Phylogenetic relationships, chromosome and breeding system evolution in *Turnera* (Turneraceae): inferences from ITS sequence data[1]

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Turnera provides a useful system for exploring two significant evolutionary phenomena—shifts in breeding system (distyly vs. homostyly) and the evolution of polyploids. To explore these, the first molecular phylogeny of *Turnera* was constructed using sequences of the internal transcribed spacer region (ITS) of nuclear ribosomal DNA for 37 taxa. We attempted to resolve the origins of allopolyploid species using single-strand conformation polymorphism and sequencing of homeologous copies of ITS. Two allohexaploid species possessed putative ITS homeologues (*T. velutina* and *T. orientalis*). A phylogenetic analysis to identify progenitors contributing to the origins of these polyploids was unsuccessful, possibly as a result of concerted evolution of ITS. Breeding system evolution was mapped onto the phylogeny assuming distyly to be ancestral in *Turnera*. Self-compatible homostyly appears to have arisen independently at least three times in *Turnera*; however, we were not able to determine whether there have been independent origins of homostyly among hexaploid species in series *Turnera*. Our phylogenetic analyses suggest that series *Turnera* is monophyletic. Neither series *Microphyllae* nor *Anomalae*, however, appear to be monophyletic. Future taxonomic revisions may require new circumscriptions of these latter series.

Key words: distyly; homostyly; ITS phylogeny; *Piriqueta*; polyploidy; *Turnera*; Turneraceae.

Both polyploidy and breeding system shifts have played significant roles in the evolution of flowering plants (Stebbins, 1974; Jain, 1976; Soltis and Soltis, 2000). *Turnera* is a useful system to explore these phenomena as breeding system variation (distyly vs. homostyly) and polyploidy (auto- and allopolyploidy) are common and have been reasonably well studied (Barrett and Shore, 1987; Solís Neffa and Fernández, 2000). While no molecular phylogenetic studies have been undertaken in *Turnera*, evolutionary relationships among various taxa have been explored using biosystematic methods (Barrett and Shore, 1985, 1987; Shore and Barrett, 1985a; Arbo and Fernández, 1987; Fernández, 1987, 1997; Fernández and Arbo, 1989, 1990, 1993a, b, 2000a, b; Shore, 1991a, b; Solís Neffa and Fernández, 1993, 2002). Base chromosome numbers within *Turnera* range from $x = 5$, 7, and 13 (Fernández, 1987; Solís Neffa and Fernández, 2000). Polyploids are common in *Turnera* and range from diploid through decaploid (Fernández, 1987; Solís Neffa and Fernández, 2000).

Genetic and microevolutionary studies of breeding system variation in the genus have been carried out for a number of species in series *Turnera* (Shore and Barrett, 1985b, 1989; Barrett and Shore, 1987; Belaoussoff and Shore, 1995; Tamari et al., 2005). Distyly and the associated dimorphic self-incompatibility system is widespread in the genus and family although self-compatible homostylous species also occur (Barrett and Shore, 1987; Belaoussoff and Shore, 1995; Arbo, 2004). Distyly in *Turnera* is determined by a single locus with two alleles (Shore and Barrett, 1985b). Recently, Athanasiou et al. (2003) and Khosravi et al. (2004) have identified proteins specific to the stylar transmitting tissue of short-styled plants

of species in series *Turnera*. The role of these proteins in distyly, if any, is presently unknown.

Although much has been accomplished over the past century with respect to the evolution and breakdown of distyly, the majority of work, until recently, has been focused at or below the species level (although see Baker, 1966). Phylogenetic analysis is necessary for hypotheses on the evolutionary pathways of distylous groups to be tested (Donoghue, 1989; Weller and Sakai, 1999). Phylogenetic analyses and character mapping in the Pontederiaceae (Kohn et al., 1996; Graham et al., 1998; Huelsenbeck et al., 2003), *Amsinckia* (Schoen et al., 1997), *Primula* (Conti et al., 2000), and *Narcissus* (Graham and Barrett, 2004) have explored hypotheses for breeding system evolution within these heterostylous taxa. Molecular data readily lends itself to phylogenetic studies of heterostyly as the characters used to examine relationships are likely to be independent of the morphological trait changes associated with breeding system evolution (Barrett, 1992). High levels of homoplasy may occur for morphological characters associated with floral evolution, complicating phylogenetic analysis (e.g., Eckenwalder and Barrett, 1986; Graham et al., 1998).

No molecular phylogenetic analyses have been undertaken for the Turneraceae (Malpighiales, eurosid I; APG, 2003), a family composed of 10 genera and approximately 190 species distributed largely in the Neotropics (Urban, 1883; Arbo, 2004). *Turnera* is the largest genus in the family, containing approximately 120 species native to the Americas (ranging from the southern USA to central Argentina), while two species are native to Africa (Urban, 1883; Arbo, 1997, 2000). *Turnera* is divided into nine series (Urban, 1883); the *Salicifoliae*, *Stenodictyae*, *Annulares*, *Capitatae*, *Microphyllae*, *Papilliferae*, *Turnera* (=*Canaligerae*, Urban), *Anomalae*, and *Leiocarpae*. Species of *Turnera* may be herbaceous annuals or perennials, shrubs, or trees. Pollination occurs by a wide diversity of bees and butterflies (Barrett, 1978).

In this paper we construct the first molecular phylogeny of species of *Turnera* using the 5.8S rDNA locus (ITS) (including ITS1, ITS2, and the 5.8S gene) to resolve relationships within and among series of *Turnera*. We explore the evolution of base chromosome number and polyploidy in an attempt to resolve the origins of allopolyploid species. Finally, we explore the number of transitions from distyly to homostyly in *Turnera*.

MATERIALS AND METHODS

Plant materials—A total of 37 species or infraspecific taxa from seven of the nine series of the genus *Turnera* have been included in this analysis, as well as five species of *Piriqueta* as an outgroup (Appendix). Efforts were made to sample as many species of *Turnera* as possible. In a few instances, we sampled more than one collection of a species, particularly when the collections differed in ploidy level, and/or there were morphological differences among the collections.

Molecular methods—Total DNA was extracted according to Doyle and Doyle (1987) using 0.5 g of fresh flowers, flower buds, or fresh leaf tissue or 0.1 g of dry leaf tissue, in 5 mL of extraction buffer. A majority of DNA samples were isolated from fresh flowers or leaf tissue of plants grown in the glasshouse at York University (Appendix). The quality of DNA extracted from dry leaf tissue varied depending on how the leaves were dried. Leaves that were air dried at room temperature usually produced good quality DNA. A small quantity of high molecular mass DNA was successfully extracted from seeds for two species (*Turnera cearensis* Urb. and *Turnera ignota* Arbo), where no other tissue was available. We pooled as many as 30 seeds for each of these species and used a reduced volume of extraction buffer.

We amplified the entire ITS region using primers ITS4 (Baldwin, 1993) and ITS5 (Sang et al., 1995), which are anchored in the 26S and 18S rDNA, respectively. PCR reactions were conducted using a Mastercycler (Eppendorf, Hamburg, Germany) following one of two protocols. For the first, we used TSG DNA polymerase and supplied reagents (Biobasic, Markham, Ontario, Canada): 1–2 μL (~50 ng) of genomic DNA was used as template and mixed with 2.5 μL (per sample) of primers ITS4 and ITS5 (10 pmoles/μL), 10 × reaction buffer containing MgCl₂. One unit of TSG DNA polymerase, 0.1 μL of dNTP mixture, and 13.65 μL of deionized water were added to a total reaction volume of 25 μL per sample. We used a second protocol for recalcitrant samples that would not amplify with the first. We used Jumpstart RED-Taq ReadyMix PCR Reaction Mix (Sigma-Aldrich, St. Louis, Missouri, USA): each sample contained 1–2 μL (~50 ng) of genomic DNA as template, 5 μL of primers ITS4 and ITS5 (10 pmoles/μL), 13 μL deionized water, and 25 μL of the Sigma Reaction Mix for a total of 50 μL. Amplification of ITS was carried out with an initial hot start of 94°C for 2 min, 35 cycles of 50 s at 94°C, 25 s at 55°C, 85 s at 72°C. Amplification was terminated with a final extension at 72°C for 3 min.

The products of amplification were run on 1% agarose gels containing 1 μg/mL ethidium bromide. We gel-purified PCR bands by excising them from the gel and purifying the DNA using a QIAQuick Gel Extraction Kit (QIAGEN, Mississauga, Ontario, Canada) following the manufacturer's protocol.

Single-strand conformation polymorphism—To identify homeologous ITS sequences within allopolyploid species, we used a single-strand conformation polymorphism (SSCP) analysis (Martins-Lopes et al., 2001). Some diploid and autotetraploid species were analyzed as well. Using SSCP, single-stranded DNA migrates through a polyacrylamide gel based upon its three-dimensional conformation. For each sample, we used 4 μL of gel-purified PCR product in 20 μL loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue). Samples were denatured at 95°C for 5 min and cooled on ice for 2 min. Samples were run (15–21 h) at 1 W on 12% polyacrylamide gels prepared as follows: 10 mL deionized water, 8.3 mL 30% acrylamide (29 g acrylamide, 1 g *N,N*′ methylene bisacrylamide, deionized water to 100 mL), 2.2 mL gel buffer (4.36 g Tris in 100 mL deionized water, pH 8.4), 60 μL 40% ammonium persulfate, 8 μL TEMED (*N,N,N*′,*N*′-tetramethyl di-amine). All gels were run using TBE buffer (0.045 M Tris-borate, 1.0 mM EDTA).

Gels were silver-stained following Montell et al. (2001). Gels were fixed with two 10-min washes in 10% acetic acid, rinsed using three 2-min washes in deionized water, all with gentle agitation. Gels were incubated for 30 min in silver solution (1 mL 10% silver nitrate solution, 150 μL formaldehyde, taken up to 100 mL with deionized water). Gels were rinsed (10 s) in deionized water and bathed in a 100 mL solution containing 3% sodium carbonate, 150 μL formaldehyde, 20 μL of 10 mg/mL sodium thiosulfate. Stain development was stopped by bathing the gel in 10% acetic acid (5 min). Gels were then rinsed with two 5 min washes in deionized water, all with gentle agitation. For storage purposes, gels were bathed in gel drying solution (10% glycerol, 30% ethanol) for 30 min, and then framed between two cellophane sheets and left to air dry for 2 d.

Cloning of homeologous ITS—When evidence from the SSCP analysis revealed more than one putative ITS homeologue, it was necessary to isolate each from the PCR products by cloning. Cloning was carried out using a pT7Blue-3 Perfectly Blunt Cloning kit (Novagen, Madison, Wisconsin, USA) following the manufacturer's protocol. Both 0.5 μL and 2 μL of gel-purified PCR product (~16.5 ng) were used in end-conversion reactions. Ligations were carried out at room temperature for 1–2 h. Both 10 μL and 50 μL of transformants were plated on S-gal plates (3,4-cyclohexenoesculetin-β-D-galactopyranoside; Sigma-Aldrich, St. Louis, Missouri, USA) containing 30 μg/mL ampicillin. Plates were incubated overnight at 37°C and left at 4°C for several hours to allow color development.

Single transformed bacterial colonies were grown overnight in 3 mL of LB broth (10 g tryptone, 5 g yeast extract, 10 g sodium chloride, in 1 L of deionized water, pH 7.5, containing 30 μg/mL ampicillin). Plasmids were isolated from overnight cultures using an alkali lysis miniprep protocol (Maniatis et al., 1989). Plasmid DNA was resuspended in 30 μL of TE buffer. Inserts in plasmids were PCR amplified as above using 1–2 μL of plasmid DNA as a template, and successful reactions were gel purified as before. These gel-purified products were run on SSCP gels along with the original allopolyploid PCR products, to verify the identity of the cloned ITS homeologues.

No plant tissue was available from *T. coerulea* var. *surinamensis*. We did, however, have DNA from an F₁ hybrid of *T. coerulea* var. *surinamensis* × *T. grandiflora* produced by one of us a number of years ago (J. S. Shore). To obtain a sequence of ITS from *T. coerulea* var. *surinamensis*, we used PCR to amplify and then clone and sequence ITS using DNA from the hybrid. We used SSCP to identify the clone containing the *T. coerulea* var. *surinamensis* × *T. grandiflora* sequence, but also sequenced the clone derived from *T. grandiflora*. We assigned identity to the clones based upon sequence similarity to the other *T. grandiflora* collection (Appendix) and phylogenetic analysis.

DNA sequencing—Cycle sequencing was performed on a Perkin-Elmer/ABI 373A sequencer in the Core Molecular Facility at York University, Toronto, Canada. Sequencing was usually performed directly using gel-purified PCR products (unless indicated otherwise) with primers ITS4 and ITS5 to sequence both strands in their entirety. If identical sequences were not obtained for both primers, additional sequencing was performed using internal primers ITS2 and ITS3 (Baldwin, 1993) to confirm the sequence. For *T. velutina* Presl., *T. orientalis* (Urb.) Arbo, and *T. coerulea* var. *surinamensis* (Urb.) Arbo & Fernández *T. grandiflora* (Urb.) Arbo, we sequenced 2–3 clones of each putative ITS homeologue. All sequences have been deposited in GenBank (Appendix).

Sequence alignment and phylogenetic analyses—DNA sequences were aligned using CLUSTALX (Thompson et al., 1997). Alignment parameters followed those recommended in Hall (2001). The alignment was adjusted manually at sites where gaps occurred using MacClade 4.05 (Maddison and Maddison, 2000). Gaps were treated as missing data for each particular sequence position in which they occurred.

Phylogenetic analyses were carried out using PAUP* version 4.0b10 (Swofford, 2001). A heuristic search was used with TBR branch swapping, random

TABLE 1.   Results of SSCP analyses indicating the number of putative homeologous copies of ITS expected (based on chromosome number) and observed for *Turnera* species.

Species	Chromosome number	Number of ITS homeologues	
		Expected	Observed
T. ulmifolia	$2n = 30$	3	1
T. campaniflora	$2n = 30$	3	1
T. velutina	$2n = 30$	3	2
T. orientalis (Corrientes)	$2n = 30$	3	3
T. orientalis (Paso de la Patria)	$2n = 30$	3	1
T. occidentalis	$2n = 30$	3	1
T. aurelii	$2n = 40$	4	1
T. cuneiformis	$2n = 40$	4	1
T. capitata	$2n = ?$	?	1
P. taubatensis	$2n = 28$	2	1

addition of sequences, and 50 replicates per search. Internal clade support was examined using bootstrapping with 1000 replicates, one random sequence addition per replicate, and restricting the number of trees to 100 per replicate. The five *Piriqueta* species were used as the outgroup.

In preliminary analyses based upon 44 sequences, one of us (Truyens, 2003) explored various weighting schemes, weighting substitutions in the coding region (5.8S rDNA) twice those in the noncoding (ITS) region, as well as the use of indels in a parsimony analysis according to Danforth et al. (1999). With the current data, we also explored analyses that excluded regions where considerable gaps occurred. Maximum likelihood analyses were also carried out using PAUP* version 4.0b10 (Swofford, 2001) with a general time-reversible model of sequence evolution, gamma distributed rates (GTR + G) and six substitution types (Truyens, 2003). Similarly, a Bayesian analysis was undertaken using MrBayes 3.01 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) and a general time reversible model with gamma-distributed rate variation and a proportion of invariable sites.

Character mapping—Using MacClade 4.05, we mapped breeding system evolution (distyly vs. homostyly) onto the majority rule consensus tree derived from our bootstrap analysis (Maddison and Maddison, 2000). We assumed

distyly to be ancestral and treated shifts from distyly to homostyly (and vice versa) as unordered. In the earlier parsimony analyses, we explored the effect of weighting transitions from homostyly to distyly more heavily (2 : 1) than the loss of distyly. To carry out this latter mapping using MacClade 4.05 (Maddison and Maddison, 2000), it was necessary to randomly resolve polytomies on a sample of the most parsimonious trees, which we did for 100 random resolutions for each tree. We then explored the subsequent mapping on each tree.

RESULTS

Single-strand conformation polymorphism (SSCP)—The ITS region was amplified, and we screened the allopolyploid species to identify putative homeologous ITS sequences using SSCP (Table 1; Figs. 1, 2). Two DNA bands should occur on silver-stained polyacrylamide gels for each ITS homeologue in the genome since the DNA is denatured prior to electrophoresis. Note that we refer to different ITS sequences within an individual as homeologues although we have not mapped these to duplicate rDNA loci, and it is possible that the variation detected may be the result of a lack of homogeneity among the tandemly repeated rDNA genes (Soltis et al., 1992). Most diploid and autotetraploid species investigated had evidence of a single ITS sequence. Plants from five of the 15 diploid species (*T. krapovickasii* Arbo, *T. weddelliana* Urb. & Rolfe, *T. hassleriana* Urb., *T. nervosa* Urb., *P. cistoides* subsp. *caroliniana*) and two of the four autotetraploid species (*T. subulata* Smith [Recife, Brazil]; *T. scabra* Millspaugh [Dagua, Colombia]) did, however, have evidence of possessing more than one sequence. We nevertheless sequenced ITS directly from the PCR products of these species without apparent ambiguity.

SSCP analysis of allopolyploid species yielded interesting results. Neither allooctaploid species (*T. aurelii* Arbo and *T. cuneiformis* Poir.) had evidence of possessing more than one ITS homeologue (Fig. 1, Table 1). Allohexaploids *T. ulmifolia* L., and *T. campaniflora* Arbo, Shore & Barrett, both had ev-
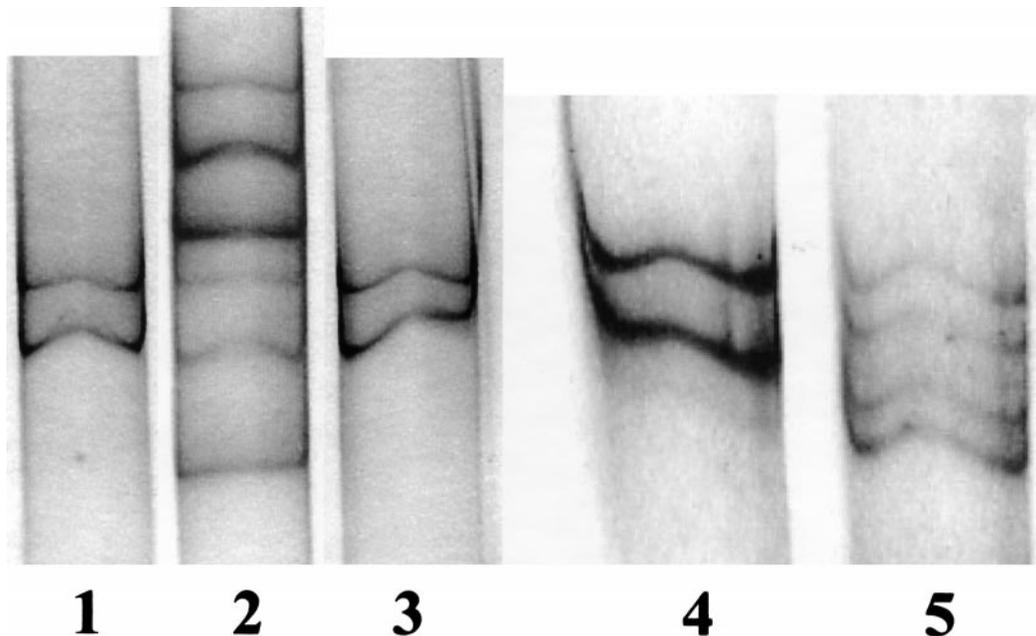

Fig. 1.   Single-strand conformation polymorphism (SSCP) analysis of allopolyploid species using polyacrylamide gel electrophoresis and silver-staining. Lane 1. *Turnera cuneiformis*. Lane 2. *T. orientalis* (Corrientes). Lane 3. *T. aurelii*. Lane 4. *T. ulmifolia*. Lane 5. *T. velutina*.
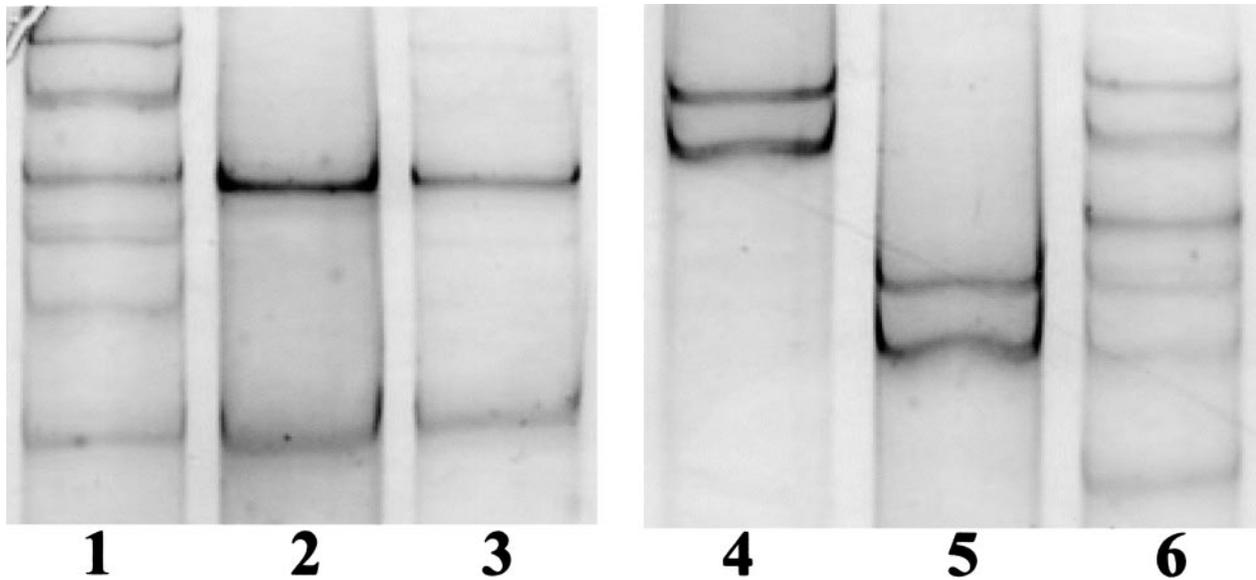
Fig. 2.   Single-strand conformation polymorphism (SSCP) analysis of allohexaploid *T. orientalis* (Corrientes) and clones of ITS homeologues. Lanes 1, 6. ITS amplified from *T. orientalis* genomic DNA. Lanes 2, 3. Clones of homeologue "c1" of ITS. Lane 4. Clone of homeologue "c2" of ITS. Lane 5. Clone of homeologue "c3" of ITS. Each clone possesses only two bands that can be found in the ITS amplicons of total genomic DNA (lanes 1, 6). Lanes (1–3) are from one SSCP gel, while lanes (4–5) are from a second SSCP gel.

idence of only a single ITS homeologue, while *T. velutina* possessed two homeologues (Fig. 1, Table 1). There were 11 single base-pair substitutions differentiating the two *T. velutina* sequences.

Interestingly, for allohexaploid *T. orientalis*, there was variation among different collections. The collection from Paso de la Patria, Argentina, had evidence of only a single homeologue, while the collection from Corrientes, Argentina, possessed three (Table 1, Figs. 1, 2). All three homeologues of ITS for this *T. orientalis* collection were cloned and sequenced (Fig. 2). There was a maximum of five single base-pair substitutions differentiating the three *T. orientalis* clones.

ITS sequence variation—Sequence lengths varied as a result of insertion/deletions, with variation in ITS1 ranging from 185–225 bp and ITS2 from 201–207 bp. We found identical sequences for some species and collections. The ITS sequences of diploid *T. subulata* (Arco Verde, Brazil), autotetraploid *T. subulata* (Recife, Brazil) and autotetraploid *T. scabra* (Dominican Republic) were identical. Likewise, sequences from allooctaploids *T. aurelii*, *T. cuneiformis*, and the sequence of allohexaploid *T. orientalis* (Paso de la Patria, Argentina) were also identical. *Turnera occidentalis* Arbo & Shore possessed a single substitution compared with the sequences for *T. aurelii*, *T. cuneiformis*, and *T. orientalis*. We included only a single representative in the phylogenetic analysis when identical sequences occurred.

Phylogenetic analysis—The alignment of 48 DNA sequences yielded 637 sites of which 341 were constant, 95 were variable but parsimony-uninformative, and 201 were parsimony informative. The data were analyzed using equal weights parsimony. This yielded 2067 equally parsimonious trees of length 834, a consistency index (CI) of 0.56, and a retention index (RI) of 0.71. A 50% majority-rule consensus tree based on 1000 bootstrap replicates of the data is shown (Fig. 3). Because both *T. velutina* and *T. orientalis* (Corrientes,

Argentina) possessed more than one ITS homeologue, we have indicated that these homeologues were derived from a single plant by drawing a vertical line joining the sequences on the tree. The phylogenetic tree derived from this analysis (Fig. 3) is similar to, but shows somewhat less resolution than, a consensus tree obtained using Bayesian methods. When posterior probabilities of clades (in the Bayesian analysis) and bootstrap support (in the case of parsimony) are considered, the trees are not substantively different.

Character mapping—We mapped breeding system evolution onto the tree, assuming distyly to be ancestral and treating shifts from distyly to homostyly as unordered (Fig. 3). Homostyly appears to have evolved on at least three occasions in *Turnera* (in *T. pumilea*, *T. candida*, and at least once in the group of allopolyploids in series *Turnera*). Within series *Turnera*, we have no basis to reject the hypothesis that the allohexaploid and allooctaploid species share a common origin of homostyly. In the outgroup, homostyly appears to have evolved in *Piriqueta viscosa*, a small-flowered species with affinities to *P. morongii*.

DISCUSSION

Systematics—Taxa from seven of the nine series of *Turnera* have been included in this study although the sampling of species was not uniform across the series. We sampled 20 of the 30 taxa within series *Turnera*, and they fall within a clade that is well-supported (bootstrap support of 95%; Fig. 3). Yellow flower color is common among species in series *Turnera* (including both distylous and homostylous species), however, a group of white/blue-flowered taxa (*T. coerulea*, *T. coerulea* var. *surinamensis*, *T. candida*, *T. fernandezii*, and *T. grandiflora*) form a clade within it (bootstrap support of 99%; Fig. 3). Species in this white/blue-flowered clade do not form viable hybrids with the yellow-flowered species (*T. subulata*, *T. scabra*, *T. krapovickasii*, *T. concinna*) at the diploid level, and
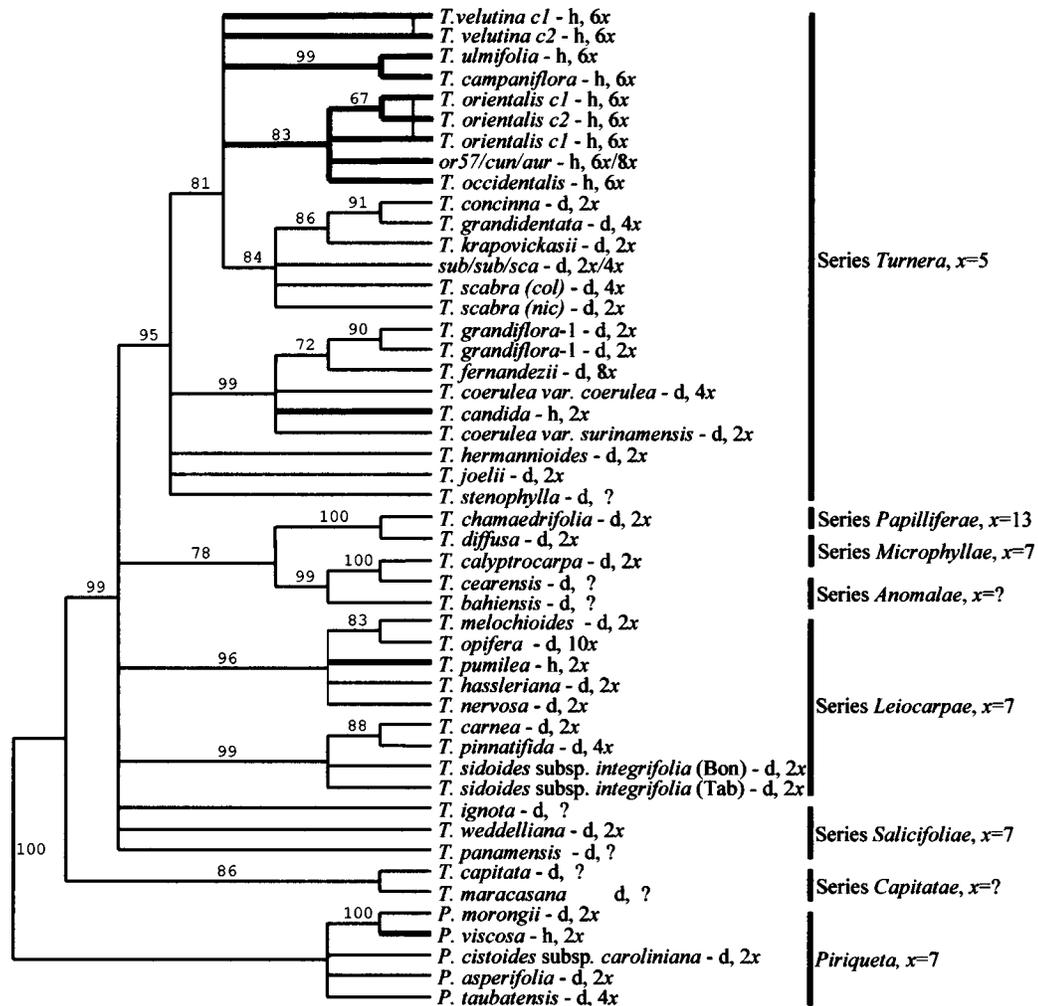
Fig. 3.   Majority rule consensus tree based upon 1000 bootstrap samples from a maximum parsimony analysis. The percentage bootstrap support is indicated on branches of the tree. In two instances, identical sequences were obtained and these are indicated at single terminal nodes (sub/sub/sca = *T. subulata* from Arco Verde/*T. subulata* from Recife/*T. scabra* from the Domincan Republic; or57/cun/aur = *T. orientalis* from Paso de la Patria, *T. cuneiformis*/*T. aurelii*). The series to which the species belong is shown to the right. The letters c1–c3 indicate particular clones of putative ITS homeologues that were sequenced for two allopolyploid species. Thin vertical lines connecting clones indicate that those clones were obtained from the same individual. Breeding system (d = distylous; h = homostylous), ploidy level, and base chromosome number are indicated. We mapped breeding system evolution on the tree using parsimony, assuming distyly to be ancestral and treating shifts from distyly to homostyly (and vice versa) as unordered. Heavy lines indicate the evolution of homostyly.

their genomes have been given different designations based upon karyotypic differences (Fernández and Arbo, 1989, 1990, 1993a, b, 1996). The white/blue-flowered species have the "C" genome, while the yellow-flowered species possess variants of the "A" genome.

Solís Neffa (1996) examined karyotypes of a number of species of series *Turnera* and suggested that *T. joelii* and *T. hermannioides* were likely more closely related to the yellow-flowered distylous species than the white/blue-flowered taxa. An examination of meiosis in hybrids led Fernández (1997) to assign the unique designation, "D," to the genome of *T. joelii*. At present, we do not have sufficient sequence information to clarify their relationships to either the white/blue or yellow-flowered clades (Fig. 3). *Turnera stenophylla* Urb. has not been well studied. It appears to share a 6-bp deletion in ITS1 with *T. hermanioides*, perhaps indicating their close affinity. Arbo (2005) has indicated that morphological and geographic distribution data provide strong support for the view

that *T. joelii*, *T. hermannioides*, *T. stenophylla*, and five additional species we have not been able to sample here, represent a monophyletic group warranting recognition as subseries *Umbilicatae* within series *Turnera*.

We sampled eight of 49 taxa within the *Leiocarpae*, which possesses the greatest number of species of any of the series. Five species (*T. melochoides*, *T. opifera*, *T. pumilea*, *T. nervosa*, and *T. hassleriana*) fall within a well-supported clade (bootstrap support of 96%; Fig. 3). The subspecies of *Turnera sidoides* investigated (*T. sidoides* subsp. *carnea*, subsp. *integrifolia*, and subsp. *pinnatifida*) fall into a separate clade that is well supported (bootstrap support of 99%; Fig. 3). Both of these clades, however, fall into a polytomy comprised of species from a number of series, and thus we can neither refute nor support monophyly of the *Leiocarpae*. Arbo (1985) indicated that the *T. sidoides* complex possesses some unusual morphological features, including seed morphology and anatomy, distinguishing it from other *Leiocarpae*. Likewise, kar-

yotype analyses (Solís Neffa and Fernández, 2002) also supported the view that the *T. sidoides* complex should perhaps be removed from the *Leiocarpae*.

Three of the 12 taxa of series *Salicifoliae* were investigated and they do not form a clade but fall within a polytomy comprised of species from various series (Fig. 3). The *Salicifoliae* warrant further study.

Turnera capitata and *T. maracasana* were the only species available representing the 11 known taxa of series *Capitatae* (Fig. 3). They form a clade which is sister to all other *Turnera* species sampled. Interestingly, the *Capitatae* may share some aspects of floral morphology possessed by *Piriqueta* species. For example, they possess ligulate petals, which could be the remnants of the corona present in *Piriqueta*. Another possibility, however, is that ligulate petals are a retained primitive condition. Both the African genus *Tricliceras*, and the only American species of *Erblichia*, also have ligulate petals.

We sampled one of the two species of series *Papilliferae* (*T. chamaedrifolia*). Interestingly, *T. chamaedrifolia* (series *Papilliferae*, $x = 13$) is sister to *T. diffusa* (series *Microphyllae*, $x = 7$), and there is strong support for this relationship (bootstrap support of 99%; Fig. 3). *Turnera calyptrocarpa* (series *Microphyllae*), on the other hand, is sister to *T. cearensis* (series *Anomalae*), and not *T. diffusa* (*T. calyptrocarpa* and *T. cearensis* sequences differ by a single base pair substitution and one indel). Based upon the taxa sampled here, neither the *Microphyllae* nor *Anomalae* are monophyletic, and both belong to a clade (bootstrap support of 78%; Fig. 3) that includes *T. chamaedrifolia* (series *Papilliferae*, $x = 13$). Future taxonomic revisions may require new circumscriptions of all three series.

We have carried out a host of additional phylogenetic analyses of the data presented here and a subset of them (Truyens, 2003). The results of those phylogenetic analyses, including various character weighting schemes in parsimony analyses, as well as Bayesian and maximum likelihood methods, are not substantively different from the results presented here (Fig. 3).

Evolution of base chromosome number—Three different base chromosome numbers have been reported in *Turnera* ($x = 5$, $x = 7$, $x = 13$). Fernández (1987) and Solís Neffa and Fernández (2000) have suggested that $x = 7$ is the ancestral base number from which $x = 5$ and $x = 13$ were derived. Of the seven series included in this study, species of three series (*Leiocarpae*, *Microphyllae*, and *Salicifoliae*) possess the apparently ancestral base chromosome number of $x = 7$, which they share with the *Piriqueta* species used as the outgroup. Series *Turnera* possesses an apparently derived base chromosome number of $x = 5$ consistent with monophyly of the series. Series *Papilliferae*, which consists of only two species, is the only known series for which a chromosome count of $x = 13$ has been reported (*T. chamaedrifolia*). The fact that *T. chamaedrifolia* (series *Papilliferae*, $x = 13$) is sister to *T. diffusa* (series *Microphyllae*, $x = 7$) suggests that the derived base chromosome number of $x = 13$ likely arose from a species with the ancestral chromosome number of $x = 7$, following polyploidy and subsequent aneuploid reduction (Solís Neffa and Fernández, 2000).

Polyploid evolution—We attempted to determine the progenitors of allopolyploid taxa by identifying, cloning, and sequencing putative homeologous copies of ITS. Such an approach has been used with success in various taxa (e.g., Soltis and Soltis, 1991; Jiang and Gill, 1994; Kim and Jansen, 1994; Dubcovsky and Dvorák, 1995; Sang et al., 1995; Waters and Schall, 1996; Campbell et al., 1997; Hughes et al., 2002; Rauscher et al., 2004), but has proven unsuccessful in others, presumably a result of concerted evolution (Hamby and Zimmer, 1992; Wendel et al., 1995; Brochmann et al., 1998). Wendel (2000) has proposed that whether the use of ITS will be successful in analyses of polyploids may be a function of the chromosomal location of the ribosomal DNA.

In cotton and soybean, homeologous ITS sequences may be partially or completely converted to one or other of the contributing genomes (Wendel et al., 1995; Rauscher et al., 2004). Kovarik et al. (2005) have demonstrated bidirectional concerted evolution within allotetraploid *Tragopon mirus* and that the homogenization of rDNA (while incomplete) has proceeded rapidly, roughly within 30 generations.

We were able to identify different putative homeologues of ITS for *T. velutina* and in one collection of *T. orientalis*. While the two *T. velutina* sequences differed from one another by 11 substitutions, we were unable to clearly identify different progenitors that might have contributed to the allopolyploid origin of that species. The *T. orientalis* sequences differed to a lesser extent, and no clear progenitors could be identified.

Interestingly, we detected only a single ITS homeologue in one of the two collections of *T. orientalis*. Rauscher et al. (2004) have shown significant biases in copy number of ITS homeologues among races of *Glycine tomentella*, and Kovarik et al. (2005) have demonstrated such biases among and within populations of *T. mirus*. Thus we may have been unable to detect minority ITS sequences in this collection of *T. orientalis* and in the other allopolyploids. Alternatively, concerted evolution of ITS likely has been responsible for a conversion of sequences within the allopolyploids (including *T. orientalis*, *T. ulmifolia*, *T. campaniflora*, *T. occidentalis*, *T. cuneiformis*, *T. aurelii*).

Identical ITS sequences were obtained for three polyploid species, including one collection of *T. orientalis* (or 57, $6x = 30$), *T. aurelii* ($8x = 40$), and *T. cuneiformis* ($8x = 40$). Fernández and Arbo (2000a, b) examined meiosis in hybrids of these three species and concluded that both octaploids share three genomes in common with hexaploid *T. orientalis*. This suggests that *T. orientalis* contributed three genomes to the octaploids and that two other species were likely involved in independent hybridizations yielding two new octaploid species (*T. aurelii* and *T. cuneiformis*). Our data support this hypothesis. We were, however, unable to discover ITS sequences of the putative 4th genome contributor to the octaploids. This could again be due to concerted evolution of ITS sequences or possibly due to the fact that the ITS from the 4th genome would be present in fewer copies in total DNA extracts and therefore was not detected in our SSCP analysis.

Our analysis suggests that polyploid species have evolved independently in *Turnera* at least six times (Fig. 3). We cannot, however, rule out a single common origin of hexaploidy in series *Turnera* (and subsequent genetic divergence giving rise to a number of hexaploid species). Interestingly, some taxa (e.g., *T. scabra*, *T. subulata*, *T. krapovickasii*, *T. coerulea*, and *T. sidoides*) are composed of diploid and polyploid populations (Arbo and Fernández, 1987; Fernández, 1987; Shore, 1991a; Solís Neffa and Fernández, 2002). A newly described autooctaploid species (*T. fernandezii*) from Paraguay, with close affinities to diploid *Turnera grandiflora* (Fig. 3), is a remarkable example of an autooctaploid species, in the appar-

ent absence of intervening ploidy levels (Fernández, 1987; Fernández and Arbo, 1993a, 1996; Arbo, 2005).

Breeding system evolution—The evolution of breeding systems has been successfully studied using molecular phylogenetics (Weller and Sakai, 1999). In particular, an increasing number of families possessing heterostylous species are beginning to be investigated (e.g., Kohn et al., 1996; Schoen et al., 1997; Graham et al., 1998; Conti et al., 2000; Mast et al., 2001; Church, 2003; Peréz et al., 2003; Graham and Barrett, 2004). Schoen et al. (1997) explored various mappings of breeding system evolution onto a molecular phylogeny and detailed arguments supporting the proposition that distyly is ancestral in *Amsinckia*. Similar arguments were made for the evolution of breeding systems in the Pontederiaceae (Kohn et al., 1996). One argument supporting the view that homostyly is commonly derived (or that transitions from homostyly to distyly are less likely), arises from evolutionary models indicating that distyly likely evolves via a multi-step process while homostyly may arise via a single genetic change (Charlesworth and Charlesworth, 1979a, b; Lloyd and Webb, 1992). Indeed, Tamari et al. (2005) have recently described the occurrence and inheritance of two mutant homostyles in *Turnera*.

In perhaps the clearest analysis to date, Graham and Barrett (2004) showed that in *Narcissus* (Amaryllidaceae), style length dimorphism arose independently from a monomorphic ancestral condition on at least four occasions. Distyly subsequently arose on a single occasion in *N. albimarginatus*, from an ancestor possessing style length dimorphism. This evolutionary analysis of *Narcissus* is in concert with models for the evolution of heterostyly proposed by Lloyd and Webb (1992).

Associations between homostyly and polyploidy occur in some lineages of *Amsinckia* (Schoen et al., 1997), *Primula* (Conti et al., 2000), *Damnacanthus* (Rubiaceae; Naiki and Nagamasu, 2004), and *Turnera*, supporting the derived nature of homostyly in those cases. For the present data set, we have assumed distyly to be ancestral in *Turnera*. If we are able to obtain a more fully resolved phylogeny taking into account reticulate evolution of the allopolyploids, a more rigorous approach to mapping breeding system evolution can be applied following Huelsenbeck et al. (2003). Under our current assumptions, the phylogeny supports the view that homostyly has arisen at least three times in *Turnera* (Fig. 3).

The genetics and compatibility behavior of distylous and homostylous species have been investigated for a number of species in the yellow-flowered clade of series *Turnera*. The allohexaploid homostyles appear to have arisen via recombination within a gene complex determining distyly (Shore and Barrett, 1985b; Barrett and Shore, 1987; Belaoussoff and Shore, 1995; Tamari et al., 2001, 2005; Athanasiou et al., 2003). The phylogenetic reconstruction using ITS (Fig. 3) provides evidence for at least two independent evolutionary events yielding homostyly in this series with one event occurring in the yellow-flowered allopolyploids and the second in the white/blue-flowered clade. *Turnera candida* is a self-compatible diploid species in the latter clade. There is variation in the relative length of styles and stamens among collections of this species with the style either somewhat exserted or equal in length to the stamens (Arbo, 1993). The only cross reported for this species was between distylous *T. grandiflora* and *T. candida*. A resulting hybrid was long-styled, but no information on compatibility relationships was obtained (Fernández and Arbo, 1996). It is not clear whether *T. candida*

has had an origin comparable with the putative recombinant origins of homostyly observed among the yellow-flowered polyploid species (Barrett and Shore, 1987; Tamari et al., 2001).

Within the yellow-flowered species of series *Turnera*, we do not have evidence that there has been more than a single origin of homostyly given the occurrence of all the homostylous polyploids in a polytomy (Fig. 3). Barrett and Shore (1987) suggested that *T. orientalis*, *T. velutina*, and *T. ulmifolia*, might have had independent origins as they exhibit F_1 hybrid sterility. We cannot, however, conclude with the present analysis that this is so. Fernández and Arbo (2000b) explored meiosis in a *T. velutina* × *T. orientalis* hybrid and suggested that these species share one similar genome. Thus, there might have been a single origin of a homostylous ancestor with subsequent divergence over time. Possibly, this ancestral homostyle might have been at a lower ploidy level ($2x$ or $4x$) and that following further hybridizations, yielded the different homostylous hexaploid species.

Outside of series *Turnera*, there has been the origin of homostyly in *T. pumilea*. No crosses with distylous species have been undertaken to explore compatibility relationships or the inheritance of homostyly. *Turnera chamaedrifolia* and three subspecies of *T. sidoides* occur both as distylous and homostylous populations (Arbo, 1985, 2000). Likewise, for the outgroup *Piriqueta* species, *P. viscosa* is homostylous and *P. morongii* is composed of both distylous and homostylous populations (Arbo, 1995; M. Arbo and J. Shore, personal observations). *Piriqueta viscosa* is a small-flowered homostyle sister to *P. morongii*. Data on the inheritance of homostyly and compatibility relationships have not been obtained for these species but warrant examination.

Conclusions—Our results provide information that might aid in the classification of *Turnera*. While our sampling of species is incomplete, our data support monophyly of series *Turnera* (species of which share an apparent derived base chromosome number $x = 5$). Two series do not appear to be monophyletic (series *Anomalae* and *Microphyllae*) and two others (series *Leiocarpae* and *Salicifoliae*) were not fully resolved. Clearly any such conclusions require analysis of additional species and the use of sequences from additional genes to resolve polytomies. The use of additional nuclear genes that do not undergo concerted evolution will be important in determining the origins of allopolyploid species. Our current analyses do provide support for multiple origins of polyploidy and homostyly. A better resolution of the phylogeny and origins of the polyploids in the future will aid in obtaining a better understanding of the breeding system evolution and its possible association with polyploidy.

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APPENDIX. Taxa, chromosome numbers, breeding system, localities, collection number, and GenBank accession numbers for ITS sequences analyzed.

Taxon; Chromosome number; Breeding system; Locality; *Source*, collection number; ITS.

Series *Turnera* (=*Canaligerae* Urb.)

Turnera aurelii Arbo; $8x = 40$*; homostyly; Presidente Hayes, Paraguay; *Krapovickas et al. 45524*; same as AY973364.

Turnera campaniflora Arbo, Shore & Barrett; $6x = 30$; homostyly; Quaco Rock, Jamaica; *Barrett 1337*; AY973367.

Turnera candida Arbo; $2n = 10$*; homostyly; Rio de Contas, Bahia, Brazil; *Jardim et al. 2588*; AY973349.

Turnera coerulea **var. *coerulea*** DC.; $4x = 20$; distyly; Tomina, Bolivia; *Wood 7989*; AY973348.

Turnera coerulea **var. *surinamensis*** (Urb.) Arbo & Fernández; $2n = 10$; distyly; Santarem, Brazil; *Barrett 1129*; AY973347.

Turnera concinna Arbo; $2n = 10$*; distyly; San Pedro, Paraguay; Arbo, Shore, *Schinini 8900*; AY973353.

Turnera cuneiformis Poir.; $8x = 40$; homostyly; Pirapora, Brazil; *Krapovickas & Cristóbal 42897*; same as AY973364.

Turnera fernandezii Arbo; $8x = 40$; distyly; Bella Vista Norte, Amambay, Paraguay; *Zardini 53286*; AY973352.

Turnera grandidentata (Urb.) Arbo; $4x = 20$; distyly; Paraguarí, Paraguay; Arbo et al. 6109; AY973354.

Turnera grandiflora (Urb.) Arbo-1; $2n = 10$; distyly; Corrientes, Argentina; *Schinini et al. 19260*; AY973350.

Turnera grandiflora (Urb.) Arbo-2; $2n = 10$*; distyly; under cultivation at University of Toronto, Canada; *unknown*; AY973351.

Turnera hermannioides Cambess.; $2n = 10$; distyly; Brumado, Bahia, Brazil; *Arbo et al. 5680*; AY973369.

Turnera krapovickasii Arbo; $2n = 10$; distyly; Salta, Argentina; *Krapovickas & Cristóbal 46355*; AY973355.

Turnera joelii Arbo; $2n = 10$; distyly; Juremal, Brazil; *Barrett and Shore 1373*; AY973368.

Turnera occidentalis Arbo & Shore; $6x = 30$; homostyly; Peru; *Sagastegui 14896*; AY973365.

Turnera orientalis 13 (Urb.) Arbo; $6x = 30$; homostyly; Corrientes, Argentina; *Arbo 1538*; AY973361, AY973362, AY973363.

Turnera orientalis 57 (Urb.) Arbo; $6x = 30$; homostyly; Paso de la Patria, Argentina; *Shore 312*; AY973364.

Turnera scabra Millspaugh—Man; $2n = 10$; distyly; Managua, Nicaragua; *Shore 308*; AY973358.

Turnera scabra Millspaugh—Dr4; $4x = 20$; distyly; San FCO de Macoris, Domincan Republic; *Shore 303*; same as AY973356.

Turnera scabra Millspaugh—Col; $4x = 20$; distyly; Dagua, Colombia; *Barrett 689*; AY973357.

Turnera stenophylla Urb.; —; distyly; Santo Inácio, Bahia, Brazil; *Nunes et al. 979*; AY973370.

Turnera subulata Smith—Bry; $2n = 10$; distyly; Arco Verde, Brazil; *Barrett and Shore 1374*; AY973356.

Turnera subulata Smith—Rec; $4x = 20$; distyly; Recife, Brazil; *Barrett and Shore 1380*; same as AY973356.

Turnera ulmifolia L.; $6x = 30$; homostyly; Falmouth, Jamaica; *Shore and Schappert 107*; AY973366.

Turnera velutina Presl.; $6x = 30$; homostyly; in cultivation, University of Missouri, Columbia MO; *Shore 309*; AY973359, AY973360.

Series *Microphyllae* Urb.

Turnera calyptrocarpa[D] Urb.; $2n = 14*$; distyly; Bahia, Brazil; *Queiroz & Crepaldi 1479*; AY973376.

Turnera diffusa Willd.; $2n = 14$; distyly; Otto Richter and Sons Ltd., Goodwood, Ont., Canada; *Shore 310*; AY973381.

Series *Anomalae* Urb.

Turnera bahiensis Urb.; —; distyly; Lençois, Bahia, Brazil; *Funch 57*; AY973378.

Turnera cearensis[S] Urb.; —; distyly; Bahia, Brazil; *Harley et al. 53491*; AY973377.

Series *Capitatae* Urb.

Turnera capitata[D] Cambess.; —; distyly; São Paulo, Brazil; *Cordeiro 2750*; AY973379.

Turnera maracasana Arbo; —; distyly; Maracás, Bahia, Brazil; *Leite et al. 224*; AY973380.

Series *Salicifoliae* Urb.

Turnera ignota[S] Arbo; —; distyly; Brazil; *Lombardi 4111*; AY973387.

Turnera panamensis Urb.; —; distyly; Gamboa, Panama; *Shore 320*; AY973389.

Turnera weddelliana Urb. & Rolfe; $2n = 14*$; distyly; Paraguarí, Paraguay; *Arbo, Shore, Schinini 8845*; AY973388.

Series *Leiocarpae* Urb.

Turnera hassleriana Urb.; $2n = 14*$; distyly; San Pedro, Paraguay; *Arbo, Shore, Schinini 8897*; AY973374.

Turnera melochioides Cambess.; $2n = 14*$; distyly; Estado do Pará, Município de Marapanim, Praia. Brazil; *Bovini 1734*; AY973371.

Turnera nervosa Urb; $2n = 14$; distyly; Corrientes, Argentina; *Arbo 2076*; AY973373.

Turnera opifera[D] Mart.; $10x = 70*$; distyly; Diamantina, Minas Gerais, Brazil; *Lombardi 4325*; AY973372.

Turnera pumilea L.; $2n = 14$; homostyly; Piaui, Brazil; *Krapovickas 38624*; AY973375.

Turnera sidoides[D] L. subsp. *carnea* (Cambess.) Arbo; $2n = 14$; distyly; Mercedes, Corrientes, Argentina; *Schinini 21711*; AY973377.

Turnera sidoides[D] L. subsp. *integrifolia* (Griseb.) Arbo; $4x = 28*$; distyly; Bonpland, Corrientes, Argentina; *Solís Neffa s.n.*; AY973385.

Turnera sidoides[D] L. subsp. *integrifolia* (Griseb.) Arbo; $2n = 14$; distyly; Tabay, Corrientes, Argentina; *Solís Neffa, Seijo 974*; AY973386.

Turnera sidoides[D] L. subsp. *pinnatifida* (Juss. ex Poir.) Arbo; $4x = 28$; distyly; Colonia Benítez, Chaco, Argentina; *Solís Neffa 306*; AY973383.

Series *Papilliferae* Urb.

Turnera chamaedrifolia Cambess.; $2n = 26$; distyly & homostyly; Corrientes, Argentina; *Noblick 3175 bis*; AY973382.

Piriqueta

Piriqueta asperifolia[D] Arbo; —; distyly; Bahia, Brazil; Franca et al. 3135; AY973394.

Piriqueta cistoides subsp. *caroliniana* (Walter) Arbo; $2n = 14$; distyly; Crawfordville, Florida, USA; *Shore 311*; AY973391.

Piriqueta morongii Rolfe; $2n = 14$; distyly & homostyly; Corrientes, Argentina; *Krapovickas 45204*; AY973393.

Piriqueta taubatensis[D] (Urb.) Arbo; $4x = 28$; distyly; Rivera, Uruguay; *Solis Neffa et al. 482*; AY973390.

Piriqueta viscosa Griseb.; $2n = 14*$; homostyly; Ilhéus, Bahia, Brazil; *Groppo et al. 1050*; AY973392.

[D] DNA extracted from dried leaves

[S] DNA extracted from seeds

* Chromosome count not from this accession