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ARTICLE

Allopolyploid origin and genome differentiation of the parasitic species *Cuscuta veatchii* (Convolvulaceae) revealed by genomic in situ hybridization

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Abstract: Interspecific hybridization and genome duplication to form allopolyploids are major evolutionary events in angiosperms. In the parasitic genus *Cuscuta* (Convolvulaceae), molecular data suggested the existence of species of hybrid origin. One of them, *C. veatchii*, has been proposed as a hybrid between *C. denticulata* and *C. nevadensis*, both included in sect. *Denticulatae*. To test this hypothesis, a cytogenetic analysis was performed with CMA/DAPI staining and fluorescent in situ hybridization using 5S and 35S rDNA and genomic probes. Chromosomes of *C. denticulata* were small with a well-defined centromeric region, whereas *C. nevadensis* had larger, densely stained chromosomes, and less CMA$^+$ heterochromatic bands. *Cuscuta veatchii* had $2n = 60$ chromosomes, about 30 of them similar to those of *C. denticulata* and the remaining to *C. nevadensis*. GISH analysis confirmed the presence of both subgenomes in the allotetraploid *C. veatchii*. However, the number of rDNA sites and the haploid karyotype length in *C. veatchii* were not additive. The diploid parentals had already diverged in their chromosomes structure, whereas the reduction in the number of rDNA sites more probably occurred after hybridization. As phylogenetic data suggested a recent divergence of the progenitors, these species should have a high rate of karyotype evolution.

Key words: allopolyploidy, CMA/DAPI bands, homogenization of 35S rDNA repeats, interphase nuclei structure, 5S and 35S rDNA sites.

Résumé : L'hybridation interspécifique et la duplication génomique menant à la formation d'allopolyploïdes constituent des événements importants dans l'évolution des angiospermes. Au sein du genre *Cuscuta* (Convolvulaceae) de plantes parasites, les données moléculaires suggèrent l'existence d'espèces d'origine hybride. Il a été proposé que l'une d'elles, le *C. veatchii*, résulterait de l'hybridation entre le *C. denticulata* et le *C. nevadensis*, toutes deux faisant partie des *Denticulatae*. Pour tester cette hypothèse, une analyse cytogénétique a été réalisée au moyen de coloration CMA/DAPI et d'hybridation in situ en fluorescence à l'aide des sondes d'ADNr 5S et 35S ainsi qu'à l'aide de sondes génomiques. Les chromosomes du *C. denticulata* étaient petits et présentaient des régions centromériques bien définies, tandis que ceux du *C. nevadensis* étaient plus grands, intensément marqués et présentaient moins de bandes hétérochromatiques CMA$^+$. Le *C. veatchii* comptait $2n = 60$ chromosomes, dont environ 30 étaient semblables à ceux du *C. denticulata* et les autres semblables à ceux du *C. nevadensis*. L'analyse GISH a confirmé la présence des deux sous-génomes au sein de l'allotétraploïde *C. veatchii*. Cependant, le nombre de sites d'ADNr et la longueur du caryotype haploïde chez le *C. veatchii* n'étaient pas additifs. Les parents diploïdes avaient déjà divergé en ce qui a trait à leur structure chromosomique, tandis que la réduction dans le nombre de sites d'ADNr est probablement survenue après l'hybridation. Puisque les données phylogénétiques ont suggéré une divergence récente des espèces parentales, ces espèces doivent présenter une évolution caryotypique rapide. [Traduit par la Rédaction]

Mots-clés : allopolyploïdie, bandes CMA/DAPI, homogénéisation des répétitions d'ADNr 35S, structure des noyaux en interphase, sites d'ADNr 5S et 35S.

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Introduction

Events of interspecific hybridization are common in the evolution of most genera of angiosperms. If diploid hybrids retain their fertility they will be able to generate a wide diversity of phenotypes through genetic introgression, and, if the hybrid lineages become reproductively isolated, they can evolve into new species (e.g., Baack and Rieseberg 2007). If, however, these original hybridization events are followed by polyploidization, the resulting allopolyploids will have less chance to introgress, becoming genetically isolated and eventually generating a new species (Abbott et al. 2013). While the polyploid nature of hybrids is relatively easy to ascertain through cytological analyses, the morphological recognition of the hybrid origin of a species can be very challenging (Soltis and Soltis 2009). Furthermore, certain groups of plants show poor morphological differentiation to start with, such as parasitic plants with reduced morphological features, making an a priori identification of possible hybrids even more difficult (e.g., García 2001). Phylogenetic analyses based on multiple unlinked markers can reveal possible cases of interspecific hybridizations even when they are not suspected based on their phenotypes (e.g., Sang and Zhong 2000). For example, strongly supported topological incongruences between organellar- and nuclear-derived phylogenies are frequently taken as indication of reticulated evolution, although other biological phenomena, such as horizontal gene transfer, undetected paralogy, or lineage sorting, may result in similar discordant gene topologies (Stefanović and Costea 2008). To determine if the interspecific hybridization was accompanied by polyploidy, a simple chromosome counting of the putative hybrids and parents may be enough to identify the change in ploidy level. However, to demonstrate that two particular diploid progenitors were involved in the formation of an allotetraploid, it is necessary to identify chromosomal markers in the karyotype of the diploids that remain in the karyotype of the allotetraploid. The most commonly used karyotype features are the chromosome size and morphology as well as the size and distribution of heterochromatic bands and rDNA sites (Vaio et al. 2005; Marasek et al. 2006; Lee et al. 2011).

Cuscuta L. (Convolvulaceae; dodders) is a genus of nearly 200 species of parasitic plants with an extreme reduction or modification of vegetative structures and difficult species delimitation (Costea et al. 2015). Dodders are herbaceous plants with little or no chlorophyll, filiform stems attached to the host by stem haustoria, reduced scale-like leaves, and vestigial, ephemeral roots (Stefanović et al. 2007; Behdarvandi et al. 2015). The genus has a nearly cosmopolitan distribution but ~75% are native to North and South America and belong to subgenus *Grammica* (Lour.) Yunck., one of the four subgenera recognized by Costea et al. (2015).

Molecular phylogenetic studies by Stefanović and Costea (2008) and García et al. (2014) revealed eight cases of strongly supported topological incongruences between plastid- and nuclear-derived trees, all of them in subgenus *Grammica*. All of those incongruences, some within sections, and some between sections, were interpreted as results of multiple hybridization events across the genus. One of these putative hybrid cases was found within section *Denticulatae*, a group of three species morphologically characterized by the spherically enlarged radicular ends of its embryos (Costea et al. 2015), considered an adaptation to vivipary in dry environments (Costea et al. 2005). Two of its species, *C. denticulata* Engelm. and *C. nevadensis* I.M. Johnst. are distributed in the southwestern United States while the third, *C. veatchii* Brandegee, is restricted to the central parts of Baja California in Mexico. Phylogenetic analyses based on plastid sequences (*trnL-F*, *rbcL*) placed *C. veatchii* as sister species to *C. denticulata*, while nuclear DNA analyses (nrITS, 26S nrDNA) placed *C. veatchii* as sister to *C. nevadensis* (Stefanović and Costea 2008; García et al. 2014). Recently, García et al. (2018) expanded the sampling of sect. *Denticulatae* to multiple individuals of all species, and using molecular, morphological, and conventional cytological data, confirmed *C. veatchii* as a tetraploid ($2n = 60$) of hybrid origin between *C. denticulata* and *C. nevadensis*.

Albeit limited, the currently available evidence indicates that *Cuscuta* may represent the genus with the broadest karyotypic and genome size diversity among all the angiosperms. It has one of the highest genome size variation in plants, with 2C values ranging among diploid species from 0.96 to 66.54 pg (McNeal et al. 2007; Kubešová et al. 2010), chromosome size varies between 0.4 and 23 μm, with highly symmetric to bimodal karyotypes, and has species with monocentric and holocentric chromosomes (Pazy and Plitmann 1995; García 2001; García and Castroviejo 2003). Most species of subgenus *Grammica* are diploids with $2n = 28$ or 30, or tetraploids with $2n = 56$ or 60 (García and Castroviejo 2003), suggesting that polyploidy is important in the diversification of species of *Cuscuta*. However, neither the autopolyploid (intraspecific) nor allopolyploid (interspecific) origins of these species have ever been cytologically investigated. Karyotype characterization of polyploids as auto- or allopolyploids is usually done by meiotic or mitotic analyses.

Mitotic analyses of allopolyploids look for structural characteristics in the karyotypes of the diploid parents to identify the presence of one or more distinct subgenomes in the polyploids (Lee et al. 2011; Moraes et al. 2013; Souza et al. 2016). In some cases, the number, size, and morphology of chromosomes may be sufficient to identify the subgenomes in the tetraploid, but in most cases it is necessary to compare other easy-to-detect structural features, such as heterochromatin distribution pattern and ribosomal DNA sites (5S and 35S rDNA)

by fluorescence in situ hybridization (FISH) (Souza et al. 2012; Moraes et al. 2013). Double staining with the fluorochromes chromomycin A3 (CMA) and 4′,6-diamidino-2-phenylindole (DAPI) is the technique most frequently used to differentiate heterochromatic bands. Since these fluorochromes bind preferentially to GC- and AT-rich sequences, they permit the identification of two main types of heterochromatin: GC-rich (CMA^+) and AT-rich ($DAPI^+$) (reviewed by Guerra 2000).

In the case of allopolyploids, the technique of genomic in situ hybridization (GISH) is particularly recommended because it allows the recognition of the diploid ancestor chromosomes, or subgenomes, present in the allopolyploid, based on the highly dispersed species-specific repetitive DNA sequences that constitute a large proportion of the plant genomes (Chester et al. 2010). Subgenome differentiation by GISH is not influenced by genetic or epigenetic factors, such as observed in meiotic analyses (Sybenga 1999), reflecting the phylogenetic distances more directly (Markova et al. 2007). In *Dahlia*, for example, some interspecific hybrids showed regular bivalent pairing although they were clearly differentiated by GISH (Gatt et al. 2000).

The objective of this study is to analyze the karyotype of *C. veatchii* and its putative parents (*C. denticulata* and *C. nevadensis*) as a conclusive evidence of the reticulation event. The karyotype characterization in our study is based on chromosome number, size, and morphology, as well as heterochromatic bands of each species using the fluorochromes DAPI and CMA. Additionally, in all three species the number of 5S and 35S rDNA sites was investigated by FISH and the chromosome composition of *C. veatchii* was analyzed by GISH.

Materials and methods

Sampling and material analyzed

Seeds of *C. denticulata*, *C. nevadensis*, and *C. veatchii* were collected from several localities in California, Arizona (USA) and Baja California (Mexico) (Table 1). After scarification with concentrated sulfuric acid for 20–30 s, seeds were rinsed several times with distilled water, and placed on wet filter paper in Petri dishes to germinate. Because *C. nevadensis* and *C. veatchii* have a narrower host range or are highly host-specific, seedlings from only one specimen of *C. denticulata* (Stefanović SS-13-46, TRTE) could be grown in the greenhouse using coleus (*Plectranthus scutellarioides* (L.) R.Br., Lamiaceae) as a host. The rest of the material for cytogenetic analyses was obtained directly and only from seedlings. Herbarium vouchers documenting the origin of analyzed accessions were deposited in TRTE and WLU herbaria (Table 1).

Slide preparation, CMA/DAPI staining, and chromosome length measurement

Slide preparation was done using young shoot tips of seedlings (*C. nevadensis* and *C. veatchii*) or cultivated plants of *C. denticulata*. The material was pretreated with 8-hydroxyquinoline for 24 h at 10 °C, fixed in 3:1 (*v*/*v*) ethanol: acetic acid for 2–24 h at room temperature, and stored at –20 °C in the fixative. Subsequently, the material was washed in distilled water, digested in an enzymatic solution containing 2% cellulase (Onozuka) and 20% pectinase (Sigma), and squashed in 45% acetic acid, as previously described in detail (Vaio et al. 2005). Finally, the coverslip was removed in liquid nitrogen.

The CMA/DAPI staining was done as described by Guerra and García (2004). The slides were aged at room temperature for three days, stained with CMA (0.1 mg/mL) for 60 min, and subsequently counterstained with DAPI (2 μg/mL) for 30 min. The slides were mounted with glycerol-McIlvaine buffer pH 7.0 (1:1) and aged again for three days at room temperature. The images were captured with a Cohu CCD camera coupled to a Leica DMLB fluorescence microscope equipped with Leica QFISH software. For chromosome length comparison, the three best metaphases of each accession were measured using Adobe Photoshop CS3 software v.10.0.

Fluorescent in situ hybridization (FISH and GISH)

All in situ hybridizations were performed according to the protocol of Pedrosa et al. (2002). For localization of the rDNA sites, a 500 bp 5S rDNA clone (D2) of *Lotus japonicus* (Regel) K. Larsen (Pedrosa et al. 2002) and a 6.5 kb 35S rDNA clone (R2) of *Arabidopsis thaliana* (L.) Heynh. (Wanzenbock et al. 1997) were used as probes. The probes were labeled by nick translation with Cy3-dUTP (Amersham) and digoxigenin 11-dUTP (Roche), respectively. The hybridization mixture, composed of 60% (*v*/*v*) formamide, 5% (*w*/*v*) dextran sulfate, 2× SSC, and 5 ng/μL of probe, was denatured at 75 °C for 10 min. The 35S rDNA probe was detected with sheep anti-digoxigenin FITC conjugate (Roche) and amplified with rabbit anti-sheep FITC conjugate (Dako). The slides were mounted into Vectashield (Vector) containing DAPI (2 μg/mL) and the images were captured as previously described.

For genomic in situ hybridization, extractions of genomic DNA from *C. denticulata* and *C. nevadensis* were done according to the protocol of Doyle and Doyle (1987). The probes were also labeled by nick translation with Cy3-dUTP (Amersham) (*C. denticulata*) and digoxigenin 11-dUTP (Roche) (*C. nevadensis*). The genomic DNA was broken using DNase I (Thermo Scientific) at 15 °C for 1 h into fragments of about 300 bp. Hybridizations were done without blocking DNA (unlabeled DNA from one of the species) or blocking at a concentration 10 times higher than the labeled DNA.

Results

All accessions of *C. denticulata* and *C. nevadensis* presented the same chromosome number, $2n = 30$, but the karyotype of these species differed in several structural characteristics. The most evident one was the chromosome size, which ranged from 1.66 to 3.68 μm in

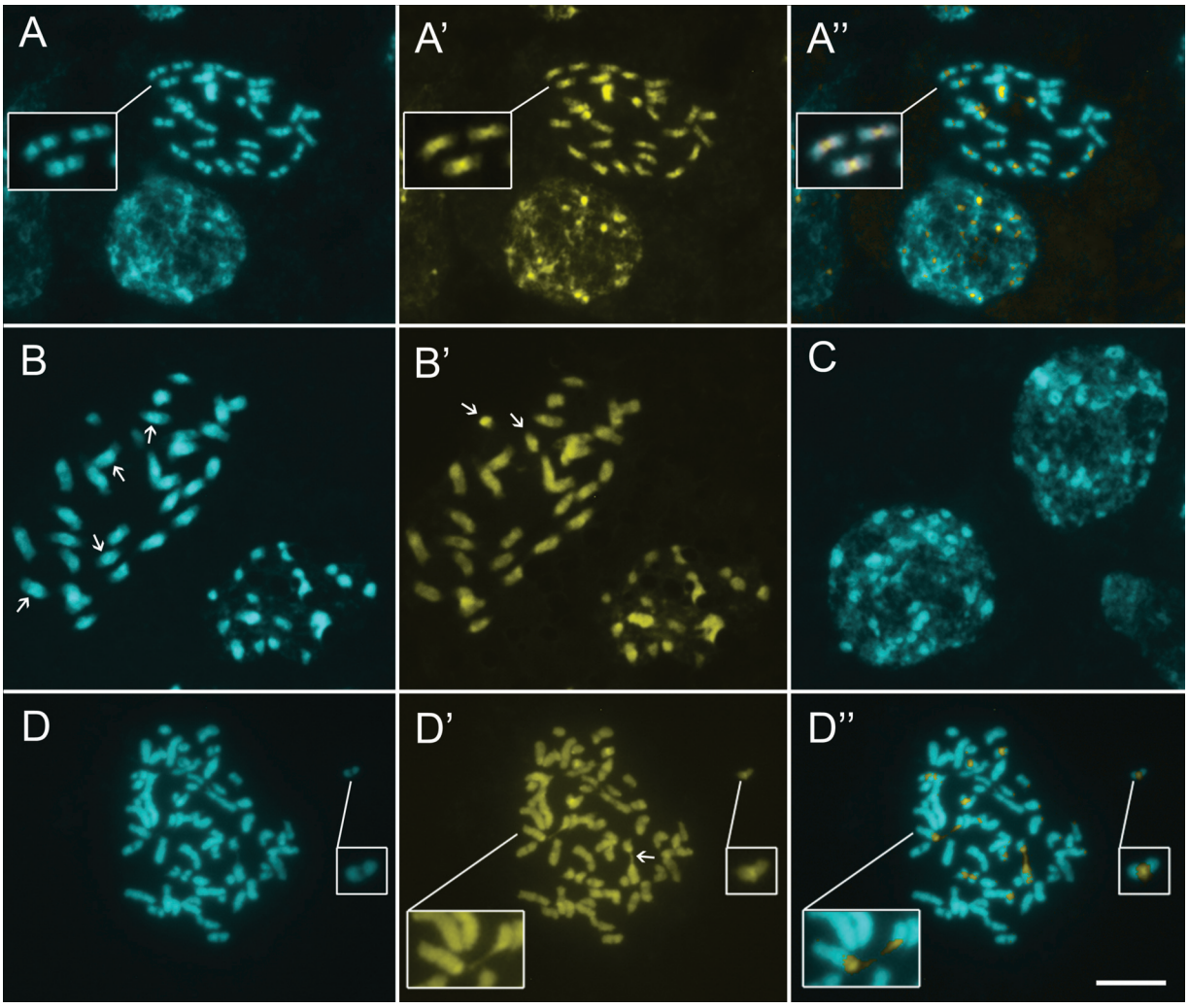
Table 1. Species of *Cuscuta* and individuals analyzed with their respective voucher information, collection locality, chromosome number (2*n*), total number of 5S and 35S rDNA sites, and number of adjacent rDNA sites.

Species and voucher (herbarium)	Locality	2*n*	5S + 35S (total number)	5S + 35S (adjacent sites only)
***C. denticulata* Engelm.**				
Stefanović SS-13-46 (TRTE)	USA, California; Inyo Co., Death Valley NP, Hwy 267, 16 mi SE of Scotty's Castle; 28 Jun 2013	30	2 + 2	2
Stefanović SS-16-24 (TRTE)	USA, Arizona; La Paz Co., Hwy 72, mile post 40, 2 mi N of aquaduct, 33°47′54″N, 113°49′53″W; 05 Apr 2016	30	2 + 2	2
***C. nevadensis* I. M. Johnst.**				
Stefanović SS-13-26 (TRTE)	USA, California; Inyo Co., Hwy 127, 45 mi N of Baker, 35°50′31.2″N, 97°14′49.14″W; 23 Jun 2013	30	6 + 10	4
Stefanović SS-13-29 (TRTE)	USA, California; Inyo Co., Hwy 190, 6 mi W of Death Valley Jct, 36°20′25.0″N, 116°35′49.5″W; 23 Jun 2013	30	6 + 10	4
Stefanović SS-13-35 (TRTE)	USA, California; Inyo Co., Hwy 190, 3.5 mi N of Olancha, 36°19′06.8″N, 117°57′54″W; 24 Jun 2013	30	6 + 10	4
Stefanović SS-13-36 (TRTE)	USA, California; Inyo Co., Saline Valley Rd., 5 mi SE of intersection w/Hwy 168, 37°09′06.9″N, 118°09′43.5″W; 25 Jun 2013	30	6 + 10	4
***C. veatchii* Brandegee**				
Bahía de Los Ángeles, Costea s.n. (WLU)	Mexico. Close to Bahía de los Ángeles, 28°58′47.1″N, 113°43′13.0″W, 298 m; 30 Apr 2014	60	6 + 4	2
Cataviña, Costea s.n. (WLU)	Mexico. Cataviña, 29°45′32.7″N, 114°45′17.5″W, 589 m; 30 Apr 2014	60	6 + 4	2
Punta Prieta, Costea s.n. (WLU)	Mexico. North of Punta Prieta, 28°57′25.4″N, 114°09′40.2″W, 240 m; 30 Apr 2014	60	6 + 4	2

C. denticulata and 2.58 to 4.99 μm in *C. nevadensis*. The total haploid chromosome length was 35.74 and 49.39 μm, respectively. In *C. denticulata*, the centromeric/pericentromeric region of the chromosomes was always weakly stained with DAPI (Fig. 1A), allowing the observation of their metacentric morphology. Around 18 chromosomes showed small CMA$^+$ proximal bands, in which the largest pair was often distended, forming a secondary constriction and a satellite (Fig. 2A). In contrast, the chromosomes of *C. nevadensis* were larger, thicker, and more densely stained, hampering the observation of the centromere position. Heterochromatic bands were hardly detected, except for a relatively large CMA$^+$ band located in the middle of the largest chromosomal pair, often forming a weakly stained secondary constriction and a satellite (Figs. 1B, 1B′). Also, a large and poorly contrasted DAPI$^+$ band was sometimes observed in the proximal region of most chromosomes of *C. nevadensis* (Fig. 1B). These two species also differed in the structure of the interphase nuclei: while in *C. denticulata* nuclei showed more uniformly distributed chromatin and only a few small chromocenters, most nuclei of *C. nevadensis* exhibited large, well-defined chromocenters, whereas the remaining chromatin was weakly stained (Figs. 1A, 1B).

The accessions of *C. veatchii* presented 2*n* = 60, with two sets of morphologically distinct chromosomes: a group of about 30 smaller chromosomes, less stained, and with a primary constriction, similar to those observed in *C. denticulata*, and another group of about 30 larger and more intensely stained chromosomes, similar to those found in *C. nevadensis*. The chromosome size in *C. veatchii* ranged from 1.59 to 4.22 μm and the haploid chromosome length was 75.20 μm. Therefore, both diploid subgenomes were reduced in size after polyploidization. Proximal CMA$^+$ bands were observed in several small chromosomes and in the largest chromosomal pair, often displaying a weakly stained secondary constriction (Figs. 1D–1D″). The interphase nuclei of *C. veatchii* were intermediate between those of the diploid species, presenting chromocenters slightly smaller than in *C. nevadensis* and the remaining chromatin denser than in *C. denticulata* (Fig. 1C). In general, the chromosomes of *C. veatchii* were smaller than in the diploid species, especially those more similar to *C. nevadensis*.

Fig. 1. Metaphases and interphase nuclei of *Cuscuta denticulata* (A–A″), *C. nevadensis* (B, B′), and *C. veatchii* (C–D″) stained with CMA (yellow) and DAPI (blue). Insets show magnified images of three chromosomes with proximal CMA+/DAPI− bands (A–A″), a chromosome with secondary constriction (D′, D″), and an isolated chromosome belonging to the metaphase, typical of *C. denticulata* (D–D″). Arrows point to DAPI+ bands (B), CMA+ satellites (B′), and secondary constriction (D′). Bar in D″ corresponds to 10 μm.

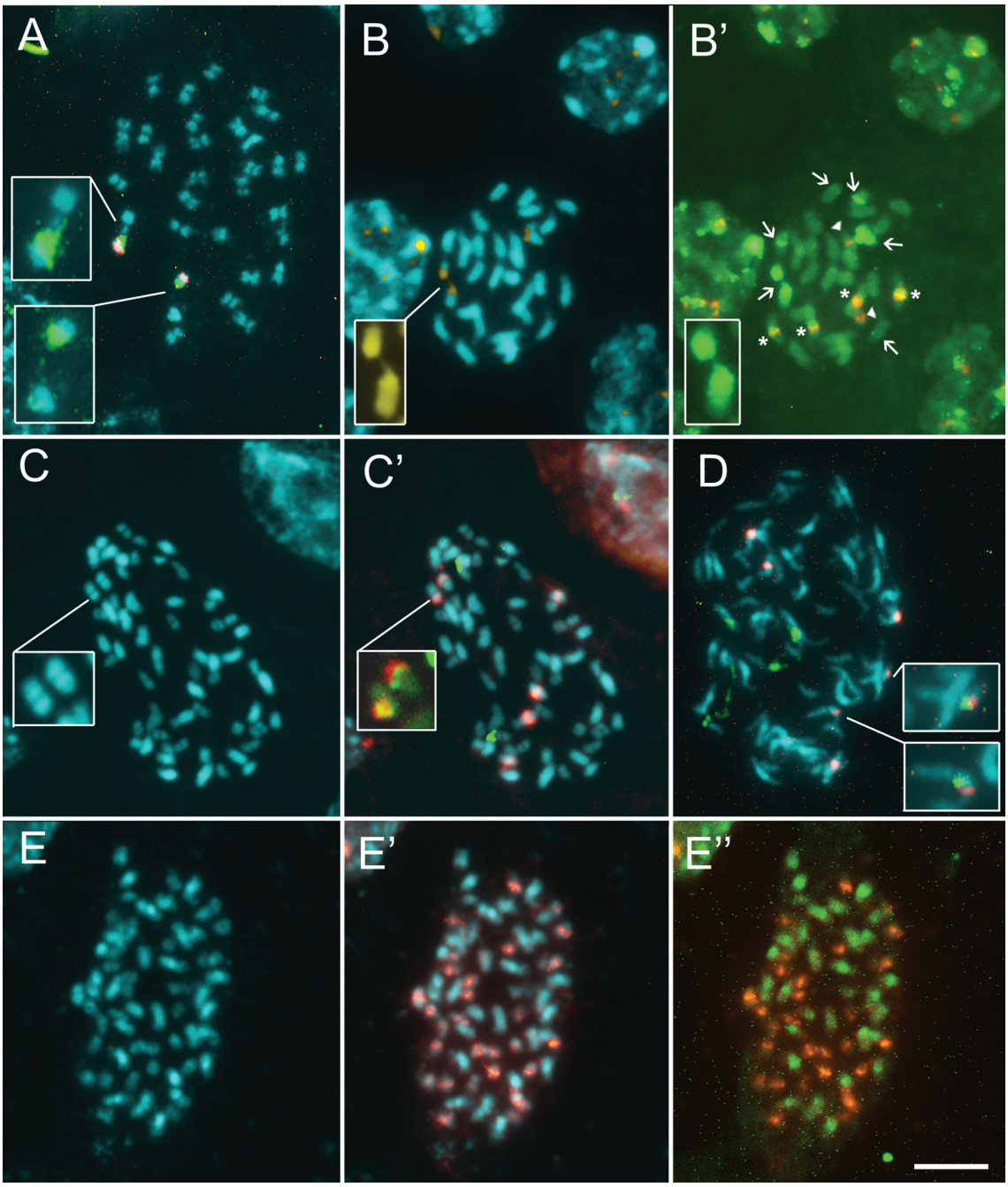


Regarding the rDNA sites, *C. denticulata* presented only two 5S rDNA and two 35S rDNA in all the individuals analyzed. These sites were located in the same chromosome pair, adjacent to each other: the 35S rDNA site was more proximal, usually distended and colocalized with a CMA+ band, whereas the 5S rDNA was more terminal (Fig. 2A). *Cuscuta nevadensis* showed three pairs of 5S and five pairs of 35S rDNA sites, with two pairs of 5S located adjacent to 35S rDNA sites (Fig. 2B′). The largest pair of 35S rDNA sites was proximally located, frequently distended, and colocalized with the CMA+ band on the largest chromosomal pair, whereas the smallest pair was not always observed (Figs. 2B, 2B′).

In *C. veatchii*, there were three pairs of 5S rDNA sites and two pairs of the 35S rDNA sites. The weakest labeled 35S rDNA and the 5S rDNA pairs were adjacent to each other (Figs. 2C′, 2D) and were located in a small chromosomal pair displaying a conspicuous centromere, very similar to the rDNA sites of *C. denticulata*. On the other hand, the largest chromosome pair of *C. veatchii* was similar to the largest chromosome pair of *C. nevadensis*, presenting the largest 35S rDNA site frequently distended and the satellite far away (Fig. 2D). The number of cells analyzed varied among samples, but at least seven complete metaphases with clear signals and good chromosome morphology were obtained for each sample.

GISH using genomic probes without blocker DNA was not sufficient to differentiate the two subgenomes. However, probes containing labeled DNA of one species plus a 10× excess of unlabeled DNA from the other species were able to distinguish the two subgenomes. Thus, the probe containing genomic DNA of *C. denticulata* labeled with Cy3 and unlabeled DNA of *C. nevadensis* stained more intensely the about 30 chromosomes morphologically similar to those of *C. denticulata*. Likewise, *C. nevadensis* genomic DNA labeled with FITC plus unla-

Fig. 2. Chromosomes of *Cuscuta denticulata* (A), *C. nevadensis* (B), and *C. veatchii* (C–E) showing the distribution of 5S (red) and 35S (green) rDNA sites in A, B′, C′, and D, and the two subgenomes of *C. veatchii* (E–E″). C′ and D illustrate the distribution of rDNA sites in chromosomes of *C. veatchii* from Punta Prieta (C′) and Bahía de los Ángeles (D). The genomic DNA of *C. denticulata* (red) labeled preferentially the smaller chromosomes (compare E and E′) whereas that of *C. nevadensis* (green) labeled the larger chromosomes (E″). Insets show the distribution of 35S rDNA signals in chromosomes bearing secondary constriction (A–B), 35S rDNA sites (A, B′), and the weak 5S/35S rDNA sites of *C. veatchii* (C–D). In B′, arrows, arrowheads, and asterisks indicate 35S, 5S, and 5S/35S adjacent rDNA sites, respectively. Bar in E″ corresponds to 10 μm.



beled genomic DNA from *C. denticulata*, hybridized more strongly with the remaining 30 chromosomes of *C. veatchii*, including all the larger and deeply stained chromosomes (Figs. 2E′, 2E″). This was observed in six complete metaphases showing 60 chromosomes. In all these metaphases and several other incomplete ones, no chromosome was labeled by both genomic probes that could indicate chromosome translocation between the two chromosome complements.

Discussion

The present work confirmed the chromosome number $2n = 30$ for *C. denticulata* and *C. nevadensis*, and $2n = 60$ for *C. veatchii* (García et al. 2018). The number $2n = 30$ is the

most frequent in *Cuscuta* (Pazy and Plitmann 1995; García and Castroviejo 2003) and represents the diploid condition for subg. *Grammica*. At least two other species of *Cuscuta* subg. *Grammica*, *C. cephalanthi* Engelm. and *C. gronovii* Willd. ex Roem. & Schult., are tetraploids with $2n = 60$, but several other species have been reported as tetraploids with $2n = 56$ (García and Castroviejo 2003).

The extent of cytological differences between *C. denticulata* and *C. nevadensis* was remarkable, considering their close evolutionary relationships (Stefanović and Costea 2008; García et al. 2014, 2018). The chromosomes of *C. denticulata* were small, mostly metacentric, and displayed three differently stained proximal chromatin bands: $CMA^+/DAPI^-$ bands colocalized with the single pair of 35S rDNA sites; other $CMA^+/DAPI^-$ bands, which are usually associated to GC-rich satellite DNA (e.g., Barros e Silva et al. 2010); and a third heterochromatin type that did not stain with DAPI but was negative or neutral for CMA. In *C. nevadensis*, the chromosomes were larger and with poorly defined morphology and heterochromatic bands, except for two CMA^+ blocks co-localized with 35S rDNA sites. Chromosome size variation among closely related species is commonly associated with genome size variation promoted by proliferation/elimination of repetitive sequences, mainly retroelements (Li et al. 2017). The extra repetitive sequences may be more concentrated in the proximal region, as in *C. nevadensis* and other species (e.g., Gaeta et al. 2010; Yuyama et al. 2012). In addition, the number of rDNA sites in *C. nevadensis*, with six 5S and 10 35S rDNA sites, was much higher than in *C. denticulata*, with only one pair of sites for each rDNA family. This extensive cytological divergence of the two parental species suggests that a high rate of chromosomal differentiation has occurred in this section.

Another important feature was the presence of several large chromocenters in the interphase nuclei of *C. nevadensis*, only few and small chromocenters in *C. denticulata*, and an intermediate condition in *C. veatchii*. Differences in the structure of the interphase nuclei are strongly associated with the distribution of acetylated H4 histones and the density of repetitive DNA sequences along the chromosomes (Feitoza et al. 2017). These structural features are regularly inherited during polyploidization and later adjusted to the tetraploid condition, similarly to the often observed DNA content reduction of each subgenome of a polyploid (Leitch and Bennett 2004). In *C. veatchii*, the haploid karyotype length was nearly 10% shorter than expected based on the total karyotype length of both diploid parents. Therefore, in terms of chromosome number, chromosome size and morphology, CMA/DAPI banding pattern, and interphase nucleus structure, *C. veatchii* clearly corresponds to what would be expected for an allopolyploid resulting from the hybridization between *C. nevadensis* and *C. denticulata*.

Also noteworthy is that the number of 5S and 35S rDNA sites in *C. veatchii* was not additive: six 5S and four 35S sites were found instead of the eight 5S and 12 35S rDNA sites that would be expected based on strict additivity. The number of rDNA sites observed in parental species is generally conserved in synthetic polyploids, but in natural polyploids it is more commonly reduced, as part of the gradual process of genome adjustment (Lee et al. 2011; Volkov et al. 2017). In synthetic allopolyploids and in a very few cases of human-influenced hybridization and allopolyploid speciation, this adjustment has evolved very quickly (e.g., Malinska et al. 2011), whereas in most polyploids it seems to demand many generations. In *Nicotiana*, for example, the reduction of 5S and 35S rDNA to a diploid number was observed in 1.5 million years old natural allopolyploids but not in allopolyploids younger than 200 000 years (Kovarik et al. 2008). Therefore, the strong reduction in the number of rDNA sites in *C. veatchii* may be an indication that this hybrid is not of a very recent origin.

It is interesting to note that in *C. veatchii* the only conserved 35S rDNA sites were the single pair originating from *C. denticulata* and the largest ones from *C. nevadensis*. These sites were often observed as secondary constrictions in the parental metaphases, meaning that they were actively transcribing rRNA genes. Analysis of the ITS sequences of approximately 25 individuals of *C. veatchii* showed no intra-individual sequence variation or clear polymorphisms were limited to only a few positions (García et al. 2018). Phylogenetic analyses of these sequences placed *C. veatchii* in the clade of *C. nevadensis*, indicating that in *C. veatchii* there was a homogenization towards *C. nevadensis* 35S rDNA repeats. Non-homologous recombination and gene conversion, the main mechanisms driving rDNA homogenization, apparently work more efficiently in active rDNA sites, where the chromatin is less condensed and most exposed to these mechanisms (Kobayashi 2006; Kovarik et al. 2008). As the 35S rDNA site inherited from *C. denticulata* and the largest one from *C. nevadensis* were active and partially decondensed in *C. veatchii*, they may have been more efficiently homogenized, while the remaining sites were less active and progressively eliminated (Kobayashi 2006; Volkov et al. 2007; Guo and Han 2014). A curious finding is that the small rDNA site observed on a *C. denticulata*-like chromosome of *C. veatchii* looks the same as the site observed in *C. denticulata* with respect to both size and position (both were small, proximally positioned, and adjacent to a 5S rDNA site). This observation indicates that the homogenization process substituted the ITS sequences of the rDNA site inherited from *C. denticulata* apparently without any visible change.

The best cytological evidence that *C. veatchii* is an allopolyploid derived from a cross between *C. denticulata* and *C. nevadensis* was provided by GISH, which revealed that half of the chromosomes of *C. veatchii* is quite similar

to the chromosomes of *C. denticulata* while the other half is similar to those of *C. nevadensis*. The impossibility to differentiate the two subgenomes of *C. veatchii* without blocking DNA suggests that in spite of the difference in chromosome size between them the main repetitive fractions of each subgenome were quite similar to each other (Kovarik et al. 2008; Lee et al. 2011; Guo and Han 2014). Thus, the differences in the CMA/DAPI bands, in the structure of the interphase nuclei, chromosome size and number of 5S and 35 rDNA sites suggest that before the formation of *C. veatchii* the two diploid species had already diverged considerably in their chromosome structure. On the other hand, after the formation of *C. veatchii* a progressive diploidization process lead to a reduction in the chromosome sizes and in the number of 5S and 35S rRNA sites, allowing the homogenization of the ITS sequences.

Conclusions

The analyses of chromosome number, morphology, and size, as well as interphase nuclear structure and GISH, clearly demonstrate that *C. veatchii* is an allopolyploid derived from *C. nevadensis* and *C. denticulata*. Because species of *Cuscuta* have one of the highest karyotype variations in plants, it is not surprising that the two parental species displayed quite differentiated karyotypes even though they are closely related. The differentiation apparently involved changes in chromosome size, number of rDNA sites, and CMA/DAPI bands before and after the polyploidization event, further confirming that the chromosome differentiation in some species of *Cuscuta* has been particularly rapid.

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