

# Confirmation of hybridity using GISH and determination of 18S rDNA copy number using FISH in interspecific F<sub>1</sub> hybrids of *Tecoma* (Bignoniaceae)

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**Abstract:** Interspecific hybridization in *Tecoma* Juss. was conducted to develop novel forms for the nursery industry. We report fertile hybrids from the cross *T. garrocha* Hieron. (pistillate parent) × *T. stans* (L.) Juss. ex Kunth. Leaf morphology of the F<sub>1</sub> hybrids of *T. garrocha* × *T. stans* was intermediate between the parents. GISH also confirmed hybridity. The F<sub>1</sub> hybrids were successfully backcrossed to both parents and self-pollinated to produce BC and F<sub>2</sub> progeny. *Tecoma garrocha*, *T. stans*, and *T. guarume* A. DC. ‘Tangelo’ were self-fertile. The F<sub>1</sub> hybrids also were crossed with *T. capensis* (Thunb.) Lindl. and *T. guarume* ‘Tangelo’, resulting in three-species hybrids. FISH conducted on F<sub>1</sub> hybrids identified four copies of the 18S internal transcribed spacer region. Further analysis using FISH has the potential to provide information on the evolution of Bignoniaceae and the potential role of polyploidy.

**Key words:** cytogenetics, *Tecoma garrocha*, *Tecoma stans*, *Tecoma guarume*, *Tecoma capensis*.

**Résumé :** Des croisements interspécifiques chez le *Tecoma* Juss. ont été réalisés afin de développer de nouvelles formes pour l’industrie horticole. Les auteurs rapportent des hybrides fertiles issus de croisements entre le *T. garrocha* Hieron. (parent femelle) × *T. stans* (L.) Juss. ex Kunth. La morphologie foliaire des hybrides F<sub>1</sub> *T. garrocha* × *T. stans* était intermédiaire entre les parents. Des analyses GISH ont également confirmé l’état hybride. Les hybrides F<sub>1</sub> ont été rétrocroisés aux deux parents et autofécondés avec succès pour produire des progénitures BC et F<sub>2</sub>. *Tecoma garrocha*, *T. stans* et *T. guarume* A. DC. ‘Tangelo’ étaient auto-fertiles. Les hybrides F<sub>1</sub> ont également été croisés avec le *T. capensis* (Thunb.) Lindl. et le *T. guarume* ‘Tangelo’, produisant des hybrides impliquant trois espèces. Des analyses FISH réalisées sur les hybrides F<sub>1</sub> ont révélé quatre copies de l’espaceur interne transcrit 18S. Des analyses FISH plus approfondies pourraient potentiellement fournir de l’information sur l’évolution des Bignoniacées et le rôle potentiel de la polyploidie.

**Mots-clés :** cytogénétique, *Tecoma garrocha*, *Tecoma stans*, *Tecoma guarume*, *Tecoma capensis*.

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## Introduction

*Tecoma* Juss. is a member of tribe Tecomeae within the Bignoniaceae. Gentry (1992) described *Tecoma* as a genus comprised of 14 species of shrubs or small trees, two being native to Africa and 12 naturally occurring in the Neotropics. However, Wood (2008) regards a number of taxa as subspecies. *Tecoma capensis* (Thunb.) Lindl. is a native of South Africa that was previously described as *Tecomaria capensis* (Thunb.) Spach. This species was separated because the apical portion of the anthers is fused. However, Gentry (1992)

justified the transfer into *Tecoma* by noting that there are other genera in Bignoniaceae that contain species with both fused and free anthers. The neotropical species range from the extreme southern US to northern Argentina, with a high concentration in Andean South America (Gentry 1992).

Gentry (1992) divided the genus into two groups based on floral morphology and pollinator type. The first has narrowly tubular orange or orange–red hummingbird-pollinated flowers and the other has campanulate yellow bee-pollinated flowers. Three hummingbird-pollinated, *T. capensis*, *T. gar-*

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*rocha* Hieron., *T. guarume* A. DC., and one bee-pollinated, *T. stans* (L.) Juss. ex Kunth, species are of interest for breeding. *Tecoma capensis* is a shrub or subshrub that has glossy foliage with 7–11 leaflets and red or red–orange narrowly tubular flowers with corollas typically 4–5 cm long, *T. garrocha* is a shrub or small tree (2–5 m) with 5–9 leaflets and very narrowly tubular flowers with yellow to orange corollas and red or orange–red lobes, *T. guarume* is a shrub (2–3 m) with 5–11 leaflets and broadly salverform-tubular flowers of variable color, and *T. stans* is a shrub or small tree to 10 m tall with 3–9 leaflets with a coarse texture and campanulate yellow flowers of 4–6 cm.

Neotropical Bignoniaceae species have been reported as self-incompatible and obligatory outcrossers (Bawa 1974; Stephenson and Thomas 1977), including *Tecoma* (Singh and Chauhan 1994). Singh and Chauhan (1994) reported *T. stans* exhibited gametophytic self-incompatibility. However, their account is unclear because it reports that autogamous pollination, meaning pollination within a single flower, aborted 5–7 days after pollination but geitonogamous pollination, or pollination resulting from pollen from one flower pollinating a different flower on the same plant, had 65% fruit set. These results are inconsistent with self-incompatibility. Regardless of the self-incompatibility mechanism (sporophytic vs. gametophytic), geitonogamous pollination should not result in production of viable seed in a self-incompatible individual (de Nettancourt 1972). The data of Singh and Chauhan (1994) indicates that *T. stans* is self-fertile, but their interpretation of geitonogamous pollinations as cross-pollination led to the erroneous conclusion that it was self-incompatible. Dutra and Machado (2001) reported that *Stenolobium stans* (Juss.) Seem. (syn. *T. stans*) was self-compatible and produced fertile seed via autogamous, geitonogamous, and xenogamous pollinations, but that this species required pollinators. Self-fertility was confirmed in *T. stans* by Raju et al. (2001). Pelton (1964) reported that while autogamy is not usually shown in *T. stans*, it was observed that in *T. stans* var. *velutina* cultivated in California, the stigma was on the same level, and within 1–2 mm, as dehiscing anthers, which may facilitate autogamous pollination.

Interspecific crosses in *Tecoma* have been reported for over a century. *Tecoma* × *smithii* Hort. is an interspecific cross between *T. velutina* (syn. *T. stans* var. *velutina* (A. DC.) Fabris) × *T. capensis* (Watson 1893; Smith 1894). Smith (1894) reported that it flowered as early as 6 months from seed and produced flowers year round in South Australia. More recently, controlled crosses have resulted in an interspecific hybrid of *T. stans* × *T. garrocha* (Kobayashi et al. 2004). Fruit set was observed when *T. garrocha* was used as the pistillate parent, but fertile seed were only obtained when *T. stans* was used as the pistillate parent (Kobayashi et al. 2004). Also, Gentry (1990) reported successful hybridization between bee-pollinated (yellow) species and hummingbird-pollinated (orange to red–orange) species. Natural hybridization between sympatric species has been reported in Bolivia, particularly where *T. tenuiflora* (A. DC.) Fabris grows with *T. stans* or *T. beckii* J.R.I. Wood (Wood 2008).

Identification of hybrids traditionally has been performed through morphological comparison, including in *Tecoma* spp. hybrids (Kobayashi et al. 2004). However, this may sometimes be difficult when the morphology of the parental

species overlaps. *Tecoma* has been described as a “...taxonomically difficult group with poorly demarcated species mostly differentiated by variable and often complexly overlapping vegetative characters” (Gentry 1992), therefore, other methods are essential in identifying hybrids. Karyotype markers such as distinctive chromosomes or specific banding pattern produced from Giemsa staining can be useful. However, the chromosomes of *Tecoma* are extremely small (Goldblatt and Gentry 1979), which could make comparison of banding patterns difficult. Genomic in situ hybridization (GISH), which uses labeled total genomic DNA as a probe (Ananthawat-Jónsson et al. 1990), has been used to successfully identify interspecific hybrids in numerous diverse crops including hybrids of teosinte (*Zea perennis* (Hitchc.) Reeves & Manglesdorf) and maize (*Zea mays* L.) (Tang et al. 2005), tomato (*Lycopersicon esculentum* Mill. syn. *Solanum lycopersicum* L.) (Ji et al. 2004), and ornamentals such as *Clivia* spp. Lindl. (Ran et al. 2001) and *Lilium* spp. L. (Karlova et al. 1999; Marasek et al. 2004).

In *Tecoma*, there are no reported species with chromosome numbers (e.g.,  $2n = 18$ ) that suggest a hybridization event occurred followed by polyploidization that would have resulted in the current complement of  $2n = 36$ , therefore, the role of polyploidy in the evolution of the genus as we know it remains unclear. Copy number of 18S rDNA, most often found with other rDNA in a cluster referred to as the nucleolar organizing region (NOR) (Long and Dawid 1980), has been associated with ploidy level. NOR copy number has been correlated to ploidy level in taxa as diverse as wheat (*Triticum aestivum* L.; Mukai et al. 1991), salmonids (*Oncorhynchus* spp. Suckley; Lozano et al. 1992), and *Musa* L. (Osuji et al. 1998). Lozano et al. (1992) and Osuji et al. (1998) used fluorescence in situ hybridization (FISH) (Bauman et al. 1980) technique, which is similar to GISH but uses specific sequence information as opposed to genomic DNA, to investigate hybridization of polyploidy events in salmonids and bananas, respectively.

Hybridization between morphologically diverse species such as *T. garrocha*, *T. guarume*, *T. stans*, and *T. capensis*, as described by Gentry (1979, 1990), offers potential to develop novel cultivars with unique combinations of flower and foliage characters. The objectives of this study were to (i) perform crosses including interspecific and self-pollinations to evaluate crossability, (ii) confirm hybridization using morphology and GISH, and (iii) determine copy number of the 18S region in hybrids using FISH.

## Materials and methods

### Plant materials

A selection of *T. capensis* that was found to be cold-hardy at The University of Georgia, Tifton Campus, was used in the current research. Two genotypes of *T. stans* were used. One form was selected because it was more compact and will be referred to as *T. stans* DS (dwarf selection). A form of *T. garrocha* with fine textured foliage and flowers with corollas and lobes of red–orange and *T. guarume* ‘Tangelo’ (Meerow and Ayala-Silva 2008) were also used. All the plant materials were maintained at The University of Georgia, Tifton Campus, (lat. 31°49'N, long. 83°53'W, USDA Zone 8b) in 2.4-L or 11.4-L containers filled with substrate containing

a pine bark : sand ratio of 8:1 amended with 0.91 kg/m<sup>3</sup> dolomitic lime and 0.45 kg/m<sup>3</sup> Micromax (The Scotts Co., Marysville, Ohio) and topdressed with 15 g (2.4-L container) or 45 g (11.4-L container) of Osmocote Plus 15-4.0-9.1 (The Scotts Co.). Plants used for controlled crosses and cytogenetic analysis were grown in a glasshouse with day/night set temperatures of 27/20 °C.

### Controlled crosses

Controlled crosses were carried out in 2008 and 2009 (Table 1). The pistillate parent was emasculated at least 2 days prior to anthesis to prevent self-pollination. Pollen of the selected staminate parent was applied by touching an anther directly to a receptive stigma. Stigmas of *Tecoma* are thigmotropic and remain closed after successful pollination, which provided a simple means of ensuring that application of pollen was not a limiting factor in the success of crosses. If pollen was not adequately applied the stigma would reopen after ~10 min. Self-pollination was conducted on the morning of anthesis in the same manner as cross-pollination. Glasshouse-grown plants of *T. garrocha* also set fruit without supplemental pollination, presumably through self-pollination.

### Comparison of leaf morphology

Ten leaves of *T. garrocha*, an F<sub>1</sub> hybrid of *T. garrocha* × *T. stans* DS, and *T. stans* DS were measured to determine if the hybrid exhibited intermediate morphology. Plants for morphological comparison were grown as described above in 11.4-L containers placed in a glasshouse. *Tecoma garrocha* and *T. stans* DS were approximately 1 year old from stem cuttings, and the F<sub>1</sub> hybrid was 1 year old from seed. Total leaf length, terminal leaflet length, terminal leaflet width, and number of leaflets were recorded. Data were subjected to analysis of variance (ANOVA) and means were separated using Duncan's multiple range test (MRT),  $\alpha = 0.05$ .

### Cytogenetic analysis

Plants that were grown for cytogenetic analysis were placed in trays filled with vermiculite and roots were allowed to grow out of containers into vermiculite for easy collection. Root tips were collected prior to 1000 HR and pretreated for 1–2 h in an aqueous solution of 2 mmol/L 8-hydroxyquinoline (Fisher Scientific Company, Suwanee, Ga.) + 0.24 mmol/L cycloheximide (Acros Organics, Morris Plains, N.J.) at 4 °C. Following pretreatment, roots were transferred to Carnoy's solution (100% EtOH : chloroform : glacial acetic acid mixture of 6:3:1 (by volume)) and fixed overnight at 25 °C. Roots were rinsed with deionized water, transferred to 70% EtOH (v/v), and stored at 4 °C.

To prepare for GISH and FISH, roots were rinsed for 15 min in deionized water and then root caps were removed with a scalpel. Root tips were placed into a previously described enzyme mixture (Akiyama et al. 2004) with modifications. The mixture consisted of 2.3% Cellulase Onozuka RS (Karlhan Research, Torrance, Calif.), 0.9% Macerozyme R-200 (Karlhan Research), 0.7% Pectolyase Y-23 (Karlhan Research), and 0.6 mmol/L EDTA adjusted to pH 4.2 at 37 °C for 1.5 h. The enzyme mixture was then removed by rinsing root tips in deionized water for 15 min. Individual root tips were then transferred to a glass slide, water was removed, and the root tip was macerated in 17  $\mu$ L of a 100% EtOH :

glacial acetic acid mixture of 3:1 (by volume) using forceps. Before the slide was allowed to dry, it was exposed to steam using a water bath set to 65 °C. Finally, the slide was dried on a heat block at 85 °C.

### Genomic DNA extraction

Genomic DNA for probe preparation was extracted from *T. stans* DS and *T. garrocha* using a DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, Calif.) according to the manufacturer's protocol. Blocking DNA was extracted from the same taxa as above using a modified version of the protocol described by Afanador et al. (1993). Approximately 150 mg of newly expanding leaves were collected on ice and ground in a mortar using liquid nitrogen. The powder was then transferred to a 1.5 mL Eppendorf tube and 600  $\mu$ L of CTAB buffer (65 °C) was added. The amount of chloroform : isoamyl alcohol (24:1) and isopropanol was increased from 400 to 600  $\mu$ L. Ten percent ammonium acetate (by volume) was added to the final precipitation step, and samples were suspended in water.

### Blocking DNA preparation

Genomic DNA from *T. garrocha* and *T. stans* DS were diluted to 50 ng/ $\mu$ L and autoclaved at 105 °C for 15 min to generate fragments of ~200 bp.

### 18S rDNA preparation

The 18S rDNA region from the obligate apomictic buffelgrass (*Cenchrus ciliaris* L.) accession B12-9 (Goel et al. 2003) was amplified using PCR. Each reaction mixture was composed of approximately 10 ng of template DNA, 0.5  $\mu$ mol/L primer (forward 5'-AACGGCTACCACATC-CAAGGAAGGC-3'; reverse 5'-GCGCGTGCGGCCCA-GAACATCTAAG-3'), 0.25 mmol/L dNTPs, 0.5 U JumpStart *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, Mo.), 1× PCR buffer (Sigma-Aldrich) in HPLC grade water for a final volume of 20  $\mu$ L. The primers were designed from the 18S sequence from maize (*Z. mays*). Reactions were conducted under the following conditions: 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 90 s, for 35 cycles. After confirmation of successful amplification using 1% agarose gel in TBE buffer, the PCR product was run on a 0.8% agarose gel and the band excised to ensure only 18S rDNA was recovered. Purification was performed using a QIAquick Gel Extraction Kit (QIAGEN Inc.), and then rDNA was diluted 1:10 in HPLC grade water and amplified under the same conditions as above. The amplification product was purified using QIAquick PCR Purification Kit (QIAGEN Inc.).

### Probe labeling and hybridization

Genomic DNA from *T. stans* DS, *T. garrocha*, and the 18S region were labeled with either biotin (Biotin-16-dUTP; Roche, Indianapolis, Ind.) or digoxigenin (DIG-11-dUTP; Roche), using a nick translation kit (Roche). Reactions were incubated at 15 °C for 4 h and unincorporated dNTPs were removed by ethanol precipitation in the presence of ammonium acetate. DNA was suspended in HB50 (2× SSC, 50% (v/v) formamide) and stored at -20 °C.

To prepare slides for hybridization, 500  $\mu$ L of 100% EtOH was applied to each slide and dried on a heat block at 85 °C and then the protocol for RNA and protein digestion de-

**Table 1.** Fruit set and seed germination from interspecific *Tecoma* spp. crosses conducted in 2008 and 2009.

Cross (♀ × ♂)	Flowers pollinated	Fruit set	Seed set / pollinated flower	Germination	Reproductive efficiency <sup>a</sup>
	no.			%	no.
<b>2008</b>					
<i>T. stans</i> × <i>T. capensis</i> UGA 4-3	13	0	—	—	—
<i>T. capensis</i> UGA 4-3 × <i>T. stans</i> DS <sup>b</sup>	10	0	—	—	—
<i>T. capensis</i> UGA 4-3 × <i>T. stans</i>	6	0	—	—	—
<i>T. capensis</i> UGA 4-3 selfed	12	0	—	—	—
<i>T. garrocha</i> × <i>T. capensis</i> UGA 4-3	5	4	36.6	0	0
<i>T. garrocha</i> × <i>T. stans</i> DS	5	5	49.6	92.7	46.0
<i>T. stans</i> DS × <i>T. capensis</i> UGA 4-3	23	0	—	—	—
<i>T. stans</i> × <i>T. capensis</i> UGA 4-3	2	0	—	—	—
<b>2009</b>					
<i>T. garrocha</i> × <i>T. capensis</i> UGA 4-3	11	11	43.7	0	0
<i>T. garrocha</i> selfed	8	7	37.4	45.5	17.0
( <i>T. garrocha</i> × <i>T. stans</i> DS) × <i>T. capensis</i> UGA 4-3	25	8	4.8	0.8	0.04
( <i>T. garrocha</i> × <i>T. stans</i> DS) selfed	7	6	23.7	67.5	16.0
<i>T. guarume</i> ‘Tangelo’ selfed	5	3	23.8	26.1	6.2
<i>T. garrocha</i> × ( <i>T. garrocha</i> × <i>T. stans</i> DS)	10	4	15.0	92.7	13.9
<i>T. garrocha</i> × <i>T. guarume</i> ‘Tangelo’	5	5	56.2	84.7	47.6
<i>T. guarume</i> ‘Tangelo’ × <i>T. capensis</i> UGA 4-3	10	2	6.1	0	0
<i>T. guarume</i> ‘Tangelo’ × ( <i>T. garrocha</i> × <i>T. stans</i> DS)	4	4	36.0	52.8	19.0
( <i>T. garrocha</i> × <i>T. stans</i> DS) × <i>T. garrocha</i>	4	2	15.0	56.7	6.8
( <i>T. garrocha</i> × <i>T. stans</i> DS) × <i>T. stans</i> DS	3	2	25.7	90.9	23.3
( <i>T. garrocha</i> × <i>T. stans</i> DS) × <i>T. guarume</i> ‘Tangelo’	6	5	46.8	78.6	36.8
<i>T. stans</i> DS × ( <i>T. garrocha</i> × <i>T. stans</i> DS)	3	2	37.0	77.5	28.7
<i>T. stans</i> DS selfed	9	4	26.4	43.7	11.6
<i>T. stans</i> DS × <i>T. capensis</i> UGA 4-3	5	0	—	—	—

<sup>a</sup>Reproductive efficiency represents the number of plants obtained per pollinated flower.

<sup>b</sup>Dwarf selection (DS) made at The University of Georgia, Tifton Campus.



scribed by Zhong et al. (1996) was followed. Two combinations of probe mixtures were used for double target hybridization including digoxigenin-labeled 18S + biotinylated *T. stans* DS DNA and biotinylated *T. stans* + digoxigenin-labeled *T. garrocha*. Hybridization experiments were also conducted by probing slides with a combination of unlabeled *T. garrocha* DNA and biotinylated *T. stans* DS DNA and also with unlabeled *T. stans* DS DNA and digoxigenin-labeled *T. garrocha* DNA using approximately 5×–10× blocking DNA. Hybridization was conducted as previously described by Goel et al. (2003) with modifications. Hybridization mixtures for each slide consisted of 1.3–2.5 ng/μL probe, 50% formamide, 5% dextran sulfate, 100–300 ng/μL salmon sperm DNA, and 2× SSC in a final volume of 15 μL. In some GISH experiments, blocking DNA was included in the hybridization mixture (described above). The hybridization mixtures were denatured at 85 °C for 10 min and chilled on ice. Slides were incubated at 37–39 °C for 16–20 h in a humid chamber. Post hybridization washes were as described by Goel et al. (2003).

### Probe detection

Digoxigenin-labeled probes were detected using a signal-amplification kit (Molecular Probes, Eugene, Oreg.), and biotinylated probes were detected using Texas Red streptavidin (Vector Laboratories, Burlingame, Calif.). All slides were blocked for nonspecific binding and washes were performed as in Goel et al. (2003) with modifications. An additional blocking step was done using blocking buffer (Roche). Incubation was conducted in the dark in three steps: fluorescein-conjugated anti-dig (Roche) and Texas Red-conjugated streptavidin, then rabbit anti-fluorescein and biotinylated anti-streptavidin, followed by goat anti-rabbit IgG and Texas Red-conjugated streptavidin. Slides were rinsed 2× for 5 min in T-PBS (0.2% Triton-X 100 (by volume) in 1× PBS; Sigma-Aldrich) and then rinsed in an alcohol dehydration series (70%, 95%, and 100% EtOH (by volume)) 1× for 5 min in each solution. Finally, slides were mounted in Vectashield (Vector Laboratories) containing 4',6-diamidino-2-phenylindole (DAPI; 1.5 ng/μL). Slides were examined using the equipment and conditions described by Goel et al. (2003). At least 20 cells from each hybridization mixture were observed.

## Results

### Crossing studies

Results of crossing studies conducted in 2008 and 2009 are presented in Table 1. In 2008, seeds were recovered from crosses between *T. garrocha* × *T. capensis* UGA4-3 and *T. garrocha* × *T. stans* DS. However, only seeds from the latter germinated. There was no fruit set from any crosses using *T. capensis* UGA4-3 as a pistillate parent in 2008; therefore, in 2009 it was only used as a staminate parent. The reproductive efficiency, calculated as the number of progeny per pollinated flower, of the cross between *T. garrocha* × *T. stans* DS was 46.0; meaning that 46 hybrids were recovered for each flower pollinated. An F<sub>1</sub> individual was selected from the above seedlings and was used in crosses in 2009. When it was self-pollinated and used as a parent in crosses with *T. capensis*, *T. garrocha*, *T. stans* DS, and

*T. guarume* 'Tangelo', the F<sub>1</sub> hybrid showed fertility similar to its parents (Table 1). When the F<sub>1</sub> hybrid was used as the pistillate parent in crosses with *T. guarume* 'Tangelo' it had a reproductive efficiency of 36.8, compared with 6.8 and 23.3 when pollinated with *T. garrocha* and *T. stans* DS, respectively. Further, the F<sub>1</sub> hybrid produced only one seedling from 25 pollinated flowers pollinated with *T. capensis* UGA4-3 pollen. The F<sub>1</sub> hybrid when used as the staminate parent in crosses with *T. garrocha*, *T. guarume* 'Tangelo', and *T. stans* DS had reproductive efficiencies of 13.9, 19.0, and 28.7, respectively. Self-pollination of the F<sub>1</sub> resulted in a reproductive efficiency of 16.0.

*Tecoma garrocha* was also self-fertile and produced 17.0 plants per pollinated flower. In addition, four flowers of *T. garrocha* produced 139 seeds with no pollination, 56 of which germinated. Crosses between *T. garrocha* and *T. capensis* UGA 4-3 produced many seeds but none germinated. However, when *T. garrocha* was pollinated with *T. guarume* 'Tangelo' it produced nearly 48 plants per pollinated flower. *Tecoma guarume* 'Tangelo' and *T. stans* DS were found to be self-fertile, producing 6.2 and 11.6 plants per self-pollinated flower, respectively.

### Comparison of leaf morphology

Leaves of the F<sub>1</sub> hybrids of *T. garrocha* × *T. stans* DS were morphologically intermediate between their parents (Table 2; Fig. 1). Length and width of the terminal leaflet was statistically different from both parents and serration of leaflets appeared intermediate.

### Confirmation of hybridity using GISH

Mitotic chromosome preparations were made for an F<sub>1</sub> hybrid of *T. garrocha* × *T. stans* DS and investigated using GISH (Fig. 2). When blocked with *T. stans* DS genomic DNA, the *T. garrocha* probe generally hybridized with 18 or 20 chromosomes. Figures 2B–2C shows chromosome spreads in which the *T. garrocha* probe hybridized with 19 chromosomes. Of the 19 chromosomes to which the *T. garrocha* probe hybridized, four were partially hybridized (Fig. 2C). When *T. garrocha* was used to block, the *T. stans* DS probe hybridized to 18–22 chromosomes (Figs. 2E–2F) and was often difficult to interpret. There was more noise in most experiments using the *T. stans* DS probe (Figs. 2E, 2I, 3C) and long exposure time during imaging was required. The *T. stans* probe appeared to partially hybridize to four chromosomes (Fig. 2F). When cells were dual probed with labeled DNA of both parents, the Dig-labeled *T. garrocha* most often hybridized to 18 chromosomes (Fig. 2H) but hybridized to 20 at times. The *T. stans* DS probe hybridized to 18–22 chromosomes (Fig. 2I). There were four chromosomes that hybridized with both probes (Figs. 2H–2I), indicating a high degree of similarity in some regions of the parental genomes.

### FISH using 18S rDNA and GISH

Mitotic chromosome preparations were probed using FISH and GISH simultaneously to investigate hybridity as well as copy number of the 18S region in F<sub>1</sub> hybrids. As in previous experiments, there was less nonspecific binding with Dig-labeled probes than biotinylated probes. However, Figs. 3B and 3D show that there was some nonspecific binding using Dig-labeled probes as well. The Dig-labeled 18S rDNA

**Table 2.** Leaf morphology of *Tecoma garrocha*, F<sub>1</sub> hybrid of *T. stans* (UGA dwarf selection, DS) × *T. garrocha*, and *T. stans* DS.

Taxon	Leaf length (cm)	Terminal leaflet length (cm)	Terminal leaflet width (cm)	No. of leaflets
<i>T. garrocha</i>	13.4b	6.4c	1.4c	7.4a
F <sub>1</sub>	16.0a	8.1b	2.5b	6.5a
<i>T. stans</i> DS	16.9a	11.4a	4.5a	4.4b

**Note:** Leaf measurements are the means of 10 leaves separated using Duncan's multiple range test (MRT). Means within columns followed by different letters are significantly different based on MRT,  $P \leq 0.05$ .

**Fig. 1.** Leaves of *Tecoma stans* (UGA dwarf selection, DS) (left), F<sub>1</sub> hybrid of *T. stans* DS × *T. garrocha* (center), and *T. garrocha* (right).



probe hybridized to four chromosomes in the F<sub>1</sub> hybrid (Figs. 3B, 3D), indicating that the 18S region is found on two chromosome pairs in the hybrid studied. Chromosomes that hybridized to the 18S probe also hybridized with the *T. stans* DS probe (Figs. 3B–3D).

## Discussion

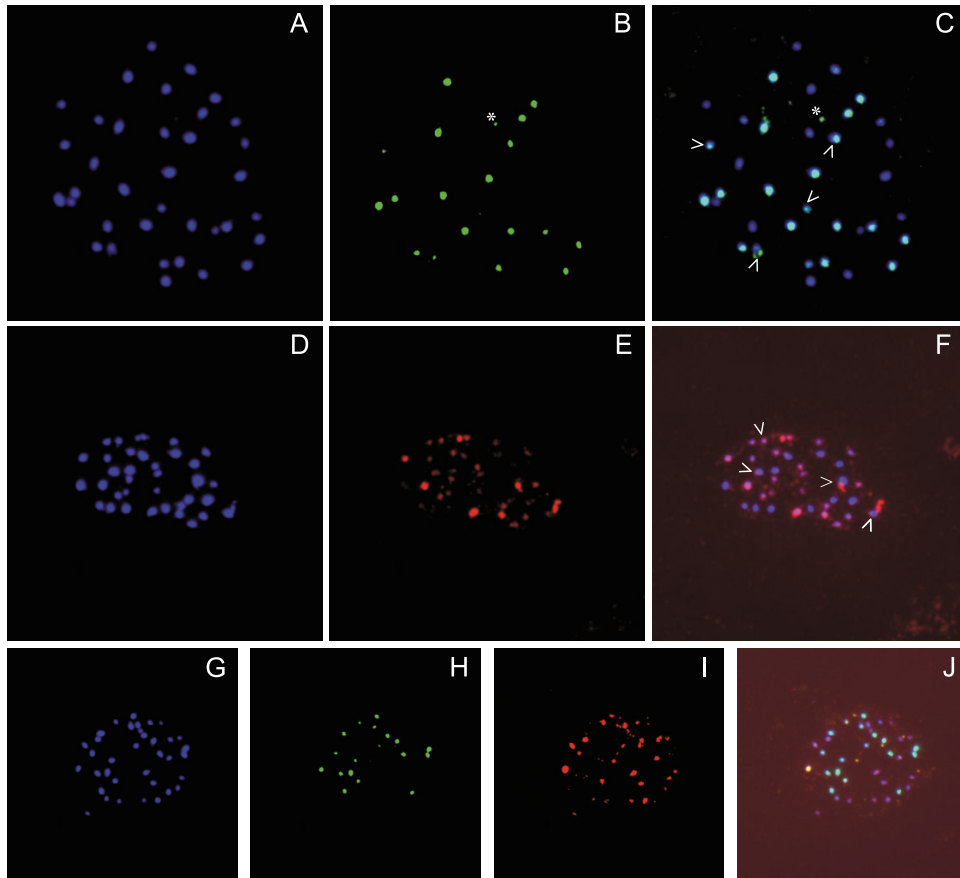
In 2008, interspecific hybrids were developed between *T. garrocha* and *T. stans* DS. A mean of 46 seedlings per pollinated flower resulted from this cross, indicating that these species are closely related. Reciprocal crosses were not conducted due to limited number of flowers produced by *T. garrocha* when *T. stans* DS was flowering. We successfully used *T. stans* as a staminate parent in crosses with *T. garrocha*, which differs from previous attempts to cross these two species. Kobayashi et al. (2004) reported viable seed set when *T. stans* was used as the pistillate parent but observed set fruit with no viable seed in reciprocal crosses.

In 2008, *T. capensis* was self-pollinated and used as a parent in crosses with *T. stans* and *T. garrocha*; however, fruit set was observed only when it was used to pollinate *T. garro-*

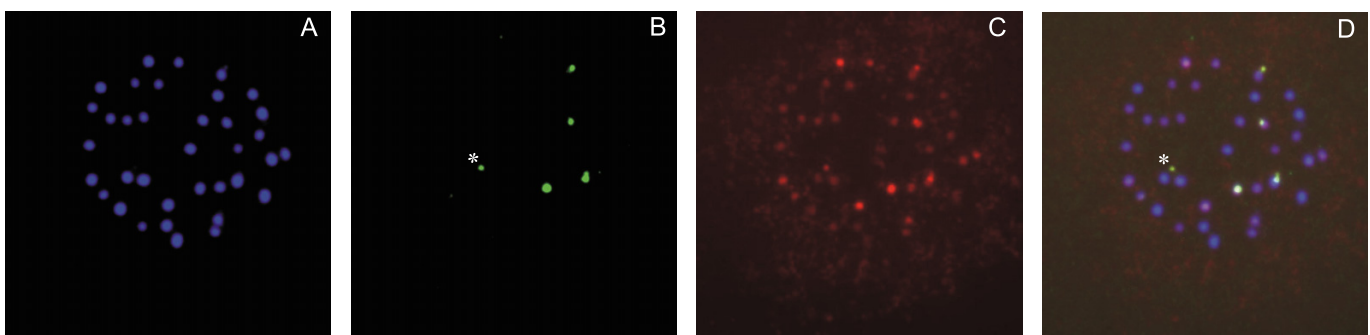
*cha*. Due to the lack of fruit set using *T. capensis* as a seed parent it was only used as a pollen parent in 2009. Crosses set seed when *T. capensis* was used to pollinate *T. garrocha*, *T. guarume* ‘Tangelo’, and the F<sub>1</sub> hybrid of *T. garrocha* × *T. stans* DS. However, only a single seedling from the latter germinated. Interspecific hybrids involving *T. capensis* have been reported (Watson 1893; Smith 1894). However, we report the first instance of its use in development of a three-species hybrid. Reciprocal crosses were also used to develop three species hybrids between the F<sub>1</sub> developed in 2008 and *T. guarume* ‘Tangelo’. *Tecoma guarume* ‘Tangelo’ was reported to produce abundant fruit in the landscape; however, no seedlings were observed (Meerow and Ayala-Silva 2008). Even though seedlings have not been observed, there is still an opportunity for non-native species such as *T. guarume* to hybridize with wild populations of *T. stans* as our research clearly demonstrates their crossability.

Self-fertility was observed in *T. garrocha*, *T. guarume* ‘Tangelo’, *T. stans* DS, and *T. garrocha* × *T. stans* DS. Our findings agree with previous reports, indicating self-compatibility in *T. stans* (Dutra and Machado 2001; Raju et al. 2001). *Tecoma garrocha* also set autogamous seed without supple-

**Fig. 2.** Results of GISH on mitotic chromosomes of an F<sub>1</sub> hybrid of *Tecoma garrocha* × *T. stans* DS probed with Dig-labeled *T. garrocha* and blocked with sheared genomic DNA of *T. stans* DS (B), biotinylated *T. stans* DS and blocked with sheared genomic DNA of *T. garrocha* (E), and probed with both parental probes without blocking DNA (H, I). Chromosome spreads counterstained with DAPI (A, D, G). \* indicates nonspecific binding of probes. > indicates partial binding of probes to only a portion of the chromosome. Chromosome spreads merged using a combination of DAPI, DIG, and TR signals (C, F, J) using Photoshop.



**Fig. 3.** Results of GISH (C) and FISH (B) on mitotic chromosomes of an F<sub>1</sub> hybrid of *Tecoma garrocha* × *T. stans* DS. The spread was dual-probed with Dig-labeled 18S rDNA from buffelgrass (*Cenchrus ciliaris*) and biotinylated genomic DNA of *T. stans* DS. Chromosomes counterstained with DAPI (A). Chromosome spreads merged using a combination of DAPI, DIG, and TR signals (D) using Photoshop. \* indicates nonspecific binding.



mental pollination in a glasshouse. Similarly, Pelton (1964) reported a close association of anthers and stigma in cultivated *T. stans* var. *velutina*, indicating the potential for autogamy. However, no reports of autogamous seed production without pollination are available for *T. garrocha*. Leaf morphology of F<sub>1</sub> hybrids of *T. garrocha* × *T. stans* DS was compared with the parents and was determined to be intermediate. Previous reports on hybrids between these spe-

cies also exhibited intermediate floral morphology (Kobayashi et al. 2004). In contrast to the report of Kobayashi et al. (2004), we found that the cross was successful using *T. garrocha* as the pistillate parent. Furthermore, it was reported that *T. stans* required uniconazole treatment to induce flowering (Kobayashi et al. 2004), however in our study *T. stans* DS flowered freely and *T. garrocha* was more reticent to flower, which prevented reciprocal crossing.



Results of GISH agreed with morphology and demonstrate the utility of this technique for identification of even closely related species. In our research parental probes often hybridized to more than half of the chromosomes in the hybrid but never to all chromosomes. For example, the *T. garrocha* probe hybridized to 19 chromosomes in the cell presented in Fig. 2B. The *T. stans* probe hybridized to all four NOR chromosomes (Fig. 3). In experiments where cells were dual probed with biotinylated 18S rDNA and Dig-labeled *T. garrocha* DNA, the *T. garrocha* probe hybridized to all four NOR chromosomes (data not shown). Even in experiments that used blocking DNA, there was often hybridization with more than 18 chromosomes. Based on these observations, it seems apparent that these species have highly homologous regions. However, it is probable that an increased ratio of block:probe may produce a better result. The amount of blocking DNA used in the previous studies varies widely from 10:1 or 20:1 (Karlov et al. 1999) to 100:1 (Ran et al. 2001). There are numerous reports on the utility of GISH to identify hybrids between closely related species used as ornamentals (Karlov et al. 1999; Ran et al. 2001; Van Laere et al. 2010), and our research confirms the utility of GISH in *Tecoma* hybrids. Molecular genetics have previously been used to investigate the phylogeny of Bignoniaceae (Spangler and Olmstead 1999) and genetic diversity at the generic level (Jain et al. 1999), but, to our knowledge, this is the first time that molecular cytogenetics have been used in the family.

We showed that ribosomal DNA appears to be located on two chromosome pairs in hybrids of *T. garrocha* × *T. stans*. Copy number of the NOR, which contains the 18S region used in the current study, is correlated to ploidy in taxa such as *Musa* (Osuji et al. 1998) and wheat (Mukai et al. 1991). Further investigations on diverse species of *Tecoma* as well as other genera in Bignoniaceae are warranted. Goldblatt and Gentry (1979) hypothesized that Bignoniaceae is originally based on  $x = 7$  due in part to the prevalence of  $n = 20$ , and perhaps more importantly on the genus *Oroxylum* Vent., which is  $n = 14$ . A more complete analysis of the family using FISH to determine copy number of the 18S region may help to identify polyploidy and provide information on the family's evolution.

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