frutescens* (76%), and five taxa of *C. annuum* (98%). The *C. annuum* complex (McLeod et al. 1982), consisting of *C. annuum*, *C. chinense*, *C. frutescens*, and *C. galapagoense*, form a weakly supported clade (bootstrap = 58%).

Both *waxy* and spacer data provided markers (nucleotide substitutions and indels) at the species level, which may be useful in species delimitation (table 2).

Table 2

Molecular Markers

	atpB-rbcL spacer		*waxy*	
Taxon	Substitutions	Indels	Substitutions	Indels
Capsicum annuum (5)	0	0	2	1
C. baccatum (2)	0	0	4	0
C. cardenasii	1	0	1	0
C. chacoense	0	0	4	1
C. chinense	0	0	1	0
C. ciliatum	2	1	20	4
C. eximium	1	0	8	1
C. frutescens (2)	0	0	0	1
C. galapagoense	1	0	1	0
C. pubescens	2	0	3	0
C. tovarii	2	0	10	0

Note. Unique substitutions and insertion/deletions not found in any other *Capsicum* or *Lycianthes* taxa, potentially useful in species delimitation within *Capsicum*. Numerals in parentheses indicate number of taxa sequenced within a species.

Discussion

Family-Level Analyses

The trees derived from the *atpB-rbcL* spacer data support with high bootstrap values (100%) a clade consisting of *Tubocapsicum*, *Aureliana*, and *Withania* and a large clade consisting of *Lycianthes* and *Capsicum* (fig. 3). Despite the presence of a possible capsaicin-like compound in *Tubocapsicum* (as detected by a taste test), this genus is not closely related to *Capsicum*. Similar results were found in earlier molecular studies of *Tubocapsicum* (Olmstead and Palmer 1997; Olmstead et al. 1999). These studies and our data indicate that capsaicin-like compounds may have arisen at least twice in the evolution of Solanaceae. This possibility is currently being explored in an evolutionary study of the functions of capsaicin (J. Tewksbury, personal communication).

A strict consensus tree of the 18 shortest trees obtained from the *atpB-rbcL* spacer data indicates that *Capsicum* is monophyletic but with relatively weak support (bootstrap = 66%; fig. 3). While the analyses of the combined *waxy* and spacer data rooted between *Lycianthes* and *Capsicum* cannot confirm the monophyly of either genus, it does indicate that if *C. ciliatum* is excluded, the monophyly of the remaining *Capsicum* species is strongly supported (bootstrap = 100%). It is clear that further work needs to be done to confirm the monophyly of both *Capsicum* s.lat. and *Lycianthes*.

Species Delimitation

Most of the variation occurs in the earliest diverging branches on the tree resulting from the combined *waxy* and *atpB-rbcL* spacer data, with *C. ciliatum*, *C. eximium*, and *C. tovarii* the most divergent species within *Capsicum* (fig. 4; table 2). All remaining *Capsicum* species appear to have diverged more recently and therefore are not so clearly delimited from each other using molecular data. The following paragraphs discuss the potential for using *waxy* and *atpB-rbcL* spacer data to delimit species. The *waxy* introns are especially useful at the species level, providing more variation at this level than is commonly found with sequence data. However, the efficacy of these markers needs to be tested with increased sampling at the population level.

Capsicum ciliatum is the only *Capsicum* species with an insertion (4 bases in length) in the *atpB-rbcL* spacer data (table 2). In addition, it has 20 unique substitutions (not found in any other *Capsicum* or *Lycianthes* taxa) and four unique gaps in the *waxy* data: three deletions (including one 12 bases long) and one insertion. D'Arcy and Eshbaugh (1974) speculated that *C. ciliatum* may belong in its own genus. The weak-to-moderate sequence support for its inclusion in *Capsicum* (figs. 3, 4) and the sequence divergence found in *C. ciliatum* (24 unique characters; table 2), combined with the unique characters of yellow flower color and the complete absence of capsaicin (D'Arcy and Eshbaugh 1974), lend some support to D'Arcy and Eshbaugh's argument.

Capsicum annuum, from five different sources and including two subspecies, is the best test of species delimitation with our data. All accessions are clearly supported as a monophyletic group on the combined phylogeny (bootstrap = 98%). In addition, all accessions share three unique characters found in no other taxon (two substitutions and one 1-base deletion; table 2). The multiple varieties or cultivars of *C. baccatum* and *C. frutescens* are moderately to hightly supported as monophyletic groups with four unique molecular markers for *C. baccatum* and one marker (a 1-base insertion) for *C. frutescens* (table 2). Other species of *Capsicum* are well defined by both unique substitutions and gaps, which may be useful in future delimitation (table 2): *C. chacoense* (four substitutions and one 1-base deletion), *C. eximium* (nine substitutions and one 4-base deletion), *Capsicum pubescens* (five substitutions), and *C. tovarii* (12 substitutions). *Capsicum cardenasii* and *C. eximium*, sometimes hypothesized as a single species (see "Introduction"), are well supported as separate species,

with at least 12 differences between them in the molecular data.

Species Relationships and Informal Classification of Capsicum

When comparing the results of this study to the enzyme studies of McLeod et al. (1979*a*, 1979*b*, 1982, 1983; fig. 1), the species of *Capsicum* in common between the two studies assume largely identical patterns of relationship. Similarly, the molecular data are somewhat congruent with hybridization studies (fig. 2). Combining all three sources of information, an informal classification was developed (see below). This is meant to be a ground plan for future studies in *Capsicum*, which will hopefully include some additional species that have been difficult to obtain. After each species name, geographic ranges and, when extensively domesticated, proposed places of origin (PPO, after Mcleod et al. 1982, 1983) are given.

***Ciliatum* group.** *Capsicum ciliatum*: southern Mexico to northern Peru.

***Eximium* group.** *Capsicum eximium*: Bolivia and northern Argentina; *Capsicum cardenasii*: Dept. of La Paz, Bolivia.

***Baccatum* group.** *Capsicum baccatum*: northwestern South America to northern Argentina (PPO: subtropical Bolivia); *Capsicum chacoense*: Bolivia, Argentina, Paraguay.

***Annuum* group.** *Capsicum annuum*: southern United States to northern Peru, Bolivia, and West Indies (PPO: Mesoamerica); *Capsicum chinense*: Central America, Caribbean, and central South America (PPO: Amazon Basin); *Capsicum frutescens*: Mexico, Central America, Carribean, and northern South America (PPO: Amazon Basin); *Capsicum galapagoense*: Galápagos Islands (Ecuador).

Unassigned to group. *Capsicum tovarii*: Dept. of Ayacucho, Peru; *Capsicum pubescens*: Andean highlands to Mexico (PPO: midelevation Bolivia).

The monotypic *Ciliatum* group is sister to all remaining *Capsicum* and, as mentioned above, is genetically distinct from the core *Capsicum* species. There is moderate phylogenetic support for its inclusion within *Capsicum*, so we retain it within the genus. This species is characterized by yellow corollas and the complete absence of capsaicin. *Capsicum ciliatum* develops small (under 1.0 cm), red, spherical fruit.

The *Eximium* group, consisting of *C. eximium* and *C. cardenasii,* is strongly supported as sister species (bootstrap = 97%; fig. 4) and moderately supported as sister to all other core *Capsicum* species (bootstrap = 73%). These two species, when crossed, are the only hybrids within *Capsicum* that produce highly fertile progeny (fig. 2). They are characterized by a "viny" habit (Eshbaugh 1976) and purple corollas with yellow-green throats. Like *C. ciliatum*, *C. eximium* and *C. cardenasii* have small (under 1.0 cm), red, spherical fruit. They inhabit low montane, xerophytic regions (Eshbaugh 1976)

The placement of *C. pubescens* and *C. tovarii* is the most problematic of all the taxa investigated in this study. Here we treat these two species as unassigned because their position within *Capsicum* is not well resolved (fig. 4). *Capsicum tovarii* has cream corollas (sometimes with purple petal margins) with a pair of yellowish spots at the base of each petal and produces small (under 1.0 cm), red, spherical fruit. *Capsicum pubescens* had previously been considered a member of the *C. eximium*/*cardenasii* complex based on hybridization studies (Heiser and Smith 1958; Lippert et al. 1966) and enzyme profile studies (Jensen et al. 1979; McLeod et al. 1979*a*, 1979*b*, 1982, 1983). *Capsicum pubescens* has purple corollas (sometimes cream with purple margins) and forms large (over 2.0 cm), globose fruit with a variety of colors. The unusual fruit size and color of *C. pubescens* is probably the result of cultivation. *Capsicum tovarii* and *C. pubescens* are both found in xerophytic regions at low to midelevations in the Andes (McLeod et al. 1982; Eshbaugh et al. 1983).

The *Baccatum* group, consisting of *C. baccatum* and *C. chacoense*, is morphologically diverse. There are no unique morphological characters that unite these species, and the molecular support for the group is weak (one substitution; bootstrap = 60%). However, isozyme data (fig. 1; McLeod et al. 1982, 1983) provide additional support for this group. *Capsicum baccatum* and its varieties have white to cream corollas (except var. *praetermissum*, which may have violet corolla margins) with a pair of yellowish spots at the base of each petal. The varieties of *C. baccatum* each have distinct fruit shapes. *Capsicum chacoense* has a dull white corolla and develops red, oblong, globose fruit under 1.5 cm long. It had been previously categorized as a species nearly equidistant between the *C. annuum* complex, *C. baccatum*, and the *C. eximium* complex (McLeod et al. 1979*b*; Moscone et al. 1993). The *Baccatum* group is believed to have originated in south-central Bolivia in drier lowland habitats (McLeod et al. 1982).

The *Annuum* group consists of *C. annuum* (including numerous varieties and cultivars), *C. chinense*, *C. frutescens*, and *C. galapagoense*. As with the *Baccatum* group, there are no morphological characters that unite this group but strong support from isozymes (fig. 1; McLeod et al. 1982, 1983) and crossing studies (fig. 2). *Capsicum annuum* typically has a white corolla but may be greenish or purple. *Capsicum annuum* var. *annuum* is the most widely cultivated chili and can develop fruit with a variety of different colors, shapes, and sizes, which are often fleshy. Some cultivars develop fruit over 20 cm in length. *Capsicum annuum* var. *aviculare*, the wild progenitor of the cultivated *C. annuum* var. *annuum*, has small (rarely exceeding 1.0 cm), red, globose or ovoid fruit. *Capsicum chinense* is a cultivated species that has a dull white corolla (rarely greenish white) that forms large (often over 1.5 cm wide and long), fleshy, variously colored and shaped fruit. *Capsicum frutescens* has a greenish white corolla and forms red (rarely orange), fleshy, globose or subconical fruit under 2.5 cm in length. *Capsicum galapagoense* has a white corolla with a faint yellow tint and develops small (under 1.0 cm), red, spherical fruit. The original geographic distribution of the *Annuum* group is believed to be moister habitats of lowland tropical South and Central America (Heiser 1976; Pickersgill et al. 1979).

The placement of *C. galapagoense* has never previously been studied using either morphological or molecular data. All data sets presented in this study, separate and combined, include *C. galapagoense* in the weakly supported *Annuum* group. Comparing the patterns of relationship in this study to the body of hybridization data, all species except *C. galapagoense* are capable of producing viable hybrids when crossed with their closest sister groups (fig. 2). The same evolutionary processes that allowed *C. galapagoense* to become morphologi-

cally distinct (extreme pubescence, dwarfed fruit, generally distinct flowers and leaves) from other *Capsicum* species may now inhibit successful crossing with other species.

Because geographic distributions within *Capsicum* are overlapping and manipulated by man, it is difficult to test their correlation with our molecular tree. However, judging from the earliest branching species on our combined tree (*C. ciliatum*, *C. cardenasii*, and *C. eximium*; fig. 4), it appears that the ancestors of *Capsicum* may have evolved in the drier regions of the present-day Andes (Peru and Bolivia) with subsequent migration north or east into tropical lowland regions. Similarly, McLeod et al. (1982) hypothesized that *Capsicum* arose in south-central Bolivia. However, they based this hypothesis on the belief that *C. chacoense* or its progenitor were ancestral in the genus. Further molecular work with more complete sampling (including many of the species that are difficult to obtain and seldom studied) is needed to test this hypothesis.

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