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A species-level phylogenetic study of the *Verbena* complex (Verbenaceae) indicates two independent intergeneric chloroplast transfers

Yao-Wu Yuan *, Richard G. Olmstead

Department of Biology, University of Washington, P.O. Box 355325, Seattle, WA 98195 5325, USA

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ABSTRACT

Two major impediments to infer plant phylogenies at inter- or intra- species level include the lack of appropriate molecular markers and the gene tree/species tree discordance. Both of these problems require more extensive investigations. One of the foci of this study is examining the phylogenetic utility of a combined chloroplast DNA dataset (> 5.0 kb) of seven non-coding regions, in comparison with that of a large fragment (ca. 3.0 kb) of a low-copy nuclear gene (*waxy*), in a recent, rapidly diversifying group, the *Verbena* complex. The complex includes three very closely related genera, *Verbena* (base chromosome number $x = 7$), *Glandularia* ($x = 5$), and *Junellia* ($x = 10$), comprising some 150 species distributed predominantly in South and North America. Our results confirm the inadequacy of non-coding cpDNA in resolving relationships among closely related species due to lack of variation, and the great potential of low-copy nuclear gene as source of variation. However, this study suggests that when both cpDNA and nuclear DNA are employed in low-level phylogenetic studies, cpDNA might be very useful to infer organelle evolutionary history (e.g., chloroplast transfer) and more comprehensively understand the evolutionary history of organisms. The phylogenetic framework of the *Verbena* complex resulted from this study suggests that *Junellia* is paraphyletic and most ancestral among the three genera; both *Glandularia* and *Verbena* are monophyletic and have been derived from within *Junellia*. Implications of this phylogenetic framework to understand chromosome number evolution and biogeography are discussed. Most interestingly, the comparison of the cpDNA and nuclear DNA phylogenies indicates two independent intergeneric chloroplast transfers, both from *Verbena* to *Glandularia*. One is from a diploid North American *Verbena* species to a polyploid North American *Glandularia* species. The other is more ancient, from the South American *Verbena* group to the common ancestor of a major *Glandularia* lineage, which has radiated subsequently in both South and North America. The commonly assumed introgressive hybridization may not explain the chloroplast transfers reported here. The underlying mechanism remains uncertain.

1. Introduction

It is a common observation that plant phylogenetics at the species or population level has lagged behind deeper-level phylogenetic studies, largely due to the lack of appropriate molecular markers (Schaal et al., 1998; Small et al., 1998; Bailey et al., 2004; Shaw and Small, 2004; Shaw et al., 2005, 2007; Hughes et al., 2006). Chloroplast and mitochondrial DNA sequences have too little variation for many plant groups at species or population level, and so do the conventional nuclear ITS sequences (Hughes et al., 2006). Single or low-copy nuclear DNA sequences may have sufficient variation, but inferences from these data are often confounded by their four times longer coalescent time in comparison with organelle DNA (Moore, 1995), which is related to the other major impediment—the potential gene tree/species tree discordance (Pamilo and Nei, 1988; Doyle, 1992; Maddison, 1997). Random lineage sorting accounts for much of this gene tree/species tree discordance as well as the incongruence among gene trees inferred from different loci (Pamilo and Nei, 1988; Maddison, 1997). It is particularly problematic for recent and rapidly diversifying species, because times between species divergences are too short for the loss of ancestral polymorphism and thus lineage sorting is largely incomplete, leading to the gene tree/species tree discordance. Despite these difficulties, the importance of a well-resolved species level phylogeny in studying organismal evolution, including hybridization, introgression, polyploidization, adaptive speciation, character evolution, etc., can never be overemphasized.

Methods for extracting information from incongruent gene trees to infer a species tree recently have been developed (Maddison and Knowles, 2006; Liu and Pearl, 2006; Ané et al., 2007; Carstens and Knowles, 2007), and these methods are likely to become more sophisticated by future improvements. Nonetheless, choice of appropriate molecular markers for inferring plant phylogenies

* Corresponding author.
E-mail address: colreeze@u.washington.edu (Y. Yuan).

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at inter- or intra-species level requires more extensive investigations. To address this issue, we are carrying out a series of studies that examine the utility of various molecular markers, including non-coding chloroplast DNA (cpDNA), large fragments of low-copy nuclear genes, and transposable element insertions, in resolving interspecies relationships in a recent and rapidly diversifying group, the *Verbena* complex (Verbenaceae). This paper represents the first part of these studies and is focused on the application of combined cpDNA sequences of relatively fast-evolving non-coding regions, in comparison with that of a large fragment of a low-copy nuclear gene.

Seven non-coding chloroplast regions were chosen for sequencing based on a previous study that examined relative utility of 21 non-coding cpDNA regions for general phylogenetic analysis (Shaw et al., 2005). These include intergenic spacers and/or introns in *trn*D–*trn*T, *trn*S–*trn*G, *trn*S–*trn*fM, *trn*T–*trn*L, *trn*G, *trn*L, and *trn*L–*trn*F, totaling ca. 5.3 kb in the *Verbena* complex. The nuclear DNA sequence data are from the granule-bound starch synthase I (GBSSI or *waxy*) gene. The intron sequences of this gene have been used to resolve relationships among closely related species in *Castilleja* (Orobanchaceae), and optimized primers to amplify this locus in Lamiales are available (Tank, 2006). The region we sequenced is from the end of exon 7 to the beginning of exon 13, which is about 3.0 kb long in the *Verbena* complex due to the relatively large introns.

The *Verbena* complex includes *Verbena*, *Glandularia*, and *Junellia*, with each genus containing 40–50 species (Botta, 1989; Sanders, 2001). The relationships among these genera are complex, and they were often combined in the genus *Verbena* in early treatments (Briquet, 1895; Perry, 1933; Troncoso, 1974). The most consistent character to separate these three groups is base chromosome number. *Verbena* has a base chromosome number of $x = 7$ and *Glandularia* has $x = 5$ (Lewis and Oliver, 1961; Umber, 1979). *Junellia*, with $x = 10$, was suggested to be more closely related to *Glandularia* than to *Verbena*, and the base number $x = 10$ was thought to be derived from $x = 5$ through polyploidy (Botta and Brandham, 1993). The underlying assumption of these hypotheses is that the smaller base number, $x = 5$, is ancestral to $x = 10$. A phylogenetic framework is highly desirable to evaluate such prior assumptions. *Junellia* is restricted to South America and contains both diploids and polyploids, whereas both *Glandularia* and *Verbena* have a disjunct distribution between the arid regions of temperate North and South America. A curious reciprocal cytogeographic pattern was pointed out by Lewis and Oliver (1961). *Verbena* is predominantly diploid in North America and mostly polyploid in South America, whereas *Glandularia* is predominantly diploid in South America and entirely polyploid in North America (Table 1). A robust phylogeny is essential to understand this intriguing cytogeographic pattern.

Chloroplast transfer (or "chloroplast capture"), the introgression of a chloroplast from one species into another, has been reported many times, including the well-known examples of *Gossypium*, *Helianthus*, *Quercus* (reviewed by Rieseberg and Soltis, 1991). Since this review, phylogenetic studies have suggested widespread chloroplast introgression in various taxa, mainly on the basis of incongruence between cpDNA and nuclear ribosomal DNA (ITS) phylogenies. The majority of these previously reported examples are interspecies introgressions, with only one case of intergeneric introgression (Soltis et al., 1991). Our study of the *Verbena* complex indicates two independent intergeneric chloroplast transfers, both from the genus *Verbena* to *Glandularia*. While one of these transfers is recent, the other is more ancient, from a source in South American *Verbena* to the common ancestor of a major *Glandularia* lineage that has radiated subsequently in both South and North America.

2. Materials and methods

2.1. Taxon sampling

Fifty species of the *Verbena* complex and two outgroup species have been included in this study. Our sampling represents both geographic distribution and ploidy level (Table 1). Outgroup species (*Lippia salsa* and *Aloysia virgata*) were chosen based on a preliminary phylogenetic study of Verbenaceae (Olmstead, unpublished data), which identified a sister group relationship between the *Verbena* complex and the *Lippia*/*Lantana*/*Aloysia* complex.

2.2. Molecular methods

Total DNA was extracted from either field-collected, silica-gel dried tissue (45 species) or herbarium specimens (7 species, Table 1) using the modified CTAB method (Doyle and Doyle, 1987). PCR primers with corresponding references are listed in Table 2. The seven cpDNA regions were amplified in four fragments. The *trn*S–*trn*G spacer and *trn*G intron were amplified together as the *trn*S–G fragment, and the *trn*T–*trn*L spacer, *trn*L intron, and *trn*L–*trn*F spacer were amplified together as the *trn*T–F fragment. However, for some of the herbarium specimens, these regions were amplified in overlapped smaller pieces. PCR procedures for these cpDNA regions were as follows: one cycle of 3 min at 94 °C, 4 cycles of 15 s at 94 °C, 15 s at 55 °C, and 90 s at 72 °C, followed by 30 cycles of 15 s at 94 °C, 15 s at 50 °C, and 60 s at 72 °C, with a final extension step for 10 min at 72 °C. Amplified PCR products were purified by precipitation from a 20% polyethylene glycol (PEG) solution and washed in 70% ethanol prior to sequencing. To ensue accuracy, both strands of the cleaned PCR products were sequenced using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, New Jersey, USA).

For the nuclear DNA data, PCR reactions were performed using PfuUltra™ II Fusion HS DNA Polymerase (Stratagene, La Jolla, CA, USA) to reduce erroneous nucleotide incorporation during PCR. Original PCR and sequencing primers used to amplify and sequence the *waxy* locus (exon 7–13) were designed for Lamiales (Tank, 2006). Once a number of sequences were obtained from species of the *Verbena* complex, specific primers were designed for generating the *waxy* data (Table 2). PCR reactions were performed under the following conditions: one cycle of 3 min at 94 °C, 4 cycles of 15 s at 94 °C, 15 s at 57 °C, and 3.5 min at 72 °C, followed by 30 cycles of 15 s at 94 °C, 15 s at 55 °C, and 3 min at 72 °C, with a final extension step for 10 min at 72 °C. The combination of PfuUltra™ II Fusion HS DNA Polymerase (Stratagene, La Jolla, CA, USA), specific primers, and our PCR conditions is such that a strong single band was produced for all samples that have good quality total DNA. PCR products were cleaned by gel excision using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA). After A-tailing and subsequent clean up, the PCR products were cloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA, USA). Depending on the ploidy level, 8–40 positive clones were screened by sequencing with one primer. Distinct clones were sequenced for the entire region in both strands. *Waxy* sequences were successfully collected from 35 out of the 52 taxa sampled in this study. There was difficulty in amplifying the entire target region (ca. 3 kb) in the remaining taxa, particularly those for which DNA was extracted from herbarium specimens. Sequences generated in this study have been deposited in GenBank (*trn*T–F, EF571518–EF571570; *trn*S–fM, EF576665–EF576717; *trn*S–G, EF583067–EF583119; *trn*D–T, EF583120–EF583172; *waxy*, EF584666–EF584737).

Table 1
List of taxa included in this study and their sampling locality, voucher information, native continents, and chromosome number counts

Taxa	Locality and voucher information	Native to	Chromosome nos.
V. bonariensis L.	Cultivation, RGO 2003-25 (WTU)	SA	2*n* = 14, 28
V. bracteata Lag. and Rodr.	Arizona, US, YYW 2005-11 (WTU)	NA	2*n* = 14
V. canescens H. B. and K.	Texas, US, YYW 2005-14 (WTU)	NA	2*n* = 14
V. halei Small.	Texas, US, YYW 2005-16 (WTU)	NA	2n = 14
V. hastata L.	UW Med. Her. Garden, RGO 2003-155 (WTU)	NA	2*n* = 14
V. intermedia Gill. and Hook.	Entre Rios, Argentina, RGO 2004-106 (WTU)	SA	2*n* = 28, 56
V. lasiostachys Link.	Oregon, US, V. Soza 1757 (WTU)	NA	—
V. litoralis Kunth.	Entre Rios, Argentina, RGO 2004-105 (WTU)	SA	2*n* = 28, 56
V. macdougalii Heller.	New Mexico, US, YYW 2005-19 (WTU)	NA	2*n* = 14
V. menthifolia Benth.	Arizona, US, YYW 2005-4 (WTU)	NA	—
V. montevidensis Spreng.	Corrientes, Argentina, RGO 2004-112 (WTU)	SA	2*n* = 21, 42
V. neomexicana var. *hirtella* L. M. Perry	Texas, US, YYW 2005-15 (WTU)	NA	2*n* = 14
V. officinalis L.	UW Med. Her. Garden, RGO 2003-156 (WTU)	Old World?	2*n* = 14,28, 42, 56
V. orcuttiana L. M. Perry	Baja California, Mexico, YYW 2005-26 (WTU)	NA	—
V. perennis Wooton.	Texas, US, YYW 2005-17 (WTU)	NA	2*n* = 14
V. rigida Spreng.	Corrientes, Argentina, RGO 2004-111 (WTU)	SA	2*n* = 42
V. urticifolia L.	UW Med. Her. Garden, RGO 2003-157 (WTU)	NA	2*n* = 14
G. aff. *pulchella* (Sweet.) Tronc.	Corrientes, Argentina, RGO 2004-117 (WTU)	SA	2*n* = 10
G. araucana (Phil.) Botta	Mendoza, Argentina, RGO 2004-177 (WTU)	SA	—
G. aristigera (S. Moore) Tronc.	Corrientes, Argentina, RGO 2004-107 (WTU)	SA	—
G. aurantiaca (Speg.) Botta	Mendoza, Argentina, RGO 2004-196 (WTU)	SA	—
G. bajacalifornica (Moldenke) Umber*	Baja California, Mexico, R. Moran and J. Reveal 20047 (US2796936)	NA	—
G. bipinnatifida Nutt.	Texas, US, YYW 2005-12 (WTU)	NA	2*n* = 30
G. canadensis (L.) Nutt.*	Louisiana, US, R.D.Thomas 88350 (US3028096)	NA	2*n* = 30
G. chiracahensis Umber	Arizona, US, YYW 2005-9 (WTU)	NA	2*n* = 20
G. crithmifolia (Gill. and Hook. ex Hook.) Schnack and Covas	Mendoza, Argentina, RGO 2004-169 (WTU)	SA	2*n* = 20
G. delticola (Small.) Umber*	Texas, US, R.J. Fleetwood 8157 (US2518783)	NA	2*n* = 30
G. dissecta (Willd. ex Spreng.) Schnack and Covas.	Misiones, Argentina, RGO 2004-122 (WTU)	SA	2*n* = 10
G. flava (Gill. and Hook. ex Hook.) Schnack and Covas	Mendoza, Argentina, RGO 2004-153 (WTU)	SA	2*n* = 10
G. gooddingii var. *gooddingii* (Briq.) Solbrig	California, US, YYW 2005-28 (WTU)	NA	2*n* = 30
G. gooddingii var. nepetifolia Tidestrom	Baja California, Mexico, YYW 2005-21 (WTU)	NA	2*n* = 30
G. guaranitica Tronc.	Misiones, Argentina, RGO 2004-124 (WTU)	SA	—
G. incisa (Hook.) Tronc.	Corrientes, Argentina, RGO 2004-108 (WTU)	SA	2*n* = 10
G. mendocina (Phil.) Covas and Schnack	Mendoza, Argentina, RGO 2004-205 (WTU)	SA	2*n* = 10
G. microphylla (Kunth.) Cabrera.	Mendoza, Argentina, RGO 2004-156 (WTU)	SA	—
G. parodii Covas and Schnack	Mendoza, Argentina, RGO 2004-149 (WTU)	SA	2*n* = 10
G. pumila (Rydb.) Umber*	Oklahoma, US, U.T.Waterfall 13073 (US2260919)	NA	2*n* = 20
G. tampensis Small*	Florida, US, P.C.Standley 52577 (US1308777)	NA	2*n* = 30
G. tenera (Spreng.) Cabrera.	Mendoza, Argentina, RGO 2004-148 (WTU)	SA	2*n* = 10
G. teucriifolia (Mart. and Galeotti.) Umber*	Veracruz, Mexico, M. Nee and G. Diggs unknown # (US3191456)	NA	2n = 30
G. verecunda Umber*	Coahuila, Mexico, Henrickson 22547 (US3405101)	NA	2*n* = 20
J. asparagoides (Gill. and Hook.) Moldenke	Mendoza, Argentina, RGO 2004-192 (WTU)	SA	2*n* = 20
J. aspera (Gill. and Hook.) Moldenke	Mendoza, Argentina, RGO 2004-163 (WTU)	SA	2*n* = 60
J. connatibracteata (Kuntze) Moldenke	Mendoza, Argentina, RGO 2004-157 (WTU)	SA	—
J. juniperina (1) (Lag.) Moldenke	Mendoza, Argentina, RGO 2004-158 (WTU)	SA	2*n* = 20
J. juniperina (2) (Lag.) Moldenke	Mendoza, Argentina, RGO 2004-175 (WTU)	SA	2*n* = 20
J. minima (Meyen) Moldenke	Mendoza, Argentina, RGO 2004-174 (WTU)	SA	2*n* = 18
J. scoparia (Gill. and Hook.) Botta	Mendoza, Argentina, RGO 2004-178 (WTU)	SA	2*n* = 20
J. seriphioides (Gill. and Hook.) Moldenke	Mendoza, Argentina, RGO 2004-147 (WTU)	SA	2*n* = 20
J. spathulata (Gill. and Hook) Moldenke	Mendoza, Argentina, RGO 2004-190 (WTU)	SA	—
J. uniflora (Phil.) Moldenke	Mendoza, Argentina, RGO 2004-155 (WTU)	SA	—
Aloysia virgata Juss.	Misiones, Argentina, RGO 2004-133 (WTU)	SA	—
Lippia salsa Griseb.	San Luis, Argentina, RGO 2004-180 (WTU)	SA	—

The taxa marked by an asterisk are herbarium specimens. All chromosome number counts are from the "Index to Plant Chromosome Numbers" (1965–1993), with an additional reference of Turner and Powell (2005). SA, South America; NA, North America; RGO, Richard G. Olmstead; YYW, Yuan Yao-Wu.

2.3. Phylogenetic analyses

Sequence alignments were performed manually using Se-Al v.2.0a11 (Rambaut, 1996) based on the similarity criterion (Simmons, 2004). Since the seven cpDNA regions are part of the haploid chloroplast genome and therefore, share the same evolutionary history, they were combined as a single coalescence gene (Hudson, 1990; Doyle, 1997) for final phylogenetic analyses. But they were partitioned into seven data sets to count phylogenetically informative characters (PICs; Shaw et al., 2005) for each region (Table 3). Both maximum parsimony (MP) and maximum likelihood (ML) analyses were performed on the combined cpDNA dataset and the nuclear *waxy* dataset, to infer cpDNA and nuclear gene phylogenies. Parsimony analyses also were performed on reduced cpDNA and *waxy* data that exclude the polyploid *Glandularia* species, to better assess the relationships among diploid *Glandularia* species (see Section 4). Parsimony-informative gaps were coded as binary characters using the simple gap coding method (Simmons and Ochoterena, 2000; Graham et al., 2000) in MP analyses of cpDNA data. All phylogenetic analyses were conducted using PAUP* v.4.0b10 (Swofford, 2002).

For MP analyses, heuristic searches were performed with 1,000 random stepwise addition replicates and TBR branch swapping with the MULTREES option in effect. Support for individual clades was determined by bootstrap analyses (Felsenstein, 1985) of 500 replicates, each with 20 random stepwise addition replicates and TBR branch swapping with MULTREES on. For ML analyses, evolutionary models were determined by using both hierarchical

Table 2
Primers used for PCR and sequencing

Gene region	Primers (PCR)	Primers (sequencing)	Source	Sequence (5′–3′, if from this study)
*trn*T–F	a, f	a, b, c, d, e, f	Taberlet et al. (1991)	
*trn*S–fM	*trn*S, *trnf*M	*trn*S, *trnf*M	Shaw et al. (2005)	
		*psbZ*F(V)	This study	AGAGATGAGAGAATTAAGGATA
		*psbZ*R(V)	This study	CCTGATGGTTGGTCAAGTAACA
*trn*S–G	*trn*S	trnS, 5′*trn*G2G, 5′*trn*G2S	Shaw et al. (2005)	
	*trn*G(V)	*trn*G(V)	This study	GGGTTATAGTCGACGTCGATTC
		*trn*G–S F(V)	This study	CTTCTTTTTGTTGTTGCTTGAATA
		*trn*G–S R(V)	This study	TATTCAAGCAACAACAAAAAGAAG
*trn*D–T	*trn*DF, *trn*TR	*trn*DF, *trn*TR	Shaw et al. (2005)	
		*trn*D2T(V)	This study	TCATTAGAATCGACATTCTCTA
		*trn*T2D(V)	This study	TAGAGAATGTCGATTCTAATGA
waxy (7F-13R)	*waxy*-7F, *waxy*-13R	*waxy*-7F, *waxy*-13R	Tank (2006)	
	waxy-7F(V)	*waxy*-7F(V)	This study	CTTCTCAATCTGCCTGATCA
		waxy-8F(V)	This study	ATGGCATGGATACTCAAGAGTGG
		waxy-8R(V)	This study	CCACTCTTGAGTATCCATGCCAT
		waxy-9F(V)	This study	GAAAGAAGCTCTTCAAGCAG
		waxy-9R(V)	This study	GCTGCTGCTTGAAGAGCTTC
		waxy-10F(V)	This study	CATGCCATGCGATATGGAAC
		waxy-10R(V)	This study	GTTCCATATCGCATGGCATG
		waxy-11R(V)	This study	ACYGTGTCAACAAGACCACC
		waxy-12F(V)	This study	AATTGCATGTCACAAGATC
		waxy-12R(V)	This study	GATCTTGTGACATGCAATT
	waxy-13R(V)	*waxy*-13R(V)	This study	ACCAGGTTCACTVCCAGCAAC

All primers denoted with "(V)" were specifically designed for the *Verbena* complex. The *trn*T–F fragment includes the *trn*T–*trn*L spacer, *trn*L intron, and *trn*L–*trn*F spacer; the *trn*S–G fragment includes the *trn*S–*trn*G spacer and the *trn*G intron.

Table 3
Number of PICs (phylogenetically informative characters) for the cpDNA regions and the *waxy* locus used in this study

	cpDNA								*waxy*
	*trn*S–*trn*G	*trn*G	*trn*T–*trn*L	*trn*L	*trn*L–*trn*F	*trn*S–*trn*fM	*trn*D–*trn*T	Total	
Number of PICs (excluding outgroup)	27	13	23	14	18	46	22	163	822
Number of aligned nucleotide sites included in analysis	811	709	727	589	388	1163	912	5299	3607
Percentage of PICs	3.33%	1.83%	3.16%	2.38%	4.64%	3.96%	2.41%	3.02%	22.79%

likelihood ratio test and Akaike information criterion as implemented in Modeltest 3.06 (Posada and Crandall, 1998). The TVM + G and TVM + I + G model were determined to best fit the combined cpDNA data and individual *waxy* data, respectively, by both hierarchical likelihood ratio test and Akaike information criterion. Sequence parameters were estimated by the iterative approach (Swofford et al., 1996), with starting parameters estimated from one of the MP trees. Heuristic searches were run with 10 replicates of random taxon addition, TBR branch swapping, and MULTREES option on. ML bootstrapping was performed with 250 replicates, each with three replicates of stepwise random taxon addition, using the same model and parameters.

2.4. Shimodaira–Hasegawa (SH) test of alternative topologies

A group of *Glandularia* species (the "*Glandularia* I" clade, Fig. 1) were resolved in an unexpected position nested within *Verbena* in the cpDNA phylogeny, so does another single species, *G. bipinnatifida* (Fig. 1). As a result, the cpDNA tree indicates neither *Glandularia* nor *Verbena* is monophyletic, in conflict with the nuclear *waxy* gene tree as well as cytological and morphological evidence (see Section 4). The Shimodaira–Hasegawa test (SH test; Shimodaira and Hasegawa, 1999), as implemented in PAUP* v.4.0b10 (Swofford, 2002), was performed on the cpDNA dataset to test whether anomalous placements of the "*Glandularia* I" clade and *G. bipinnatifida* are significantly favored over the phylogenetic hypothesis that other evidence suggest. This test assigns a *P* value to the difference in likelihood between the optimal ML tree found and that ML trees with constrained relationships, based on the same data set. We compared the best ML tree to three topologies: (1) "*Glandularia* I and II" (Fig. 1) form a clade (i.e., the "*Glandularia* I" clade was constrained where other evidence suggest), (2) *G. bipinnatifida* and "*Glandularia* II" form a clade (i.e., *G. bipinnatifida* was constrained where other evidence suggest), (3) "*Glandularia* I and II", and *G. bipinnatifida* together form a clade (i.e., the "*Glandularia* I" clade and *G. bipinnatifida* were both constrained where other evidence suggest).

3. Results

3.1. Sequence alignment

The cpDNA regions were aligned unambiguously, although numerous short gaps were introduced and a 150-bp deletion was found in the *trn*D–*trn*T region of all *Glandularia* and *Verbena* species. The total aligned length of the cpDNA data was 5282-bp long, and 17 gaps were included in the MP analyses as binary characters. Most *waxy* sequences of the *Verbena* complex, from the end of exon 7 to the beginning of exon 13, are ca. 3.0 kb long. However, the sequences of a few *Junellia* species are notably shorter than others. The difference in length was identified as a MITE (miniature inverted-repeat transposable element) insertion in the *waxy* intron 8 of all the *Verbena* complex species except one *Junellia* clade. Alignment of the *waxy* exon sequences was straightforward. However, it was more difficult to align the intron sequences. It was necessary to introduce numerous large and small gaps throughout the six introns sequenced. The final alignment of the *waxy* sequences

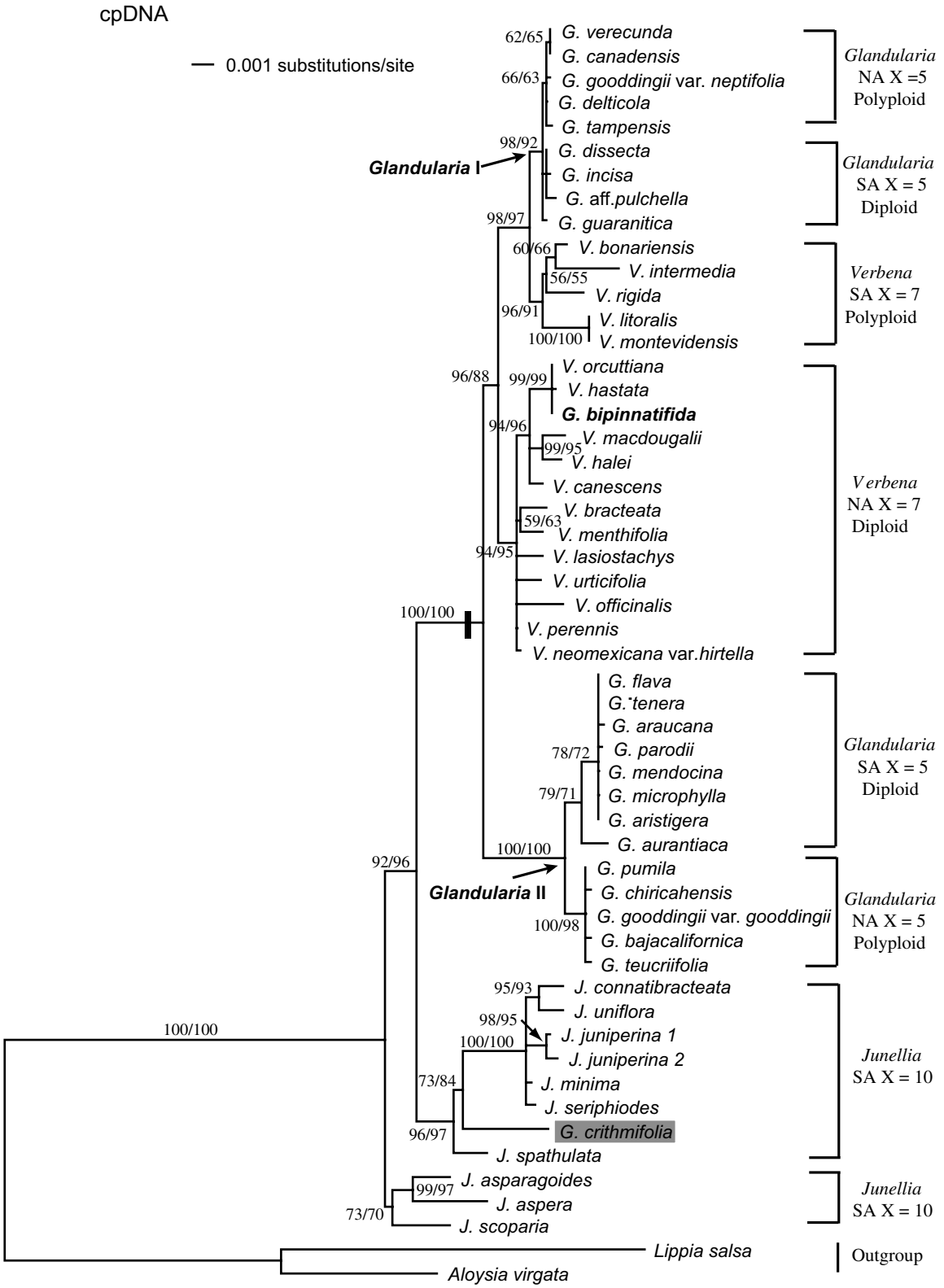

Fig. 1. Maximum likelihood tree resulted from the combined cpDNA data. The topology is identical to the single MP tree. Bootstrap values of both ML and MP analyses supporting the corresponding branches are shown when greater than 50% (MP/ML). The black bar represents a 150-bp deletion in the *trn*D–*trn*T region, supporting the close relationship between *Verbena* and *Glandularia*. The two arrows point to the two *Glandularia* clades, "*Glandularia* I" and "*Glandularia* II". *Glandularia bipinnatifida* and *G. crithmifolia* is in bold and shaded, respectively, to highlight their phylogenetic positions. Geographic distribution, base chromosome number, and ploidy level of smaller groups are indicated on the right. SA, South America; NA, North America.

was 3687 bp long. Five short portions of the introns that could not be aligned unambiguously were excluded from phylogenetic analyses. The *waxy* intron sequences were too variable to be aligned with the outgroup species. Consequently, the intron sequences from the outgroup species were not included in the data matrix.

3.2. Phylogenetic analyses

Maximum likelihood and parsimony analyses gave very similar results. Figs. 1 and 2 represent the cpDNA and nuclear *waxy* gene phylogenies, respectively. Although most of the seven non-coding cpDNA regions were suggested to be better suited for low-level phylogenetic studies than many other commonly employed cpDNA regions (Shaw et al., 2005), their divergences are very low in the *Verbena* complex (Fig. 1), which implies a very recent diversification of this group. Although with little variation, the combination of these seven regions resolved the relationships among several major lineages with strong support (Fig. 1). None of these three genera is monophyletic in the cpDNA phylogeny. *Junellia* is paraphyletic and ancestral to the other genera. *Glandularia* includes two major clades, "*Glandularia* I" and "*Glandularia* II", as well as two additional species, *G. bipinnatifida* and *G. crithmifolia*. *G. bipinnatifida* is nested within the North American *Verbena* group and *G. crithmifolia* belongs to a *Junellia* clade. *Verbena* is paraphyletic in relation to the "*Glandularia* I" clade (Fig. 1), and the two *Verbena* clades are associated with geographic distribution (i.e., North America or South America).

The nuclear *waxy* gene phylogeny is congruent with the cpDNA phylogeny in the basal paraphyly of *Junellia* and the inclusion of *Glandularia crithmifolia* in *Junellia* (Fig. 2). However, both *Verbena* and *Glandularia* (excluding *G. crithmifolia*) are strongly supported as monophyletic groups, with bootstrap value of 99/83 (MP/ML) and 97/93 for each clade, respectively, and these two genera are more closely related to each other than to *Junellia*. All six alleles of the hexaploid *Glandularia bipinnatifida* occur in the *Glandularia* clade (Fig. 2). The MITE insertion occurring in *waxy* intron 8 was a molecular synapomorphy defining a clade composed of *Glandularia*, *Verbena*, and one group of *Junellia*.

Fig. 3 shows the results of MP analyses on the dataset that excluded polyploid *Glandularia* species. The cpDNA phylogeny inferred from this dataset is very similar to that inferred from the full dataset in terms of phylogenetic relationships among the diploid *Glandularia* species (Fig. 1). The *waxy* gene tree on the reduced dataset, however, gives a more lucid phylogenetic structure among diploid *Glandularia* species than the phylogeny inferred from the full dataset. The diploid *Glandularia* species were grouped into two clades in the *waxy* tree. All species in the "shaded" clade are in the "*Glandularia* II" clade, and all species but one (*G. aurantiaca*) in the "bold" clade are in the "*Glandularia* I" clade.

3.3. Shimodaira–Hasegawa test

The *P* values for "*Glandularia* I" constrained, *G. bipinnatifida* constrained, and both "*Glandularia* I" and *G. bipinnatifida* constrained from the SH tests are 0.005, 0.001, and 0.000, respectively. This indicates that there is significant difference in –ln score between the best ML tree and the three topologies with constrained relationships.

4. Discussion

4.1. Monophyly of Verbena and Glandularia

Several morphological and cytological characters have been suggested to separate *Verbena* and *Glandularia* (Schnack and Covas, 1944; Schnack, 1964; Umber, 1979), including seed morphology, the ratio of style length to the ovary length, reproductive modes, and the most consistent character, base chromosome number. However, these characters have never been evaluated in a phylogenetic context, and therefore whether they can be used as synapomorphies to define the monophyly of *Verbena* and/or *Glandularia* remained ambiguous.

Our phylogenetic analysis based on the nuclear *waxy* gene sequences strongly suggests that both *Verbena* and *Glandularia* (excluding *G. crithmifolia*) are monophyletic (Fig. 2). If this is true, base chromosome number $x = 7$ and $x = 5$ are putative synapomorphies that define the monophyly of *Verbena* and *Glandularia*, respectively. In contrast, the cpDNA phylogeny suggests neither of these two genera is monophyletic and indicates an unexpected but strongly supported sister relationship between South American *Verbena* and a *Glandularia* clade ("*Glandularia* I", Fig. 1) composed of both South and North American species. Multiple independent origins of base chromosome numbers and all major morphological characters used to separate *Verbena* and *Glandularia* (Schnack and Covas, 1944; Schnack, 1964; Umber, 1979) have to be invoked to explain this cpDNA phylogeny. In addition, cytological and morphological evidence are consistent with the *waxy* gene data. Our *waxy* gene tree is more likely to reflect the organismal phylogeny than the cpDNA tree, and both *Verbena* and *Glandularia* (excluding *G. crithmifolia*) are monophyletic.

4.2. Chloroplast transfers from Verbena to Glandularia

If the "*Glandularia* I" (Fig. 1) clade of the cpDNA tree is pruned and regrafted with the "*Glandularia* II" clade, monophyletic *Verbena* and *Glandularia* (excluding *G. bipinnatifida* and *G. crithmifolia*) will result, which is consistent with the nuclear *waxy* gene phylogeny. This observation suggests that the conflict between the cpDNA and nuclear *waxy* phylogeny is due to an ancient chloroplast transfer from the South American *Verbena* group to the common ancestor of a *Glandularia* lineage ("*Glandularia* I"), which subsequently has radiated in both South and North America (Fig. 1). This conflict also could be caused by incomplete lineage sorting instead of chloroplast transfer. However, the coalescence of organelle DNA is four times faster than nuclear genes (Moore, 1995), and therefore it is unlikely that the lineage sorting for nuclear genes had been completed before the divergence of these two genera from their common ancestor, while polymorphism of chloroplast genes were retained in that common ancestor.

If the conflict is indeed caused by a chloroplast transfer from *Verbena* to the common ancestor of the "*Glandularia* I" clade (Fig. 1) and no other factors (e.g., incomplete lineage sorting, hybridization) further confound phylogenetic inference within *Glandularia*, then the "*Glandularia* I" clade also should be recovered in the nuclear gene tree. Since the polyploid *Glandularia* species may be of hybrid origins, the inclusion of polyploid species is likely to obscure the phylogenetic pattern within the genus *Glandularia*. Therefore, only diploid *Glandularia* species are discussed here for clarity. Fig. 3 shows that the diploid species of "*Glandularia* I" group with *G. aurantiaca* and three alleles of species from "*Glandularia* II", *G. flava* c.17, *G. parodii* c.1, and *G. aristigera* c.3, to form a monophyletic group (the "bold" clade, Fig. 3) in the *waxy* phylogeny. The diploid species of "*Glandularia* II", except *G. aurantiaca*, also form a monophyletic group (the "shaded" clade, Fig. 3) in the *waxy* phylogeny, albeit with some alleles of these taxa occurring in the "bold" clade, most likely due to hybridization or incomplete lineage sorting. Since the relationship between *G. aurantiaca* and species of "*Glandularia* I" was not resolved in the strict consensus *waxy* gene tree (Fig. 3), the prediction that diploid species of "*Glandularia* I" are monophyletic in the nuclear gene phylogeny, based on the ancient chloroplast transfer hypothesis, cannot be tested unambiguously. In fact, two of the four MP trees obtained from the phylogenetic analysis of the reduced dataset of *waxy* sequences suggest that *G. aurantiaca* is sister to a clade composed of the diploid species of "*Glandularia* I" (with the three alleles from "*Glandularia* II"). Although the results from analyses of the reduced datasets seem

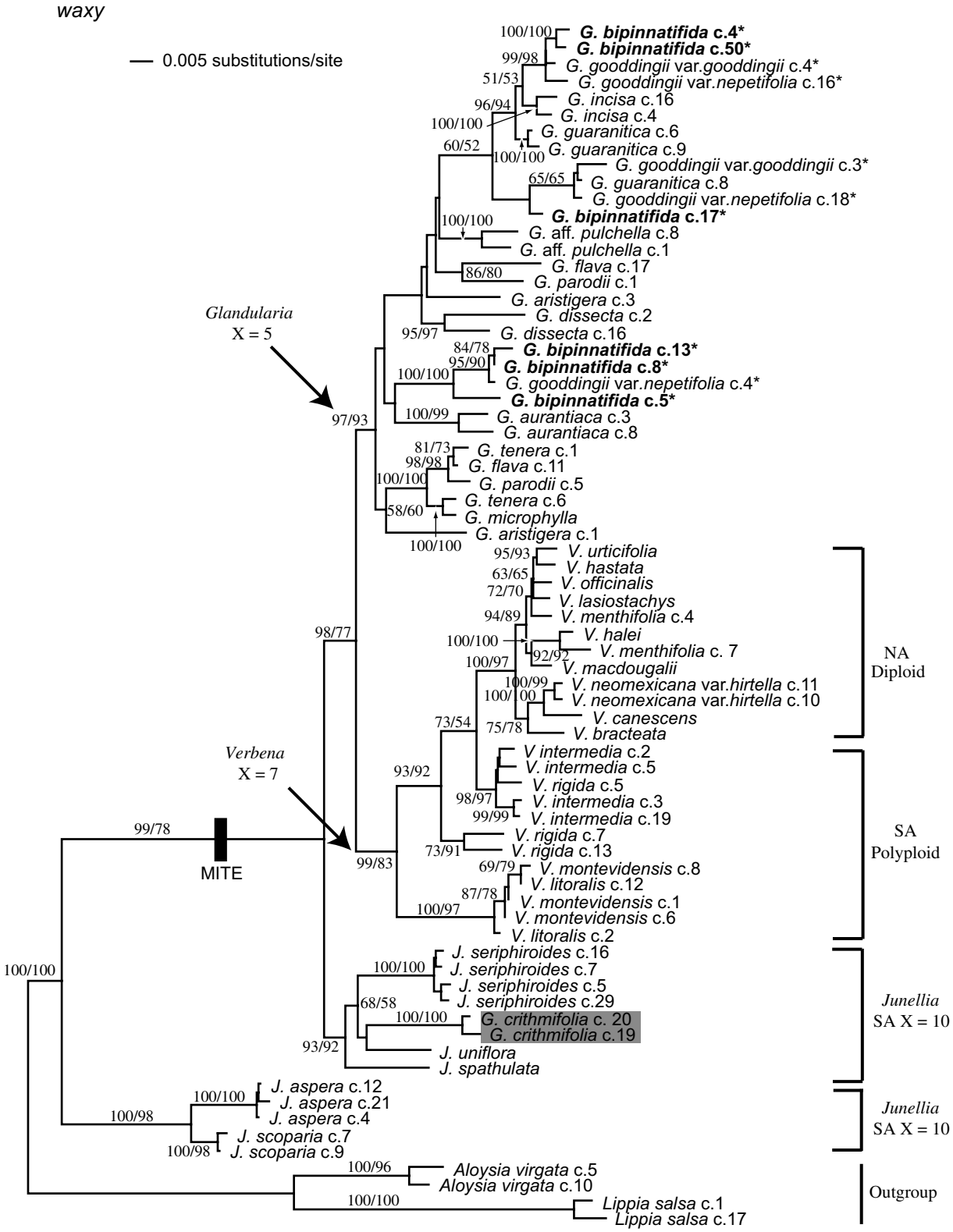

Fig. 2. Maximum likelihood tree resulted from the *waxy* sequences. The topology is identical to one of the 4 MP trees. Bootstrap values of both ML and MP analyses supporting the corresponding branches are shown when greater than 50% (MP/ML). Clone numbers are designated after species names when the individual sampled is heterozygous at the *waxy* locus. The black bar represents the MITE (miniature inverted-repeat transposable element) insertion occurring in *waxy* intron 8. The two arrows indicate the monophyly of *Glandularia* (excluding *G. crithmifolia*) and *Verbena*. *Glandularia bipinnatifida* and *G. crithmifolia* is in bold and shaded, respectively, to highlight their phylogenetic positions. Within the *Glandualaria* clade, North American taxa (polyploids) are marked with asterisks, the rest are South American taxa (diploids). distribution of the *Verbena* species is indicated on the right. SA, South America; NA, North America.

only weakly to support the ancient chloroplast transfer hypothesis, they have an interesting implication. The close relationship between *G. aurantiaca* and diploid species of "*Glandularia* I" uncovered from the *waxy* phylogeny (Fig. 3) indicates *G. aurantiaca*

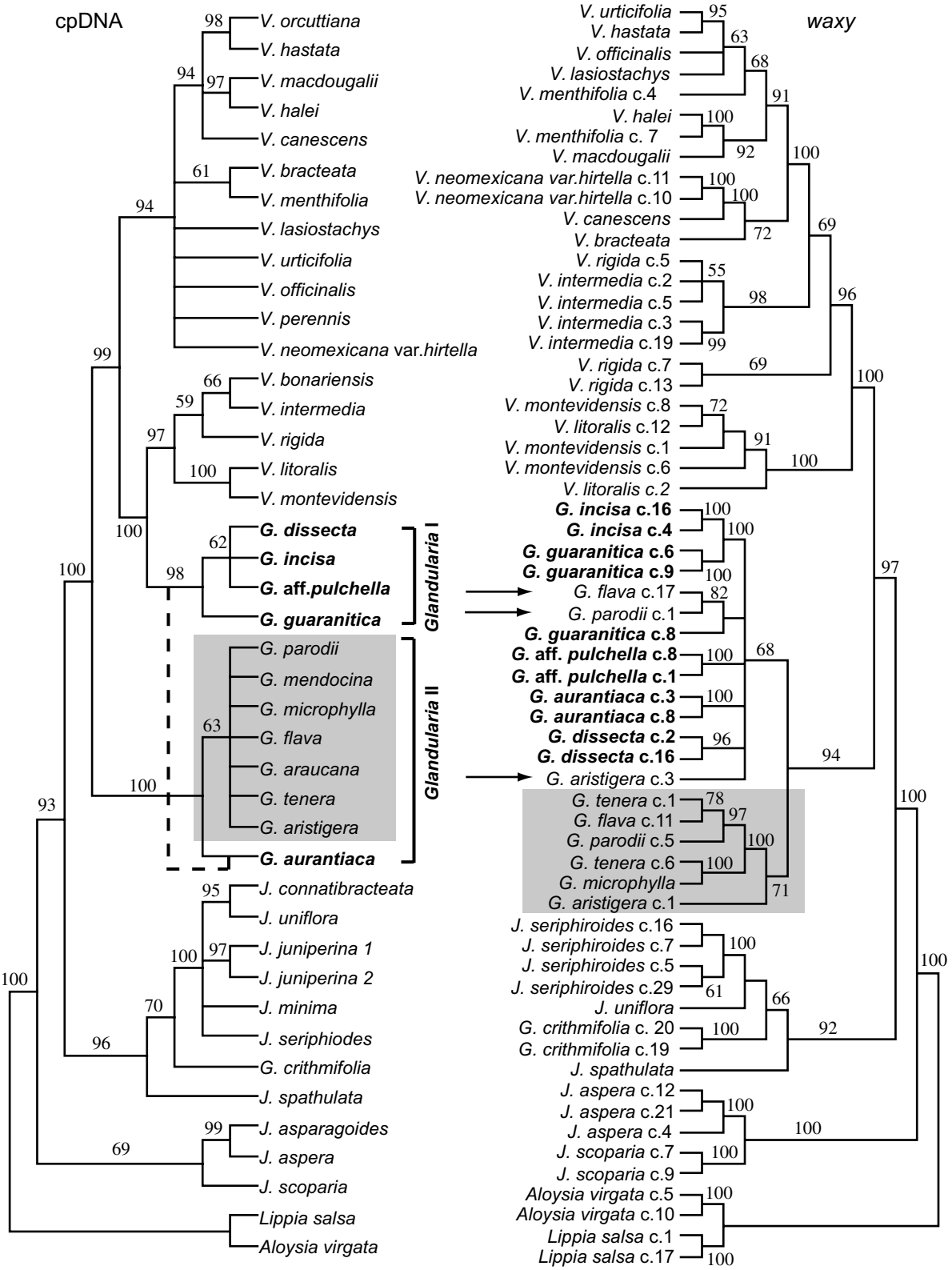

Fig. 3. Strict consensus trees from MP analyses of the reduced cpDNA and *waxy* datasets that excluded polyploid *Glandularia* species. Numbers along the branches are MP bootstrap supports. The *waxy* phylogeny shows diploid *Glandularia* species are divided into two clades. All species in the "shaded" clade are from the "*Glandularia* II" clade (corresponding to the labels in Fig. 1). The "bold" clade include all diploid species of the "*Glandularia* I" clade and one species from the "*Glandularia* II" clade, *G. aurantiaca*, as well as three alleles (indicated by the three arrows) of taxa in the "shaded" clade. The dashed line in the cpDNA phylogeny indicates the hypothetical position of the "*Glandularia* I" clade, had the inferred chloroplast transfer not taken place.

might be an extant close relative of the extinct "common ancestor" that had been involved in this ancient chloroplast transfer (Fig. 3) and led to the extant lineage "*Glandularia* I".

The North American species, *Glandularia bipinnatifida*, represents another chloroplast transfer. The cpDNA phylogeny suggests this species is nested within the North American *Verbena* clade

(Fig. 1), whereas evidence from morphology, cytology, and the nuclear *waxy* gene sequences, all concur that this is a *Glandularia* species. All six *waxy* alleles of this hexaploid species were found in the *Glandularia* clade. We have re-extracted the DNA and re-sequenced those cpDNA regions for this species (same accession) to rule out the possibility of contamination. The remarkable cpDNA sequence similarity between this *Glandularia* species and the North American *Verbena* species, *V. orcuttiana* and *V. hastata* (Fig. 1), also strongly suggests this conflict between cpDNA phylogeny and other evidence is due to a recent chloroplast transfer instead of incomplete lineage sorting. Considering *G. bipinnatifida* is a widespread North American species (Umber, 1979), it would be interesting to sample multiple populations to investigate whether this chloroplast transfer occurs in only some extant populations or in the common ancestor of the entire species.

These results highlight the pitfall of exclusively relying upon organelle DNA to address low-level phylogenetic questions (Rieseberg and Soltis, 1991), because it may often cause erroneous phylogenetic conclusions, considering that the phenomenon of chloroplast transfer is so widespread (Rieseberg and Soltis, 1991).

Introgressive hybridization has been commonly assumed as the underlying mechanism for chloroplast transfer in other plant groups, but it may not explain the two cases we report here. Both of these transfers occurred between different genera, and both were from *Verbena* to *Glandularia*. The only previously reported intergeneric introgression that we are aware of is between *Mitella* and a monotypic genus, *Tellima* (Soltis et al., 1991). It is not surprising, however, to discover introgressive hybridization between these two genera, given the fact that they are very closely related (Soltis et al., 1991) and have the same chromosome number, $2n = 14$ (the Missouri Botanical Garden TROPICS database, http://mobot.mobot.org/W3T/Search/ipcn.html). Nevertheless, in the case of *Verbena* ($x = 7$) and *Glandularia* ($x = 5$), it is hard to imagine how the two genera with different basic chromosome numbers hybridized leading to a hybrid lineage (“*Glandularia*” I) with the same base chromosome number ($x = 5$) of one of the parental lineages. Furthermore, although artificial hybridization within each genus was fairly readily attained, attempts to cross species between *Verbena* and *Glandularia* have been unsuccessful (Dermen, 1936; Solbrig et al., 1968). Given these concerns, we may need to consider that there may be some unknown mechanism other than introgressive hybridization that can also cause chloroplast transfer.

4.3. Paraphyly of Junellia and the phylogenetic position of G. crithmifolia

Both cpDNA and nuclear *waxy* gene phylogenies (Figs. 1 and 2) suggest that *Junellia* is paraphyletic and ancestral within the complex and that *G. crithmifolia* belongs to one clade of *Junellia*. *G. crithmifolia* and a morphologically very similar species, *G. hookeriana* (not sampled in this study), are quite different from other *Glandularia* species in two aspects. All other *Glandularia* species are herbaceous, whereas these two species are woody, as is *Junellia*. In addition, *G. crithmifolia* and *G. hookeriana* are the only two South American *Glandularia* species reported as polyploids, with chromosome number $2n = 20$. Our results suggest that these previously recognized polyploid *Glandularia* species are actually diploid *Junellia* species. A MITE insertion found in the *waxy* intron 8 (Fig. 2) corroborates the paraphyly of *Junellia* as indicated by phylogenetic analyses of both cpDNA and nuclear *waxy* gene sequences. While these results certainly have significant implications for taxonomic treatment (e.g., generic redefinition of *Junellia*, and new combinations for *G. crithmifolia* and *G. hookeriana*), our insufficient sampling of *Junellia* renders taxonomic revisions at this point premature. A future study with extensive sampling of *Junellia* will be necessary to provide a solid basis for taxonomic revisions of these taxa.

4.4. Chromosome number evolution and biogeography

The base chromosome number of *Junellia*, $x = 10$, has been suggested to be derived from $x = 5$ via polyploidy (Botta and Brandham, 1993). Based on this hypothesis, Botta and Brandham (1993) suggested that *Junellia* and *Glandularia* are more closely related to each other than either is to *Verbena* ($x = 7$). This study suggests that *Junellia* is paraphyletic and that *Glandularia* and *Verbena* are sister groups derived from within *Junellia*, which implies that $x = 10$ is the ancestral state, and that $x = 7$ and $x = 5$ are derived from $x = 10$. That the base chromosome number of the *Lippia*/*Lantana*/*Aloysia* complex, sister group of the *Verbena* complex, is 9–12 (the Missouri Botanical Garden TROPICS database, http://mobot.mobot.org/W3T/Search/ipcn.html), also suggests that the ancestral state of the *Verbena* complex is $x = 10$ instead 5. Our conclusion contradicts the assumption that $x = 5$ is ancestral to $x = 10$ and highlights the potential peril of such prior assumptions about character evolution without a phylogenetic framework.

Junellia is restricted to southern South America, which suggests a South American origin of the *Verbena* complex. Lewis and Oliver (1961) and Umber (1979) cited the reciprocal cytogeographic pattern of *Verbena* and *Glandularia* (Lewis and Oliver, 1961) as evidence that each group is monophyletic and originated on the continents with the primarily diploid species diversity, subsequently spreading to the other continents, presumably by long distance dispersal. The cpDNA phylogeny is inconsistent with the organismal history due to the putative ancient chloroplast transfer, and hence, not very informative with respect to the biogeography of *Glandularia* and *Verbena*. Our phylogenetic analyses of the *waxy* sequences suggest that both *Glandularia* and *Verbena* originated in South America, given that the basal lineages of both genera have a South American distribution (Fig. 2). A South American origin for *Glandularia* is consistent with prior interpretation by other authors (Lewis and Oliver, 1961; Umber, 1979), but it seems counterintuitive that the diploid North American *Verbena* may have been derived from South American species that are predominantly polyploids, as suggested by the *waxy* phylogeny (Fig. 2). The cpDNA phylogeny suggests that both North and South American *Verbena* are monophyletic, whereas the *waxy* phylogeny suggests that North American *Verbena* are monophyletic but South American *Verbena* are paraphyletic and ancestral to the North American group. If *Verbena* originated in South America, then extinctions of diploid ancestors in South America need to be assumed to reconcile our results. The monophyly of South American *Verbena* in the cpDNA tree can be explained by the four times faster coalescence of organelle DNA than nuclear genes. This more rapid coalescence of cpDNA could result in extinction of polymorphic ancestral gene lineages and lead to the transition from paraphyly to monophyly (Avise et al., 1983). However, these hypotheses need to be tested with more data from other nuclear loci and a thorough taxon sampling.

4.5. Utility of non-coding cpDNA and large fragments of nuclear DNA in species-level phylogenetics

Lack of variation is a well-known limitation to the use of cpDNA in plant species-level or population-level phylogenetics (Small et al., 1998; Schaal et al., 1998; Shaw and Small, 2004; Shaw et al., 2005; Hughes et al., 2006). In an effort to remedy this, greater attention has been paid to the more rapidly evolving nuclear genome for studies at this level (see reviews in Sang, 2002; Small et al., 1998; Hughes et al., 2006). The application of seven relatively

fast-evolving non-coding cpDNA regions (Shaw et al., 2005) to the *Verbena* complex, an apparently recently diversified group, confirms their inadequacy in resolving relationships among closely related species. The ca. 3.0 kb (ca. 3.6 kb aligned nucleotide sites) nuclear *waxy* sequences possess five times more phylogenetically informative characters than the >5.0 kb non-coding cpDNA (Table 3). This suggests that to address low-level phylogenetic problems, especially in those recent, rapidly diversifying groups, nuclear DNA will be an indispensable source of variation, though the use of multiple loci will be critical to overcome the lineage sorting problem. Perhaps the ideal way to resolve relationships among closely related species is to sample multiple nuclear loci that possess sufficient variation to give well-resolved gene trees (Small et al., 1998; Cronn et al., 2002; Linder and Rieseberg, 2004). A species tree can then be inferred by extracting information from these independent gene trees (Maddison and Knowles, 2006; Liu and Pearl, 2006; Ané et al., 2007; Carstens and Knowles, 2007). However, general phylogenetic practices tend to employ multiple nuclear loci that are often too short to give well-resolved gene trees. Our experience of the *waxy* locus suggests that relatively large nuclear DNA fragment (at least as large as 3 kb) can be readily employed in routine phylogenetic studies, provided that good quality total DNA is available. This study included only one nuclear locus and its gene tree is likely to be different from the organismal tree within the major clades (e.g., North American *Verbena*), assuming insufficient time for complete lineage sorting. It is noticeable that non-monophyletic alleles at the *waxy* locus were recovered from several *Glandularia* species, even diploid species (e.g., *G. parodii*, *G. flava*, *G. aristigera*, *G. tenera*, Fig. 2). The non-monophyly of these alleles might be caused by incomplete lineage sorting or recent gene flow or the combination of both effects. These results indicate an interpretation of relationships between closely related species based on a single nuclear gene tree (the *waxy* tree, in this case) could be quite misleading. Therefore, we will leave the discussion of interspecies relationships within those smaller groups for future studies that include data from more nuclear loci as well as more individuals representing each species.

On the other hand, despite the lack of variation, cpDNA are still useful, and sometimes even indispensable, to achieve a comprehensive understanding of the evolutionary history of a recent, rapidly diversifying group. For example, the combined data of seven non-coding cpDNA regions employed in this study are sufficient to resolve relationships among the major lineages with fairly strong support (Fig. 1). The evolutionary history of the uni-parentally inherited organelles cannot be unmasked without cpDNA data. Similarly, cpDNA also is indispensable in distinguishing maternal and paternal lineages in case of hybridization.

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