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On the Brink of Holoparasitism: Plastome Evolution in Dwarf Mistletoes (*Arceuthobium*, Viscaceae)

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Abstract Chloroplast sequences spanning *rps7* to 23S rDNA in *Arceuthobium campylopodum* and *A. pendens* were generated and compared to *Arabidopsis* and seven other parasitic plants. Pseudogenes for *trnV*, *trnI* (GAU), and *trnA* (UGC) were seen in both *Arceuthobium* species, paralleling the situation in the holoparasite *Epifagus* (Orobanchaceae). These tRNA genes were intact, however, in two other members of Santalales (*Ximenia* and *Phoradendron*). The 16S–23S rDNA intergenic spacer was sequenced for 13 additional species of *Arceuthobium* representing both Old and New World taxa. All species examined had pseudogenes for *trnI* and *trnA*, however, deletions in these tRNAs have occurred in different regions among various lineages of the genus. The aligned 16S–23S rDNA intergenic spacer was analyzed using maximum parsimony and compared with nuclear ITS rDNA using a similar suite of species. Overall species relationships were generally congruent, although two cases of potential lineage sorting or chloroplast capture were detected. *Arceuthobium* is a valuable genetic model to constrast with holoparasites because, despite significant alteration and truncation of its plastome, it still maintains photosynthetic function.

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Introduction

The number of complete chloroplast genome (plastome) sequences for green algae and land plants (Streptophyta) deposited with NCBI GenBank continues to increase in both depth (e.g., Brassicaceae) and breadth (see Jansen et al. 2005). In December 2004 only 41 plastome sequences existed (18 from angiosperms), whereas in July 2008 there were 105 Streptophyte sequences, 85 of which are angiosperms. Among the latter, 16 are from monocots and 69 from dicots distributed across 31 orders and 42 families. Among the fully photosynthetic, nonparasitic plants, plastome sizes range from 124 kb in *Medicago* with 109 genes to 217 kb in *Pelargonium* with 220 genes (Chumley et al. 2006). The mean genome size in nonparasitic angiosperms is 154 kb with 136 genes. Despite this variation, this organellar genome is amazingly collinear, with very few rearrangements of the same basic suite of genes.

Parasitic plants have served as important model systems for understanding the fate of the plastome in heterotrophic organisms that lose photosynthesis. Complete plastome sequences currently present in GenBank are from asterid parasites in Orobanchaceae (*Epifagus virginiana*) and Convolvulaceae (*Cuscuta exaltata*, *C. gronovii*, *C. obtusiflora*, and *C. reflexa*). Additional asterid sequences that are complete but not yet available are from *Conopholis* (Orobanchaceae [dePamphilis et al. 2005]) and *Pholisma* (Lennoaceae [dePamphilis et al. 2005]). The 70-kb plastome reported for *Epifagus* (dePamphilis and Palmer 1990; Wolfe et al. 1992b) attained this small size via the loss of all photosynthetic and chlororespiratory genes. Despite

this, empirical evidence was obtained that indicated functionality (Ems et al. 1995). Likely similar in gene content to *Epifagus*, its relative *Conopholis* has lost one copy of the inverted repeat (Colwell 1994), thus its plastome may be only 43 kb in size, the smallest to date among angiosperms. *Cuscuta* contains a range of species that have different degrees of reduction of their plastomes and substantial physiological differences exist among them (Krause et al. 2003; Stefanovic et al. 2002). The photosynthetic *Cuscuta reflexa* genome is smaller than that of a typical angiosperm (121 kb), wheareas its nonphotosynthetic relative *C. obtusiflora* is reduced to 85 kb. These results have prompted much speculation as to how far such reductional trends can go (Nickrent et al. 1997b), but complete plastome loss has never been observed and arguments favoring selective retention have been made (Barbrook et al. 2006; Bungard 2004).

The asterid parasites provide excellent opportunities to examine plastome evolution because both groups contain taxa representing transitional series from photosynthetic hemiparasites to nonphotosynthetic holoparasites. All Orobanchaceae are parasitic plants whose seedlings respond to host roots by attaching to them via a haustorium that rapidly develops from the radicle apex. *Cuscuta* seeds also germinate in the soil but it is their aerial stems that twine and attach to host tissues. After this first haustorial attachment, the seedling radicle degenerates, thus more mature parasites have no connection to the soil.

In contrast to the above groups, the sandalwood order (Santalales) has both root parasites and stem parasites (the latter includes mistletoes). Although some members of Santalales (in the strict sense) approach holoparasitism, this trophic mode does not exist in the order, i.e., all members are photosynthetic during at least some stage of their life cycle. More recent studies indicate that the root holoparasites in Balanophoraceae are related to Santalales (Nickrent et al. 2005; Su and Hu 2008), but the exact topology remains to be determined. Some mistletoes such as *Arceuthobium* have reduced photosynthesis and adult shoots may fix only 30% of the carbon required by the plant (Hull and Leonard 1964a, b; Miller and Tocher 1975). But unlike root holoparasites of Orobanchaceae, mistletoe seedlings begin development not in a moist rhizosphere but on the comparatively xeric surface of a host branch. The time from germination to successful attachment to the host is more protracted in mistletoes, thus the seedling obtains photosynthates from its hypocotyl, radicle, and endosperm. With this life history constraint in place, mistletoes cannot undergo the extreme plastome reorganization seen in true holoparasites. This limitation immediately raises the question, "How far can plastome modifications proceed while still maintaining photosynthetic function?" It is this distinction that makes santalalean parasites valuable natural genetic mutants to contrast with fully holoparasitic angiosperms.

Arceuthobium (dwarf mistletoes, Viscaceae) has been the focus of much study from many disciplines including taxonomy, ecology, pathology, morphology, anatomy, and physiology (Hawksworth and Wiens 1996); however, to date no genomic work has been conducted. This genus contains 26 Old and New World species, some of which are important pathogens of commercially valuable conifers in Pinaceae and Cupressaceae. *Arceuthobium* is characterized by leaves reduced to scales, an explosively dehiscent fruit, and a root system that consists of an endophyte within the host branch that may or may not (depending upon the species) induce host deformations called witches' brooms. Molecular phylogenetic studies have focused on broad relationships within Santalales where *Arceuthobium* was included (Der and Nickrent 2008; Nickrent 1996; Nickrent et al. 1998; Nickrent and Malécot 2001) or on interspecific relationships within the genus (Nickrent et al. 2004; Nickrent et al. 1994). Chloroplast gene sequences for *Arceuthobium* include *rbcL*, *matK*, and the *trnT-L-F* region. These sequences reveal that nucleotide substitution rates are increased compared with nonparasitic plants, a result paralleling the trend seen in nuclear ribosomal genes (Nickrent et al. 1998; Nickrent and Starr 1994).

Increased substitution rates plus the life history constraints on holoparasitism discussed above provided the impetus to further investigate the *Arceuthobium* plastome. Although methods have been reported to rapidly obtain complete plastome sequences (Jansen et al. 2005; McNeal et al. 2006; Moore et al. 2006), as yet these have not been applied to *Arceuthobium*. The plastome sequences of *Ximenia americana* (Olacaceae) and *Phoradendron serotinum* (Viscaceae) are now known (Moore et al. 2008) and the *rps7*-to-23S portion was obtained to allow comparisons of trends within Santalales. Herein we compare a portion of the inverted repeat of *Arceuthobium* with sequences of seven other parasitic plants in terms of gene presence and absence, formation of pseudogenes, and fate of tRNA genes that are known to be volatile in *Epifagus* and *Orobanche* (Lohan and Wolfe 1998). In addition, the region between the 16S and the 23S rDNA was sequenced for 15 species of *Arceuthobium*, thus allowing a detailed examination of mutations within two tRNA genes, *trnI* (GAU) and *trnA* (UGC). Finally, the molecular evolution of additional plastome regions is compared and contrasted between Santalales and other parasitic plants.

Materials and Methods

A variety of *Arceuthobium* tissues was used for genomic DNA extraction including frozen seeds, shoots, and

herbarium samples. A 2× CTAB miniprep protocol (Nickrent 1994) was used, as well as the E.Z.N.A. Plant MiniPrep Kit (Omega-Biotech, Doraville, GA, USA). The plastid *trnT-L-F* region (hereafter referred to as the *trnL* region) as well as nuclear ITS (internal transcribed spacer of the rDNA cistron) sequences were obtained from GenBank following Nickrent et al. (2004). GenBank numbers for all sequences used in this study are given in Tables 1 and 2.

The *rps7*-to-23S rDNA region in *A. campylopodum* (voucher 2161) and *A. pendens* (voucher 2014) was PCR amplified in several pieces. The first used *rps7* reverse (CCA MCA TGT TAA CTA ATC GAT T) and 16S 7 reverse (TGA GCC AGG ATC GAA CTC TCC). Cycling parameters for this amplification were 94°C for 3 min and 35 cycles of 94°C for 40 s, 52°C for 40 s, and 72°C for 40 s, followed by a final extension for 10 min at 72°C. 16S rDNA was amplified and sequenced using primers reported by Nickrent et al. (1997a), whereas the 23S rDNA gene was amplified and sequenced with primers given by García et al. (2004). Additional primers used to fill gaps include *rps12* forward (GTT AGC CAT ACA CTT CAC ATG), 16S 942 forward (ACA TGC CGC GAA TCC TCT TGA), Arc 16S-*trnI* reverse (TTG GGT CTT CAC CGT CGA GAA), *trnI* intron (GAA AGG GGT GAT CTC GTA GTT), and 23S 909 reverse (CTA GTA TTC AGA GTT TGC CTC G). The larger PCR products were gel-purified using Gel Elute spin columns (Sigma Aldrich, Inc., St. Louis, MO, USA). The products were then cloned using the TOPO TA cloning kit with the XL-Topo vector (Invitrogen Corporation, Carlsbad, CA, USA) following the recommended protocol. The plasmids were purified using the QIAprep Spin Miniprep Kit (QIAGEN Inc., Valencia, CA, USA). The presence of inserts was checked by restriction digestion (*Eco*RI) followed by electrophoresis of the fragments in an agarose gel. Cycle sequencing reactions were conducted on the plasmids using the universal primers M13 forward and reverse as well as primers on the 16S and 23S rDNA inserts. The latter primers were 16S 717 forward (CGA GAC GGG TGA ATG GG) and 16S 1457 forward (CTG GAA GGT GCG GCT GGA TCA CC), as well as the following reported by García et al. (2004): 23S 42 forward, 23S 245 reverse, 23S 815 forward, 23S 1294 forward, and 23S 1686 forward.

The 16S–23S rDNA intergenic region was amplified for the 15 dwarf mistletoe species using the following two primers (both 5′ to 3′): 16S 1457 forward (CTG GAA GGT GCG GCT GGA TCA CC) and 23S 42 reverse (CTG GGT GCC TAG GTA TCC ACC). The reactions were conducted in a Perkin-Elmer Thermal Cycler, with one cycle at 94°C for 5 min and 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by a final extension for 10 min at 72°C. These products were purified using the QIAquick PCR purification kit (QIAGEN Inc) and sequenced directly using the PCR primers.

Electropherograms were edited using 4Peaks (Griekspoor and Groothius 2006) and Sequencher (Gene Codes Corp., Ann Arbor, MI, USA) and the sequences ported into SeAl (Rambaut 2004), where manual alignments were conducted. For the *rps7*-to-23S rDNA region, this alignment included sequences obtained from GenBank for *Arabidopsis* (Brassicaceae, a photosynthetic nonparasite), two species of *Cuscuta* (Convolvulaceae), and *Epifagus*, *Orobanche*, and *Conopholis* (all Orobanchaceae). Currently unpublished sequences for this region were also obtained for *Phoradendron serotinum* (Viscaceae) and *Ximenia americana* (Olacaceae) from M. Moore (Oberlin College). Pseudogene status was assigned to tRNAs if their sequence was reported as such on GenBank flatfiles, in the original publication, or by direct inspection demonstrating losses of crucial structural features. Insertions or deletions were mapped onto tRNA secondary structure to further examine details of pseudogene formation. Phylogenetic analyses of the *Arceuthobium* 16S–23S rDNA intergenic region was conducted with PAUP* (Swofford 2002) using a branch-and-bound maximum parsimony search of the matrix (without gap coding). A similar search was conducted using ITS sequences (all derived from GenBank [see Nickrent et al. 2004]) of the same suite of species to examine the phylogenetic utility of these plastid sequences. One thousand bootstrap replicates were performed to assess support for the clades.

Results

Despite the large phylogenetic distances and high rates of sequence evolution, the general conservation of cpDNA allowed essentially the entire *rps7*-to-23S rDNA region to be aligned. Although a small number of highly variable regions could not be aligned, these sites were flanked by conserved regions such that the majority of the greater than 10,000 positions could be unambiguously aligned across all species (Supplementary Data S1). Table 1 gives the lengths of the various spacers, ribosomal protein genes, tRNA genes, and introns for *Arceuthobium campylopodum* and *A. pendens*, as well as for seven other parasitic plant species and *Arabidopsis*. For *rps7*, the length is conserved at 468 nucleotides (nt) for all species with complete plastome sequences (the *Arceuthobium* sequences are incomplete). Exon 3 of *rps12* is 27 nt in most taxa but likely pseudogenes in *Epifagus* (by deletion) and *Arceuthobium* (by substitutions in the stop codon). The second exon of *rps12* is uniformly 231 nt in length for all species examined. The spacer sequence between *rps12* exon 2 and *trnV* is variable in length among the plants for which we have sequence

Table 1 Sizes of the chloroplast rps7-to-23S rDNA region for nine parasitic plants and *Arabidopsis*

	GenBank no.	Plastome size	*rps7*	Spacer	*rps12* exon 3	Intron	*rps12* exon 2	Spacer	*trnV*	Spacer	16S	Spacer	*trnI* 5′	Intron	*trnI* 3′	Spacer	*trnA* 5′	Intron	*trnA* 3′	Spacer	23S
Arceuthobium campylopodum	FJ040857	N/A	N/A	56	27 ΨI	568	231	1,329	67 ΨD	185	1,489	295	17 ΨD	659	35	46	32 ΨD	405	35	159	2,755[a]
Arceuthobium pendens	FJ040858	N/A	N/A	61	27 ΨI	569	231	1,731	54 ΨD	196	1,483	299	21 ΨD	692	35	28	29 ΨD	698	35	159	2,753[a]
Phoradendron serotinum	Unpubl.	?	468	62	27	539	231	1,820	72	221	1,501	300	37	886	35	43	39	817	35	154	2,816
Ximenia americana	Unpubl.	?	468	53	27	543	231	1,816	72	219	1,493	294	37	950	35	62	39	809	35	169	2,805
Cuscuta reflexa	NC009766	121,521	468	53	27	531	231	1,152	72	220	1,491	303	37	944	35	62	39	781	35	144	2,811
Cuscuta gronovii	NC009765	86,744	468	45	27	0	231	588	72	116	1,492	168	26 ΨD	0	0	0	0	0	30 ΨD	98	2,828
Epifagus virginiana	NC001568	70,028	468	51	26 ΨD	545	231	1,380	0	146	1,492	262	50 ΨD, I	885	34	51	44 ΨD	474	29 ΨD	180	2,805
Orobanche minor	AJ007728	N/A	N/A	N/A	N/A	N/A	N/A	N/A	72	252	1,487	235	8 ΨD	0	0	0	0	305	35	167	N/A
Conopholis americana	X58864, X59768	~43,000	N/A	N/A	N/A	N/A	N/A	N/A	N/A	93	1,487	244	42 ΨI	55	0	0	0	0	0	50	2,811
Arabidopsis thaliana	NC000932	154,478	468	53	27	537	231	1,915	72	231	1,491	298	37	729	35	61	38	802	35	152	2,811

N/A not available, *ΨD* pseudogene by means of deletion, *ΨI* pseudogene by means of insertion

[a] Incomplete (missing ca. 38 nt at the 3′ end)

Table 2 *Arceuthobium* species used in this study

Arceuthobium species	Abbreviation	16S–23S NCBI	ITS NCBI	*trnT-L-F* NCBI	Locality	Voucher no. (herbarium)
A. abietis-religiosae Heil	ABR 2010	FJ040856	L25787	AY288201	Mexico	Nickrent 2010 (SIU)
A. americanum Nutt. ex Engelm.	AME 1966	FJ040854	N/A	AY288204	Colorado, USA	Nickrent 1966 (ILL)
A. americanum Nutt. ex Engelm.	AME 1911	N/A	L25788	N/A	California, USA	Nickrent 1911 (SIU)
A. azoricum Hawksw. & Wiens	AZO 4333	FJ040843	AY288253	N/A	Azores Islands	Sequeira s.n. (SIU)
A. blumeri A. Nelson	BLU 4345	FJ040848	AY288254	AY288209	Arizona, USA	Mathiasen 9813 (FPF)
A. campylopodum Engelm.	CAM 2161	FJ040847	L25685	AY288211	California, USA	Nickrent 2161 (ILL)
A. douglasii Engelm.	DOU 1955	FJ040850	L25687	AY288217	New Mexico, USA	Nickrent 1955 (ILL)
A. globosum ssp. *globosum* Hawksw. & Wiens	GLO 2053	FJ040851	L25690	AY288223	Mexico	Nickrent 2053 (ILL)
A. hondurense Hawksw. & Wiens	HON 4316	FJ040853	AY055215	AY288228	Mexico	Mathiasen 0061 (FPF)
A. juniperi-procerae Chiovenda	JUN 4238	FJ040844	AY288265	N/A	Kenya	Wiens 4456 (FPF)
A. minutissimum J.D. Hooker	MIN 4077	FJ040841	AY288269	N/A	Bhutan	Glätzel s.n.
A. nigrum Hawksw. & Wiens	NIG 2019	FJ040852	AY288271	AY288233	Mexico	Nickrent 2019 (SIU)
A. oxycedri (DC) Bieb.	OXY 4234	FJ040846	AY288275	N/A	Spain	Rios s.n. (FPF)
A. oxycedri (DC) Bieb.	OXY 4335	FJ040845	AY288277	N/A	Morocco	Wiens 4437 (FPF)
A. pendens Hawksw. & Wiens	PEN 2014	FJ040849	L25695	AY288238	Mexico	Nickrent 2014 (SIU)
A. sichuanense (H.S. Kiu) Hawksw. & Wiens	SIC 4243	FJ040842	AY288281	N/A	China	Dao 3847 (FPF)
A. verticilliflorum Engelm.	VER 2065	FJ040855	L25700	AY288247	Mexico	Nickrent 2065 (ILL)

N/A not available

(588 nt in *Cuscuta gronovii* to 1915 in *Arabidopsis*), but most regions are unambiguously alignable. The *trnV* gene is 72 nt in *Arabidopsis* and most parasitic species except for *Epifagus*, where it is absent, and the two *Arceuthobium* species, where the genus is truncated and likely a pseudogene (Table 1). The plastid small-subunit (16S) and large-subunit (23S) rDNA genes are extremely conservative in length (Table 1) and in sequence (Supplementary Data S1) across all species included here. The 16S–23S rDNA intergenic region for *A. campylopodum* and *A. pendens* is similar in sequence and in length, and both have undergone similar mutational changes in *trnI* (GAU) and *trnA* (UGC). For both of these tRNA genes, the 5′ portion experienced deletions, whereas the 3′ portion remained intact.

Despite the presence of numerous insertions and deletions, the alignment of the 16S–23S rDNA intergenic region for 15 *Arceuthobium* species was nearly unambiguous (Supplementary Data S2). Conservation was also sufficient to align the dwarf mistletoe and the more divergent *Arabidopsis* sequences. The mean size of the region was ca. 1500 nt (range, 986 in *A. azoricum* to 2027 in *A. nigrum*), compared with *Arabidopsis*, with 2175 nt. Insertions (mostly tandem duplications of flanking regions) as well as numerous deletions can be seen (see Discussion). Several large deletions appear to be characteristic of particular clades, such as for the Old World *Arceuthobium* species (in the first spacer, first intron and second intron). Three deletions are shared among the New World species in the first intron.

Variation in the lengths of the tRNA genes, introns, and spacers among *Arceuthobium* species is reported in Table 3. The spacer between the 16S rDNA and the *trnI* 5′ exon is relatively consistent in length (ca. 250 to 300 nt), however, it is marked by numerous indels across the species, many of which are phylogenetically informative. We consider indels homologous if they share identical boundaries. For example, a tandem duplication of five nucleotides (at alignment positions 36–40) is shared by section *Americana* taxa (*A. abietis-religiosae*, *A. americanum*, and *A. verticilliflorum*). Similar regions can be seen throughout the entire 16S–23S rDNA spacer region. In comparison to *Arabidopsis*, the *trnI* 5′ exon is truncated in all dwarf mistletoes except *A. douglasii*. Some of the deletions appear to be phylogenetically informative, i.e., are shared by related taxa, however, others are unique to particular species and have likely arisen independently. The *trnI* 3′ exon has also been impacted by indel events, although it has remained relatively intact for several species in subgenus *Arceuthobium* (e.g., *A. juniperi-procerae*, *A. oxycedri*, and *A. minutissimum*). The intron separating these two exons varies widely in length (118 to 692 nt), with the smaller sizes being found in subgenus *Arceuthobium*. In contrast, the spacer between the *trnI* 3′ exon and the *trnA* 5′ exon is longer in that subgenus. The *trnA* 5′ exon is generally shorter in the *Arceuthobium* species than in

Table 3 Sizes of spacers, tRNAs, and introns in the 16S–23S rDNA intergenic region for 15 species of *Arceuthobium*

Arceuthobium species	Spacer	*trnI* 5′	Intron	*trnI* 3′	Spacer	*trnA* 5′	Intron	*trnA* 3′	Spacer
JUN 4238	277	8	134	37	64	36	280	35	162
AZO 4333	263 (+14)	8	128	37	59	36	268	35	138
OXY 4234	276	7	134	37	60	36	277	35	162
OXY 4235	277	7	134	37	60	36	277	35	162
SIC 4243	281	18	125	37	52	32	274	35	158
MIN 4077	285	29	118	37	52	34	276	35	163
ABR 2010	296	25	653	35	37	29	667	35	151
AME 1966	309	25	664	27	41	29	426	35	157
VER 2065	303	25	430	0	0	0	497	35	154
BLU 4345	292	17	422	0	0	0	442	35	159
CAM 2161	282	17	659	35	46	32	405	35	159
PEN 2014	299	21	692	35	28	29	698	35	159
GLO 2053	284 (+4)	28	686	26	46	26	713	35	150
HON 4316	293	25	660	26	50	34	668	35	150
NIG 2019	293	25	393 (+278)	42	50	34	685	35	150
DOU 1955	285	42	671	35	43	26	684	35	140

Note. See Table 2 for species name acronyms. For species with incomplete sequences, numbers in parentheses indicate possible additional length based on the length of a related species

Arabidopsis, reflecting numerous different deletion events. The intron separating this exon from the *trnA* 3′ exon is quite variable in length, ranging from 268 nt in *A. azoricum* to 713 nt in *A. globosum*. In stark contrast to the previous three exons, the *trnA* 3′ exon is 35 nt long in *Arabidopsis* and all *Arceuthobium* species. Indeed only two species show single-nucleotide substitutional changes in this region. Finally, the remaining spacer between the *trnA* 3′ exon and the 23S rDNA gene is relatively uniform in length (138 to 163 nt).

As has been previously documented for asterid parasitic plants, major sequence changes in the 16S–23S rDNA intergenic spacer region have rendered the two internal tRNA genes nonfunctional. Information about the functionality of these molecules in *Arceuthobium* can be inferred by plotting the indel events on potential secondary structures. This was done for *trnI* (GAU) and *trnA* (UGC) as shown in Figs. 1 and 2, respectively. All *Arceuthobium* species have indel or substitutional changes that affect the anticodon loop, thus rendering all pseudogenes (Fig. 1). Most species also have deletions or insertions at various other locations on the molecules. All species except *A. douglasii* have deletions that remove most or all of the D loop. Insertions between the D loop and the anticodon loop can be seen, as well as a 6-nt insertion between the anticodon loop and the variable loop in *A. nigrum*. The most unusual variant is found in *A. douglasii*, which has insertions that appear to add length to the D loop helix, however, it is likely that the molecule is still nonfunctional owing to the GAG (instead of GAU) present in the anticodon loop. As with *Arceuthobium*, all other parasites except *Cuscuta reflexa* appear to have pseudogenes for *trnI* (Table 3).

The structural diagrams for *trnA* (Fig. 2) indicate that changes are more subtle than observed for *trnI*, however, all are likely pseudogenes. Modifications of the anticodon loop occurred in some species (e.g., *A. juniperi-procerae*, *A. oxycedri*, and *A. campylopodum*), whereas most deletions occurred in the D loop region. The variable and TΨC loops have remained intact, owing to lack of modification of the *trnA* 3′ exon.

Results of the maximum parsimony analysis of the plastid 16S–23S rDNA intergenic spacer sequences are shown in Fig. 3. The consensus tree is compared with the nuclear ITS rDNA tree using the same suite of taxa. Overall, the two trees are highly congruent, where both capture the two major clades corresponding to the Old and New World species. Conflicts between the two gene partitions are shown by lines connecting the positions of *A. douglasii* and *A. pendens*.

Discussion

Rate Increases in the *Arceuthobium* Plastome

Previous molecular phylogenetic studies have shown that *Arceuthobium* exhibits higher substitution rates in plastome genes than relatives in Viscaceae and Santalaceae. The first evidence of this came from *rbcL* (Nickrent 1996; Nickrent

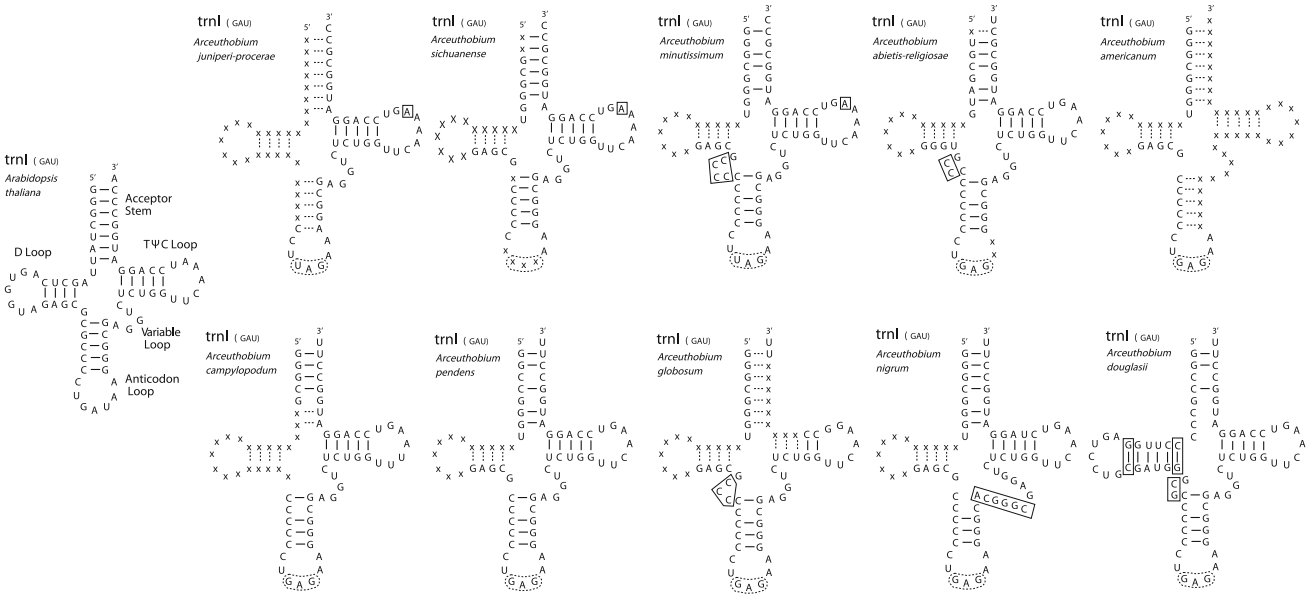

Fig. 1 Structural diagrams for *trnI* (GAU). The tRNA molecule of *Arabidopsis* is compared to that of various *Arceuthobium* species, all of which have pseudogenes but via different mutational histories. The "X" symbols in these diagrams represent nucleotides present in *Arabidopsis* but deleted in *Arceuthobium*. Nucleotides enclosed in polygons represent notable insertions

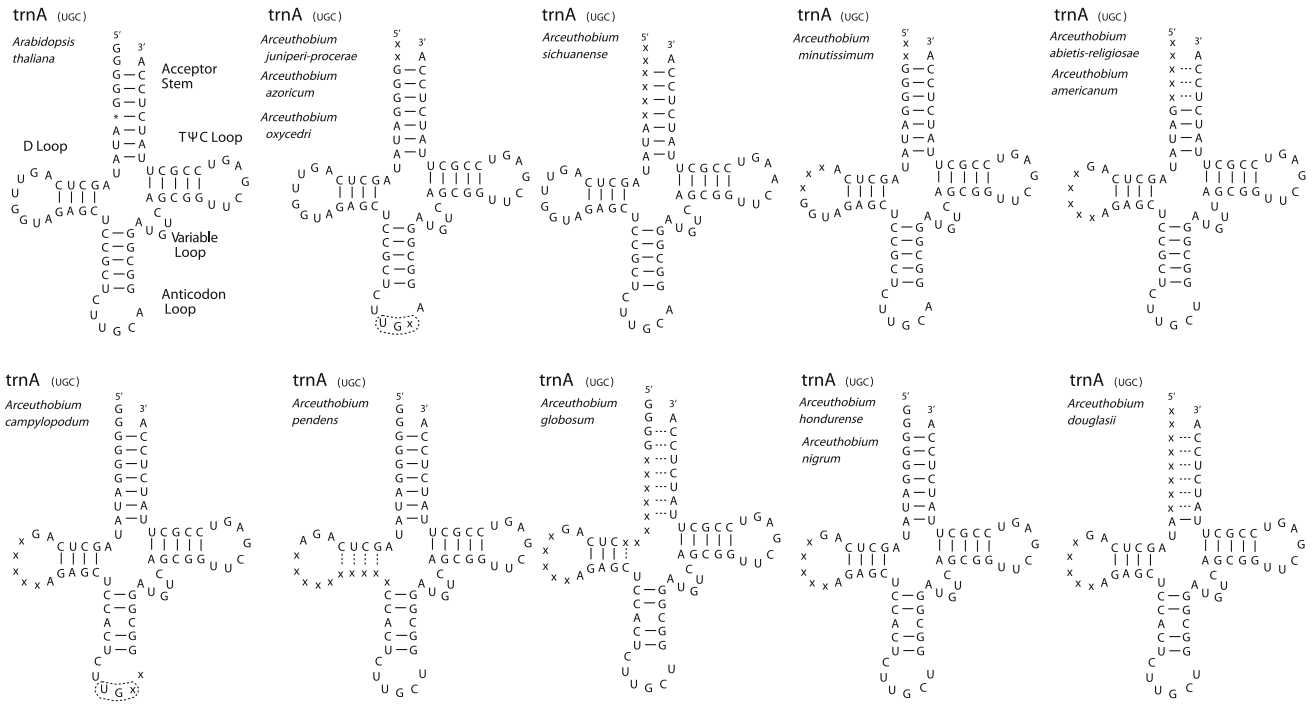

Fig. 2 Structural diagrams for *trnA* (UCG). The tRNA molecule of *Arabidopsis* is compared with that of various *Arceuthobium* species, all of which have pseudogenes but via different mutational histories. The asterisk in *Arabidopsis* indicates a deletion that may be a sequencing error. Labels are as in Fig. 1

and Soltis 1995), specifically where twice as many substitutions (relative to the outgroup *Santalum*) were seen in *A. verticilliflorum* compared with *Antidaphne* (Santalaceae). Although sequences of *matK* from other members of Viscaceae have been obtained (Der and Nickrent 2008), to date no sequences for this gene have been obtained from *Arceuthobium*. This negative result could stem from (1) the absence of the gene, (2) rate acceleration leading to divergence at primer binding sites, or (3) relocation of the gene, as has occurred in *Epifagus* (Wolfe et al. 1992b).

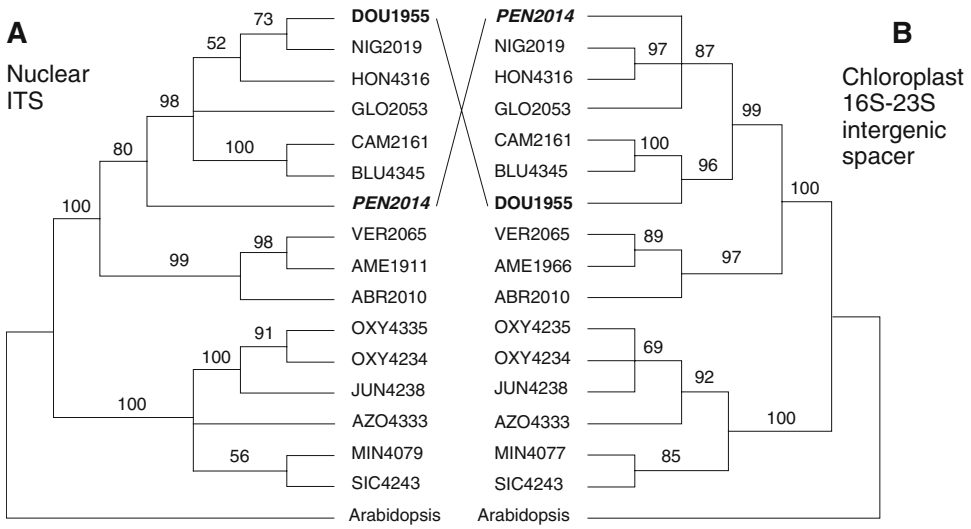

Fig. 3 Maximum parsimony trees with boostrap values (1000 replications) above the branches. **A** Strict consensus of two trees derived from analysis of nuclear ITS: length, 769; consistency index minus uninformative sites, 0.6766. This tree was constructed using 262 variable and parsimony informative characters from the total 744 characters. **B** Strict consensus of three trees derived from analysis of the chloroplast 16S–23S intergenic spacer: length, 413; consistency index minus uninformative sites, 0.8203. This tree was constructed using 95 variable and parsimony informative characters of the total 2840 characters. For species name abbreviations see Table 1. The lines indicate apparent incongruence between the nuclear and the plastid gene trees

Another chloroplast region that exhibits increased evolutionary rates is *trnT-L-F* (Fig. 4). Attempts to PCR-amplify this region failed for all members of the Old World clade (Nickrent et al. 2004). The majority of species have lost the *trnL* 5′ and 3′ exons entirely, whereas for others this region is intact. For *A. douglasii*, the 5′ exon is missing, thus rendering the entire tRNA molecule a pseudogene. The arrangement of the taxa in Fig. 4 is according to the similarity of the genic region, and in many cases this reflects phylogenetic similarity. One exception to this is *A. bicarinatum* and *A. pusillum*. Despite different indel mutational patterns, the sequences that remain in common to the two species are sufficiently similar (including sharing unique insertions and deletions) to place

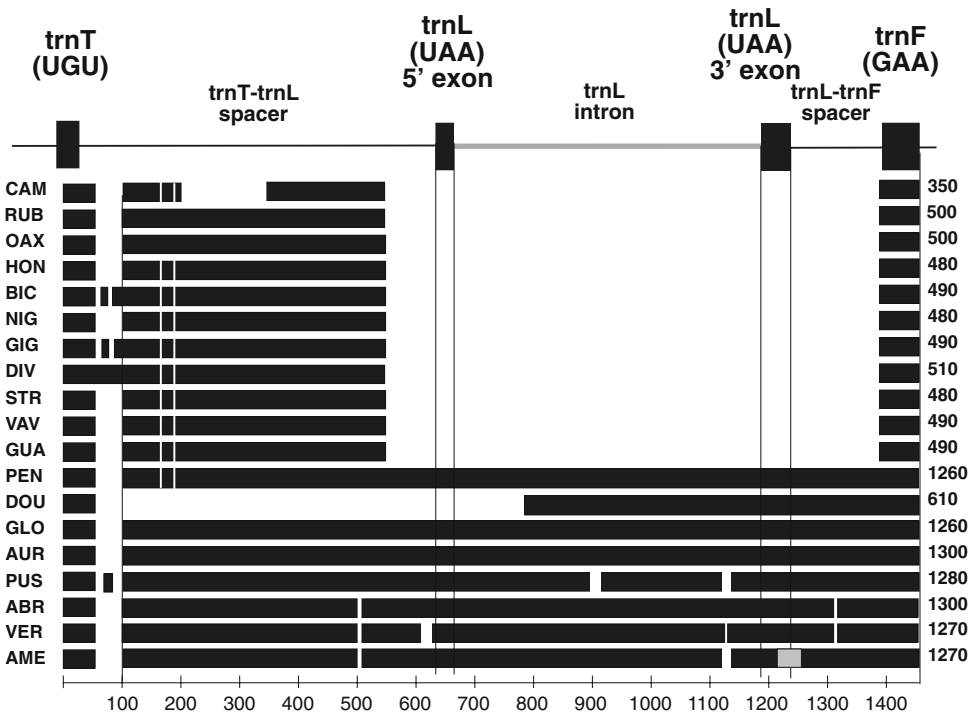

Fig. 4 The *trnT-L-F* region for New World *Arceuthobium* species. Full species names for the abbreviations not given in Table 1 are as follows: AUR, *A. aureum*; BIC, *A. bicarinatum*; DIV, *A. divaricatum*; GIG, *A. gillii*; GUA, *A. guatemalense*; OAX, *A. oaxacanum*; PUS, *A. pusillum*; RUB, *A. rubrum*; STR, *A. strictum*; VAV, *A. vaginatum* ssp. *vaginatum*. The gray region in the AME sequence represents an inversion

these taxa in the same clade (Nickrent et al. 2004). By examining the indel patterns in a phylogenetic context it is seen that superficially homologous changes (i.e., those sharing identical borders) may be evolving in parallel. This suggests that "hot spots" may exist in the plastome that are more likely to experience mutational changes.

tRNA Pseudogenes

As reported in Table 1, tRNA genes are frequent targets for the molecular processes that ultimately result in pseudogenes. These genes are also the site of nonhomologous recombination and inversions in the chloroplast (Haberle et al. 2008). *Arceuthobium* parallels the situation seen in *Epifagus*, where the 16S and 23S genes remain full-length and functional whereas the two split tRNA genes have become pseudogenes (Wolfe et al. 1992a). Across their entire plastomes, *Epifagus* has 17 intact and 5 tRNA pseudogenes, whereas *Orobanche* has 14 intact and 6 pseudogenes (Lohan and Wolfe 1998). A comparison between these genera showed that nine tRNA genes have escaped deletion in both parasites, are conserved in sequence, are not imported from the nucleus, and are likely functional and maintained by selection (Lohan and Wolfe 1998). Looking at another example, the parasitic green alga *Helicosporidium* has a small plastome (37.5 kbp) that encodes exactly the minimal number of tRNAs (25) required to translate the universal genetic code (de Koning and Keeling 2006). The evolutionary "strategy" here appears to be one that has eliminated redundancy, as evidenced by the loss of tRNA isoacceptors and the inverted repeat. This differs from holoparasitic angisperms such as *Epifagus* that have lost essential rRNA genes and must import those tRNAs from the nucleus. The three tRNA pseudogenes (A, I, and V) in *Arceuthobium* reported here represent a class of molecules that are considered nonessential (Lohan and Wolfe 1998) and are likely also imported from the nucleus. It is not clear why some tRNA genes remain undeleted even though they are nonessential. If mutational hits accumulate in a stochastic manner, this may simply relate to the time since the lineage began moving toward a holoparasitic nutritional mode. Alternatively, retention or loss of chloroplast genes may relate to the overall metabolic efficiency of the chloroplast, which is tied to environmental factors encountered over the organism's lifetime.

Utility of the 16S–23S Intergenic Region for Species-Level Phylogenetic Studies

Sequence divergence among plastid rDNA genes (16S and 23S) is only ca. 3%, with a K_0 value for the former of only 3 (Olmstead and Palmer 1994). For this reason, it is not unexpected that these genes have only been used to examine deep phylogenetic relationships (Nickrent et al. 2000; Soltis and Soltis 1998). Apparently less attention has been focused on the 16S–23S rDNA intergenic region as a potential source of sequences for phylogenetic studies of angiosperms. The 16S–23S intergenic spacer tree shown in Fig. 3, although constructed with fewer taxa, has resolution comparable to that of the *trnL* region sequences employed by Nickrent et al. (2004), plus this region allows the inclusion of the Old World species. A relationship between *A. douglasii* and the *A. campylopodum* complex was recovered with both chloroplast partitions but this is in conflict with the nuclear ITS tree that places the former taxon with *A. nigrum*. This incongruence suggests the possibility of confounding biological processes such as lineage sorting or chloroplast capture following hybridization (Wendel and Doyle 1998).

Concerning the biogeography of the genus, the existing theory that *Arceuthobium* originated in the Old World (Hawksworth and Wiens 1972) is not supported by the 16S–23S intergenic spacer data. All Old World species have significantly shorter spacer sequences owing to numerous deletions, yet these regions are present in the New World species. Moreover, the regions that are present show high sequence similarity to the outgroup santalalean genera *Phoradendron* and *Ximenia*, thus they are assumed to be homologous. A recent molecular phylogenetic analysis placed the genus *Arceuthobium* (two New and one Old World species) as sister to *Phoradendron* (Mathiasen et al. 2008). These data, together with the fact that *Arceuthobium* and *Phoradendron* are both most diverse in the highlands of central Mexico, support a New World origin for the dwarf mistletoes. Thus, the previously proposed easterly migration across the Bering Strait during the Tertiary (Hawksworth and Wiens 1972) may actually have been in the opposite direction.

Plastome Evolution in Santalales

Although the present study compares only a portion of the plastome among Santalales representatives, sequences from this part of the inverted repeat provide the first evidence useful in addressing molecular evolutionary trends in the order. As reported in Table 1, tRNA pseudogenes in this region have only evolved in *Arceuthobium*. The green, leafy mistletoe *Phoradendron* is a close relative of *Arceuthobium*, yet this portion of its plastome has not experienced comparable deletions. Molecular phylogenetic analyses (Der and Nickrent 2008; Malécot and Nickrent 2008; Vidal-Russell and Nickrent 2008) unambiguously support the following topology: (*Arabidopsis* (*Ximenia* (*Phoradendron*, *Arceuthobium*))). A closer examination of the 16S–23S rDNA intergenic region (Supplementary Data

S2) shows that of the ~160 deletions (≥2 nt), 120 of these occur in *Arceuthobium*, not *Phoradendron*, *Ximenia*, or *Arabidopsis*. Approximately half of these deletions are unique to a particular species. In addition to deletions, 48 tandem duplications can be seen, 40 of which occur in *Arceuthobium* and 37 of which are unique to a species. The plastome sequence of *Ximenia*, and even *Phoradendron*, is markedly more similar to that of the outgroup *Arabidopsis*. Taken together, these data indicate that the predominant mode of sequence evolution has been via deletions and that these have occurred at an increased rate in the more evolutionarily derived taxon (*Arceuthobium*) relative to its santalalean ancestors.

There is an obvious relationship between the loss of photosynthesis and the absence of photosynthetic genes in the plastome of various holoparasitic plants. This trend can be seen in a single genus, *Cuscuta*, where plants with larger plastomes are photosynthetic, whereas those with small plastomes are nonphotosynthetic holoparasites (Berg et al. 2003). In addition to these extreme cases, there are many parasitic plant lineages that show intermediate conditions, i.e., still retain some photosynthetic genes in their plastome but have reduced rates of photosynthesis. In Santalales, other examples of plants "intermediate" between fully photosynthetic hemiparasites and nonphotosynthetic holoparasites include the mistletoes *Tristerix aphyllus* (Loranthaceae), *Misodendrum* (Misodendraceae), and *Daenikera corallina* (Santalaceae), an amphiphagous species (see Der and Nickrent 2008). Reduced rates of CO_2 fixation may stem from fewer chloroplasts per cell, lower chlorophyll concentrations, lowered photosynthetic efficiency (e.g., from reduced density of thylakoids), or combinations of these factors (De la Harpe et al. 1979; Tuohy et al. 1984).

Because gene losses and overall plastome size reductions are occurring in *Arceuthobium* but not the related mistletoe *Phoradendron*, two assumptions can be made: (1) that the selectional environments acting on the two plastomes are different and (2) that the cost of retaining "expendable" genes is sufficiently high for them to be eliminated under particular conditions. Photosynthesis levels in adult shoots are considerably lower in *Arceuthobium* than *Phoradendron*, the former fixing only 30% of the carbon required by the plant (Hull and Leonard 1964a, b; Miller and Tocher 1975). It has been suggested that leafy species of *Phoradendron* differ from *Arceuthobium* in that they are mainly water parasites, obtaining little carbon from their hosts (Hull and Leonard 1964a). In contrast, *Phoradendron juniperinum* (a scale-leaf species) derives a significant amount (62%) of its carbon from host sources (Marshall and Ehleringer 1990). It is likely that photosynthetic rates and host carbon uptake levels vary across a continuum among the viscaceous mistletoes and that this correlates with the degree of leaf development. It would be informative to have complete plastome sequences across a range of *Arceuthobium* and *Phoradendron* species to better understand trends in plastome evolution.

Chloroplast Biogenesis, Plant Developmental Stage, and Plastome DNA

In addition to photosynthesis and starch production, plastids are involved in myriad other cellular functions such as biosynthesis of fatty acids, nucleic acids, and amino acids as well as storage of lipids (Tetlow et al. 2005). The developmental state of the plastid is highly coordinated with cell and organ development (Sulmon et al. 2006) and is mediated by plastid-nucleus signaling (Gray et al. 2003; Ruppel and Hangarter 2007; Sugimoto et al. 2007). Plastids are most often maternally inherited in angiosperms and exist in a variety of forms that may interconvert via differentiation and dedifferentiation. In tissues such as shoot meristems, proplastids occur that may give rise to other forms such as chloroplasts, leucoplasts, chromoplasts, amyloplasts, and etioplasts (Waters and Pyke 2005).

Ultrastructural studies of various *Arceuthobium* species have provided evidence that various tissues contain different plastid types. Ultrastructural studies of *Arceuthobium oxycedri* bract mesophyll (Dodge and Lawes 1974) showed large (4- to 6-μm) lens-shaped chloroplasts with a complex lamellar system, numerous grana, and varied numbers of thylakoids, i.e., a type typical of many angiosperms. In seedlings of *A. pusillum* just prior to and during host attachment, the radicular promeristem contained numerous chloroplasts, but with relatively little stacked grana and a variable number of parallel lamellae (Tainter 1971). The endophyte of *Arceuthobium*, which forms from the seedling radicle, exists as cortical strands (in proximity to host cambium and phloem) and sinkers (in contact with host xylem). The endophyte of *A. occidentale* was shown to contain etioplasts and pregranal plastids, the former with typical prolamellar bodies and numerous plastoglobuli (Alosi and Calvin 1984). Other plastids showed early stages of thylakoid biogenesis as seen in greening etioplasts. These studies suggest that the plastid types found in dwarf mistletoes are functionally correlated with tissue type, as seen in nonparasitic angiosperm roots, leaves, and seeds.

Selective Forces Acting to Alter Plastome Structure

Given that more than 3000 proteins are imported into the typical photosynthetic chloroplast, the question can be asked, "Which genes must be retained in both nonphotosynthetic and photosynthetic organisms?" Parasitic plants such as *Arceuthobium* beg the second question, i.e., "What is the minimum number of plastome genes that must be

retained while still maintaining a photosynthetically functional chloroplast?"

Several genes have been suggested to be indispensible (such as *trnE*), and various hypotheses have been proposed for their maintenance (Barbrook et al. 2006; Bungard 2004; Pfannschmidt et al. 1999). The general argument for why plastome truncation occurs is relaxed selection, i.e., less demand for photosynthesis in parasites receiving host carbon (Young and de Pamphilis 2005). But the selectional environment of an organism is the sum total of all conditions it experiences throughout its life cycle, and these may vary significantly. In the case of *Arceuthobium*, the seedling phase must exist independently of the host prior to the time haustorial attachments are made. *Arceuthobium* seedlings actively photosynthesize, from both the radicle and the endosperm (Muir 1975; Tocher et al. 1984). It is possible that only during this seedling stage are high photosynthetic rates required. During other life cycle phases *Arceuthobium* receives sufficient host carbon to grow and develop. In fully photosynthetic plants such as *Arabidopsis* and *Medicago*, the number of plastome molecules per plastid varies according to the plant developmental state (Fujie et al. 1994) and light conditions (Shaver et al. 2008). Given the differences in plastid types observed in various tissues of *Arceuthobium*, it is probably that similar variations in plastome number also occur in this mistletoe. It has been proposed that selection pressure for a small genome (i.e., one with increased deletions) is higher in life stages that require rapid cell division (Duminil et al. 2008; Selosse et al. 2001). In *Arceuthobium*, we propose that the endophyte, whose cells are rapidly dividing and obtaining essentially all nutrients from the host, may be the most likely life cycle stage where major plastome modifications occur.

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