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ORIGINAL ARTICLE

Natural hybridization among subspecies of *Turnera sidoides* L. (Passifloraceae) revealed by morphological and genetic evidence

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Abstract *Turnera sidoides* is a complex of outcrossing, perennial, rhizomatous herbs that is widely distributed in southern South America. Five subspecies are recognized taxonomically based on morphological features and geographical distribution. In certain regions, the areas of distribution of the subspecies overlap partially. In such contact zones, the extent of reproductive barriers among subspecies is still largely unknown, but morphologically intermediate individuals have been found in the field, indicating that hybridization may actually occur between subspecies. Crossability among subspecies of *T. sidoides* has been shown by experimental studies with cultivated plants, but the mechanisms involved in natural populations are still unknown. To investigate the mechanisms that underlie gene flow within the *T. sidoides* complex, in this paper we analyze the morphological and genetic variation, as well as the crossability among taxa in a contact zone between subspecies *pinnatifida* and *sidoides*, in southeastern Uruguay. Our results constitute the first evidences of ongoing natural hybridization between subspecies of *T. sidoides* and suggest that, although hybridization may not have been of significance in the early phase of the species differentiation, reticulate evolution is ongoing enhancing the current morphological and genetic variability of the complex.

Keywords *Turnera sidoides* · Hybridization · Gene flow · RAPD

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Introduction

Natural hybridization has played an important role in plant evolution (Stebbins 1959; Harrison 1990; Rieseberg and Wendel 1993; Arnold 1997; Rieseberg 1997). This is particularly true for Angiosperms, where hybridization seems to have influenced the evolutionary history of 30–80 % of extant taxa (Grant 1981; Rieseberg and Ellstrand 1993). It has long been recognized that one consequence of hybridization is the melding of two previously isolated taxa (Stebbins 1959; Grant 1981). Different views have considered hybridization one of the processes that may lead to new species or ecotypes as well as to an increase of genetic diversity and either reinforcement or breakdown of reproductive barriers among closely related groups (Rieseberg and Wendel 1993; Arnold 1997; Rieseberg 1997; Rieseberg and Carney 1998). In particular, if reproductive barriers are weak, extensive introgression may contribute to increased genetic variation in populations and to the spread of novel adaptations (Stebbins 1950; Barton and Hewitt 1985; Grant and Grant 1996; Arnold 1997).

Contact zones among closely related taxa provide a natural setting to investigate evolutionary processes, such as adaptation, speciation, hybridization, and introgression (Arnold 1997; Avise 2004). Understanding the genetic structure and introgression mechanisms within hybrid

zones is critical to predict the potential evolutionary outcomes of natural hybridization (McCauley 1995; Ouborg et al. 1999; Avise 2004).

The *Turnera sidoides* L. complex (Passifloraceae) represents an ideal system to analyze natural hybridization and its evolutionary significance. *Turnera sidoides* is an autopolyploid complex of heterostylous obligate outcrossing, perennial, rhizomatous herbs that is widely distributed from the southern regions of Bolivia and Brazil, through Paraguay to Uruguay and Argentina, reaching 39 °S (Arbo 1985; Solís Neffa 2000). Five subspecies are recognized taxonomically based on morphological features and geographical distribution (Arbo 1985). The subspecies possess different ploidy levels based on $x = 7$, ranging from diploid ($2n = 2x = 14$) to octoploid ($2n = 8x = 56$) (Fernández 1987; Solís Neffa and Fernández 2001; Solís Neffa et al. 2004; Elías et al. 2011; Kovalsky and Solís Neffa 2012).

At a geographical scale, the subspecies of *T. sidoides* mostly occur at contiguous areas; however, at certain regions of the species range, the subspecies partially overlap (Solís Neffa 2000). In contact zones, the extent of reproductive barriers among subspecies is still largely unknown, but individuals with intermediate morphology have been found in the field, indicating that hybridization may actually occur between subspecies (Solís Neffa 2000). Moreover, analysis of cpDNA variation in *T. sidoides*, based on the sequence of the *trn*L—*trn*F spacer, identified three haplotypes, the distribution of which was strongly structured geographically irrespective of subspecific or ploidy boundaries. Based on this, it has been suggested that introgression of cytoplasmic haplotypes must occur at contact zones (Speranza et al. 2007). Crossability among subspecies of the *T. sidoides* complex has been shown by experimental studies with cultivated plants (Solís Neffa 2000; Solís Neffa et al. 2008), but the mechanisms involved in natural populations are still unknown.

To investigate the mechanisms that underlie gene flow within the *T. sidoides* complex, in this paper we analyze the morphological and genetic variation in a recently identified contact zone in southeastern Uruguay, between *T. sidoides* subsp. *pinnatifida* (Juss. ex Poir.) Arbo and *T. sidoides* subsp. *sidoides* (herein referred to as subspecies *pinnatifida* and *sidoides*). This contact zone is particularly suitable for the study of gene flow across subspecific and ploidy boundaries because of three main reasons. First, the two subspecies are morphologically distinguishable; second, in this location, the two subspecies show different ploidy levels; and third, and most interesting, the area is precisely located on the macro-geographical boundary between two of the cpDNA haplotypes of the complex and the two subspecies have different cpDNA haplotypes at this site (Speranza et al. 2007).

Materials and methods

Study site

Fieldwork was conducted in Aguas Blancas (34°30′46″S and 55°21′15″W) in the Lavalleja Department, Uruguay (Fig. 1a).

Sampling

Route 81 was used as a base line for the sampling of *T. sidoides* individuals (Fig. 1b). A total of 112 individuals were randomly sampled on both roadsides along a transect of 10 km. Voucher specimens (Solís Neffa and Speranza 2000, 2001 and 2002) have been deposited in the

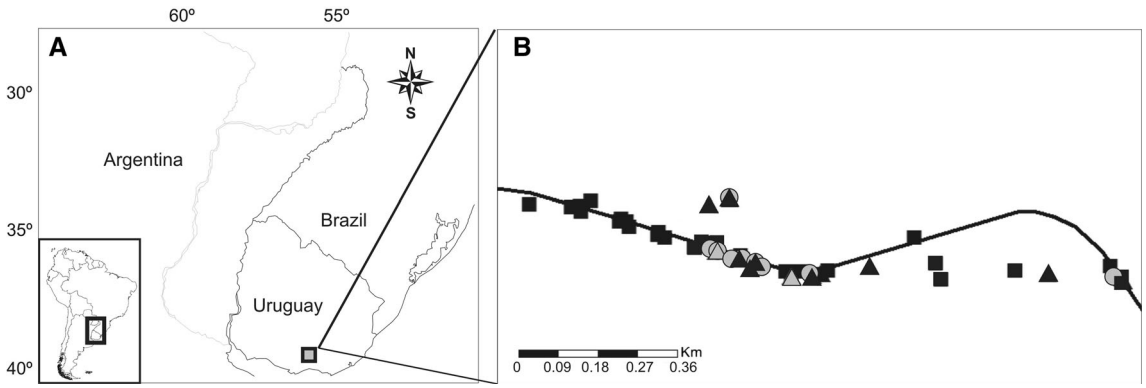

Fig. 1 Study area. **a** Map showing the location of the Aguas Blancas contact zone. **b** Spatial distribution of the individuals of *Turnera sidoides* subspecies and putative hybrids in the contact zone. *Circles represent* individuals assigned as *T. sidoides* subsp. *pinnatifida*, *squares* represent those from *T. sidoides* subsp. *sidoides*, and *triangles* represent putative hybrids between *T. sidoides* subsp. *pinnatifida* and *T. sidoides* subsp. *sidoides*. *Colors* indicate different cpDNA haplotypes: *gray symbols* represent haplotype B and *black symbols* represent haplotype C

Herbarium of the Instituto de Botánica del Nordeste (CTES). Selected representative plants were transported to Corrientes (Argentina), where they were grown in a greenhouse. The location of each individual in the field was mapped from a database of the geo-referenced collections (Supplementary material S1) using the software gvSIG Desktop 1.11 (www.gvsig.com).

Morphological analyses

Taxonomic determinations were based on the treatment of Arbo (1985), and specimens deviating from the typical subspecies morphological circumscription were marked as putative hybrids. A set of 13 morphological characters was scored for each individual. Characters were selected based on the traits used by Arbo (1985) to diagnose subspecies. Leaf quantitative traits (area, total length, average width, and maximum width) were measured in the three largest leaves of each specimen using a portable leaf area measurer Li-3000 Li-COR. Other leaf traits studied included shape (entire, pinnatifid and pinnatisected) and indumentum type. The indumentum was analyzed using a Jeol 5800 LV scanning electron microscope (SEM) at an acceleration voltage of 20 kV. Sampled leaves from herbarium specimens were dried at critical point (Denton Vacuum DCP-1) and sputter-coated with gold–palladium (Denton Vacuum sputter coater). For each individual, we recorded the color of flowers (light pink, pink, and dark pink), anthers (yellow or green), filaments (yellow or redish), style (yellow or redish), and stigma (yellow or redish). We also recorded the presence or absence of a basal macula on the petals and the number of seeds per fruit.

For statistical analyses of morphological traits, we used the software Infostat (2012). Qualitative and quantitative characters were analyzed together with a Principal Coordinates Analysis (PCoA) and using Gower's similarity coefficient. Significance of differences for each trait among the three entities was evaluated with a one-way ANOVA at a significance level of 5 % ($\alpha = 0.05$) after Bartlett's test of homogeneity. Significance of differences between each pair of means was evaluated with the Tukey's test 5 %.

Cytogenetic analyses

Ploidy level was inferred from chromosome counts in meiosis and from estimates of the relative DNA content using flow cytometry. Meiotic chromosomes of some plants of both subspecies were examined in pollen mother cells of young buds after fixation in 5:1 absolute ethanol: lactic acid (Fernández 1973) for 12 h at 4 °C and storage in 70 % ethanol at 4 °C. Pollen mother cells were extracted in a drop of 2 % aceto-orcein by cutting the tip of pollen sacs, and slightly squashed. Slides were made permanent in Euparal using Bowen's (1956) method. For flow cytometry analyses, we used the Partec kit CySatin UV Precise P (05-5002). Briefly, 0.5 cm^2 of leaf material was placed in a Petri dish with a comparable amount of tissue from an internal standard. After adding 0.5 ml of extraction buffer, the tissue was chopped with a razor blade. Following a 2-min incubation, samples were filtered through a 50-µm nylon mesh into the sample tube with 1.5 mL of DAPI (4′,6-diamidino-2-phenylindole) staining solution. The mixture was incubated for 2 min at room temperature and analyzed. The fluorescence intensity of DAPI-stained nuclei was determined using a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) with the detector operating at 355 nm. About 3,000 nuclei were measured per sample. Ploidy levels were estimated by comparing the DNA peak of the samples to the internal standard. Data analysis was performed using PA II's Partec FloMax software.

Pollen viability was estimated based on the ability of carmine–glycerin 1:1 to stain pollen. At least 300 grains per flower were scored.

Molecular analyses

Total DNA was extracted from silica-gel dried leaves using a modified hexadecylmethylammonium bromide (CTAB) protocol taken from Doyle and Doyle (1987) and Cullings (1992).

PCR–RFLP analyses of cpDNA

Polymerase chain reaction (PCR) was carried out in a 25 µL final volume containing 4 ng/µL of DNA, 0.8 µM of primers for the cpDNA intergenic spacer $trnL^{UAA}$–$trnF^{GAA}$ (Taberlet et al. 1991), 100 µM of each dNTPs (Amersham Biosciences, Buckinghamshire, UK), and 1 U of Taq polymerase (Amersham Biosciences, Uppsala, Sweden) in 1× reaction buffer.

Amplification was performed in an Eppendorf Mastercycler Gradient thermocycler, according to the parameters for the region proposed in Shaw et al. (2004). The program consisted of an initial cycle of 5 min at 95 °C, followed by one cycle of 1 min at 94 °C, 1 min at 58 °C, and 2 min 30 s at 72 °C. The annealing temperature was then decreased by 1 °C for 6 cycles and the following 32 cycles were carried out with an annealing temperature of 52 °C, followed by a final extension step of 5 min at 72 °C.

Amplification was confirmed by electrophoresis in 1.4 % agarose gels (Bio-Rad Laboratories, Spain) in 1× TAE buffer, stained with ethidium bromide (10 mg/mL), and photographed under UV light in a Bio-Rad Gel Doc™ XR. Product concentration was estimated visually by comparison with known standards (0.1–10.0 kb ladder,

BioLabs, New England). Aliquots containing approximately 100 ng of the amplified products were digested with 2 U of Mse I (Invitrogen) for 3 h at 37 °C. Restriction fragments were resolved by electrophoresis in 2 % agarose gels (Bio-Rad Laboratories, Spain) at 6 V/cm for 120 min in 1× TAE buffer. After electrophoresis, gels were stained with ethidium bromide (10 mg/mL), rinsed for 1 h in deionized water, and photographed under UV light.

RAPD analyses

A total of ten RAPD primers (kit OP: OPO2, OPO3, OPO4, OPO5, OPO7, OPO8, OPO9, OPO13, OPO15, OPO18) supplied by Operon Technologies (Alameda, CA, USA) were screened. The PCR reactions were carried out in a final volume of 12 μL containing approximately 8.33 ng of DNA, 0.2 μM of primer, 100 μM of each dNTP (Amersham Biosciences, Buckinghamshire, UK), and 1 U of Taq polymerase (Amersham Biosciences, Uppsala, Sweden) in 1× reaction buffer. Amplification was performed in an Eppendorf Mastercycler Gradient thermocycler programmed with one cycle of 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 1 min at 37 °C, 2 min at 72 °C, and a final extension step of 5 min at 72 °C. Amplified products were resolved by electrophoresis in 2 % agarose gels (Bio-Rad Laboratories, Spain) at 2.3 V/cm for 160 min in 1× TAE buffer. After electrophoresis, gels were stained with ethidium bromide (10 mg/mL), rinsed for 30 min in deionized water, and photographed under UV light. A negative control was included in all reactions.

Amplified products were scored as present (1) or absent (0) to construct a binary matrix. An AMOVA (Excoffier et al. 1992) was performed, with the software GenAlEx 6.3 (Peakall and Smouse 2006), using both parental subspecies and the putative hybrids as groups. Within-group variation was calculated as the unbiased expected heterozygosity (Hj), according to Lynch and Milligan (1994).

Relationships among individuals in the contact zone were analyzed with a PCoA of the genetic distance values using the software GenAlEx 6.3 (Peakall and Smouse 2006). Also, Bayesian methods implemented in the software STRUCTURE version 2.3.1 (Pritchard et al. 2000; Falush et al. 2003) were used to further support the distinctness of the parental gene pools and genetically characterize putative hybrids. We chose the admixture model and the option of correlated allele frequencies between populations, and we let the degree of admixture alpha be inferred from the data. A priory subspecies assignment was taken into account in the analysis. A burn-in of 50,000 steps followed by 100,000 iterations was used to assign individual multilocus genotypes to one of the predefined K populations. Different K values were tested; ranging from 1 to 8, with five independent replicates for each value of K. For the final analysis, we selected the value of K with the highest posterior probability of the data for a given K, Pr ($X|K$) [called 'Ln P(D)' in STRUCTURE output] as reported by STRUCTURE (Pritchard et al. 2000).

Artificial crosses

To verify crossability between the subspecies and between them and the putative hybrids, a controlled crossing program was performed. All crosses consisted of legitimate combinations between long-styled and short-styled plants. Crossings were made, according to Fernández and Arbo (1989), under greenhouse conditions to avoid undesired insect pollination. Open flowers used as females were emasculated prior to pollination with anthers of plants selected as males. The number of crosses for each parental combination varied according to the availability of plants and on the occurrence of simultaneous flowering. Maturing capsules were wrapped in small tulle bags to prevent loss of seeds during dehiscence. Development of the seed capsules lasted approximately 20 days, after which seeds were collected, and the number of viable and unviable seeds was recorded.

Results

Morphological analyses

Most of the 112 sampled individuals in the study area could be unambiguously assigned either to subspecies *pinnatifida* (27.68 %) or to subspecies *sidoides* (50.90 %) based on morphology. However, the remaining 21.42 % showed clear intermediate morphology and were treated as putative hybrids. Individuals of the subspecies *sidoides* were randomly distributed over the studied area; those belonging to subspecies *pinnatifida* were less common and occurred in small patches, while putative hybrids were scarce and tended to appear near the patches of subspecies *pinnatifida* (Fig. 1b).

Individuals of subspecies *pinnatifida* had pinnatisect leaves with a relatively smaller leaf area than those of subspecies *sidoides* which had entire leaves with a larger leaf area. Putative hybrids had pinnatifid leaves and intermediate values of leaf area (Fig. 2a–c; Table 1). The indumentum consisted of simple hairs in subspecies *pinnatifida* and of stellate hairs in all individuals of subspecies *sidoides*; additionally, in some individuals of subspecies *sidoides*, single long hairs of different lengths along the leaf margin or bifid and trifid hairs in individuals 26 and 33, respectively, were observed. The

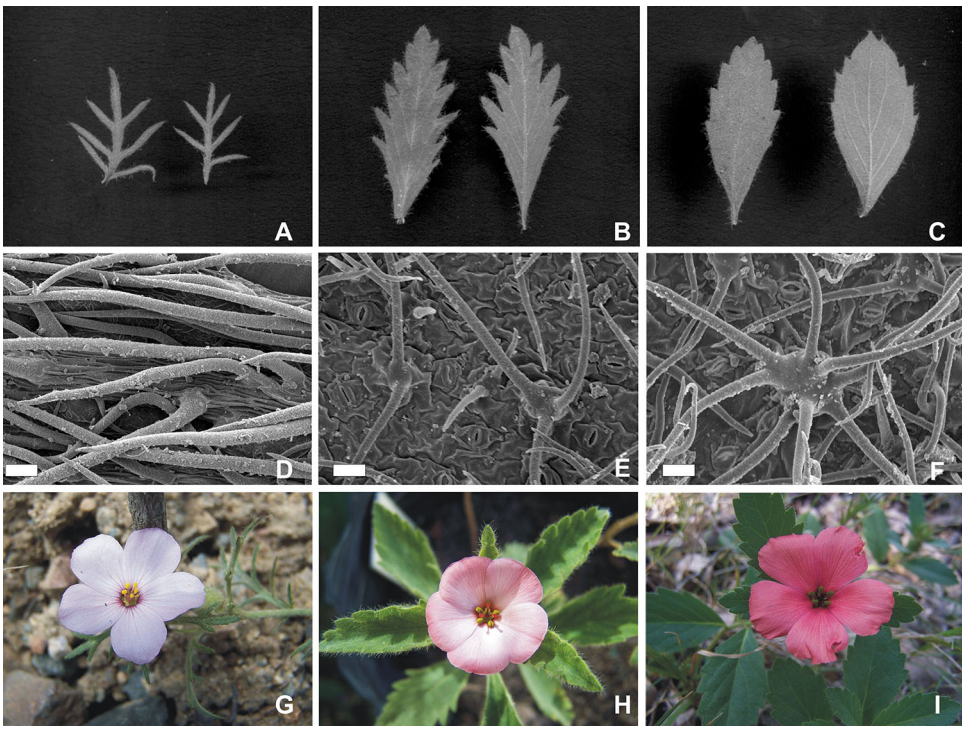

Fig. 2 Morphological variation observed among individuals of *Turnera sidoides* from the studied contact zone. **a**, **d**, **g** *T. sidoides* subsp. *pinnatifida*. **b**, **e**, **h** Putative hybrids. **c**, **f**, **i** *T. sidoides* subsp. *sidoides*. **a–c** Variation in the degree of incision of the leaf shape. **a** Pinnatisected leaf. **b** Pinnatifid. **c** Entire leaf. **d–f** Indumentum. **d** simple hairs. **e** Bifid and trifid hairs. **f** Stellated hairs; **g–i** Flower *color* variation. **g** *light pink* flowers. **h** *Pink* flowers. **i** *Dark pink* flowers. *Bar* 10 μm

Table 1 Leaf traits analyzed in the subspecies and putative hybrids of *Turnera sidoides* from the contact zone

T. sidoides	Area	Average length	Average width	Maximum width	Margin	Indumentum
subsp. *pinnatifida*	$0.65^{a} \pm 0.19$ 0.34–1.07	$2.04^{a} \pm 0.34$ 1.25–2.87	$0.31^{a} \pm 0.07$ 0.20–0.50	$0.67^{a} \pm 0.15$ 0.40–1.00	Pinnatisected	Simple hairs
subsp. *sidoides*	$2.89^{c} \pm 0.86$ 1.13–4.99	$3.29^{b} \pm 0.59$ 2.00–4.50	$0.90^{c} \pm 0.23$ 0.55–1.83	$1.40^{c} \pm 0.23$ 0.80–1.83	Entire	Stellate hairs
Putative hybrids	$2.10^{b} \pm 0.62$ 1.14–2.98	$3.08^{b} \pm 0.32$ 2.43–3.80	$0.67^{b} \pm 0.15$ 0.40–0.90	$1.24^{b} \pm 0.22$ 0.87–1.55	Pinnatifid	Simple, bifid, and/or trifid hairs
F (ANOVA)	109.12^{**}	66.31**	104.04**	118.12**		

$^{**}p < 0.05$

indumentum of putative hybrids consisted of single, bifid, and trifid hairs, and also, sparse stellate hairs in individual 54 and long simple hairs along the leaf margin in some other individuals (Fig. 2d–f). The indumentum density varied both within subspecies and among putative hybrids.

Flowers of the individuals of subspecies *pinnatifida* were light pink with diffuse basal maculae, pink filaments, and yellow anthers, style, and stigma. Flowers of subspecies *sidoides* were dark pink without basal maculae, and with green anthers and reddish filaments, style, and stigma. Flowers of putative hybrids were pink with a diffuse basal spot, green anthers, reddish filaments and style, and yellow stigma (Fig. 2g–i). Individuals of both subspecies produced a mean of 20 seeds per fruit, whereas hybrids produced one or two seeds per fruit (Fig. 2j–l).

A PCoA based on morphological data inferred three resolved and clearly separated groups corresponding to individuals identified as subspecies *pinnatifida*, individuals of subspecies *sidoides*, and the putative hybrids (Fig. 3). The first two coordinates of the PCoA accounted for 80 % (58 and 22 %, respectively) of the variation.

Cytogenetic analyses

Individuals identified morphologically as subspecies *pinnatifida* were all diploid, $2n = 2x = 14$, and those identified as subspecies *sidoides* were tetraploid, $2n = 4x = 28$, while all hybrids were triploid, $2n = 3x = 21$ (Fig. 4). The percentages of pollen viability were high in both subspecies *pinnatifida* (96.79–97.00 %) and subspecies *sidoides* (71.49–94.00 %). In hybrids, pollen viability varied between 0.00 and 14.30 %.

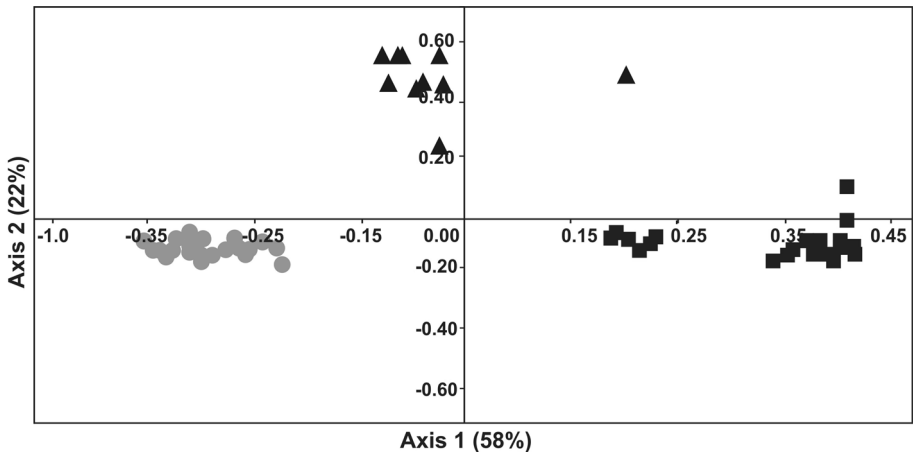

Fig. 3 PCoA ordination plot of the sample points in the plane of the first two principal axes based on morphological data of plants of *Turnera sidoides* from the contact zone. *Circles* represent individuals assigned as *T. sidoides* subsp. *pinnatifida*, *squares* represent those from *T. sidoides* subsp. *sidoides*, and *triangles* represent putative hybrids between both subspecies

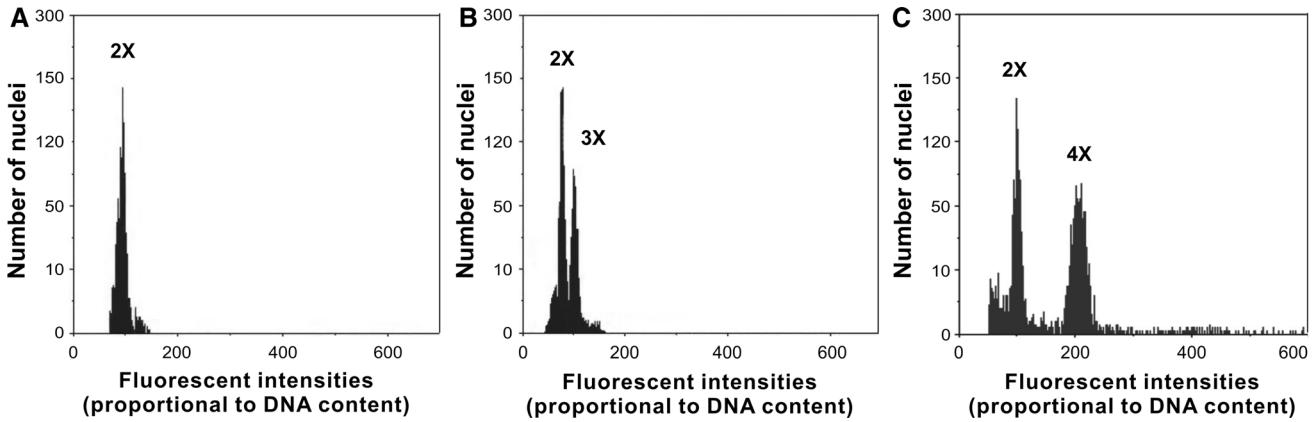

Fig. 4 Flow cytometric profiles of plants of *Turnera sidoides* from the contact zone. **a** Flow cytometry histogram of a diploid plant of *T. sidoides* subsp. *pinnatifida* and the internal standard with a high peak at 2X. **b** Flow cytometry histogram of a putative hybrid between both subspecies showing a peak at 3X and the standard (2X). **c** Flow cytometry histogram of a tetraploid of *T. sidoides* subsp. *sidoides* plant with a high peak at 4X and the diploid standard (2X)

PCR–RFLP analyses of cpDNA

The two cpDNA haplotypes expected according to Speranza et al. (2007) were identified in the contact zone. All the individuals of subspecies *pinnatifida* presented haplotype B and those of subspecies *sidoides* presented haplotype C. The putative hybrids presented both haplotypes C (73.90 % of the individuals) and B (21.74 % of the individuals). Hybrids with haplotype B were distributed near individuals of subspecies *pinnatifida*; while those with haplotype C occurred near individuals of subspecies *sidoides* (Fig. 1b).

RAPD profile

A total of 90 RAPD markers were scored. A summary of the results from the two subspecies and the putative hybrids are listed in Table 2. Of the total molecular variability found, 66 % remained within groups (Table 3). The first two coordinates of PCoA based on RAPD data accounted for 74.15 % of the variation (63.05 and 11.11 %, respectively). Individuals of the two subspecies, *pinnatifida* and *sidoides*, and the putative hybrids formed clearly distinct groups, except two individuals with intermediate morphological traits (individuals 50 and 51) that were grouped with individuals of the subspecies *pinnatifida* (Fig. 5).

These gene pools were divided into two mostly homogeneous subspecies *pinnatifida* and subspecies *sidoides* subpopulations and a group of individuals with gene pools composed of elements from both subspecies which corresponded to the individuals with intermediate morphology. The analysis also indicated that some individuals of subspecies *pinnatifida* (individuals 2, 4, 7, 11, 17, 21) and of subspecies *sidoides* (individuals 25, 33, 34, 38, 41, 42, 43, 45, 47, 48) had a proportion of their genome assigned to the "*sidoides* genome type" and to the "*pinnatifida* genome type", respectively (Fig. 6).

Artificial crosses

Of the 24 crosses performed to verify the crossability of the parental subspecies with the hybrids, 56 % produced fruits. There were differences in reciprocal crossability, since certain combinations set more seed than their reciprocal crosses. In crosses between subspecies, fruits were produced only when subspecies *sidoides* was used as a maternal parent (mean of 15 seeds per fruit). Crosses between subspecies *sidoides* and the hybrids were effective only when subspecies *sidoides* was the maternal parent (mean of 15 seeds per fruit). Crosses between hybrids and subspecies *pinnatifida* were effective in both directions, although more seeds were produced when hybrids were used as the maternal parent (4–9 seeds per fruit) than the reverse (6 seeds per fruit).

Table 2 Summary of the RAPD markers found in *Turnera sidoides* subsp. *pinnatifida*, *T. sidoides* subsp. *sidoides*, and their putative hybrids

RAPD markers	subsp. *pinnatifida*	subsp. *sidoides*	Hybrids
Total number of bands	50	65	51
Polymorphic markers (%)	52.22	71.11	54.44
Unique bands to subspecies present or not in hybrids	18	33	–
Unique bands to hybrids absent in both subspecies	–	–	7
Unbiased expected heterozygosity	0.13 ± 0.02	0.13 ± 0.02	0.15 ± 0.02

Table 3 Hierarchical distribution analysis of genetic variation estimated by AMOVA

Source	*df*	SS	MS	CV	%	*Φ*
Among groups	2	152.21	76.10	3.81	34	
Within groups	55	405.43	7.37	7.37	66	
Total	57	557.64		11.18	100	0.34 ($p = 0.001$)

df Degrees of freedom, *SS* sum of squares, *MS* mean square, *CV* component of the variance, % percentage of the total variance, *Φ* phi value

Discussion

Results from the secondary contact zone in Aguas Blancas suggest the presence of hybridization between *T. sidoides* subsp. *pinnatifida* and *T. sidoides* subsp. *sidoides*, and constitute the first evidence of ongoing natural hybridization between subspecies of the *T. sidoides* complex.

Individuals with an intermediate phenotype detected in the contact zone are supported as hybrids by several lines of evidence. First, the most direct evidence is the intermediate morphology of traits in such individuals (Rieseberg et al. 2000). In Aguas Blancas, most of the putative hybrids are intermediate in morphology suggesting that they represent F_1 hybrids rather than later-generation hybrids or backcrosses. Second, the intermediate ploidy level ($2n = 3x = 21$) also support the recent F_1 hybrid origin of such individuals. Finally, the additive patterns of RAPD markers observed in triploids reflect the contribution of both parental subspecies to the hybrids which was also consistently inferred by the Structure analysis.

Because hybrids display both cpDNA haplotypes that are diagnostic of the respective subspecies *pinnatifida* and *sidoides* at this site, it can be inferred that these hybrids resulted from reciprocal crosses between the subspecies. However, since a higher number of hybrids displayed the diagnostic haplotype of subspecies *sidoides*, this

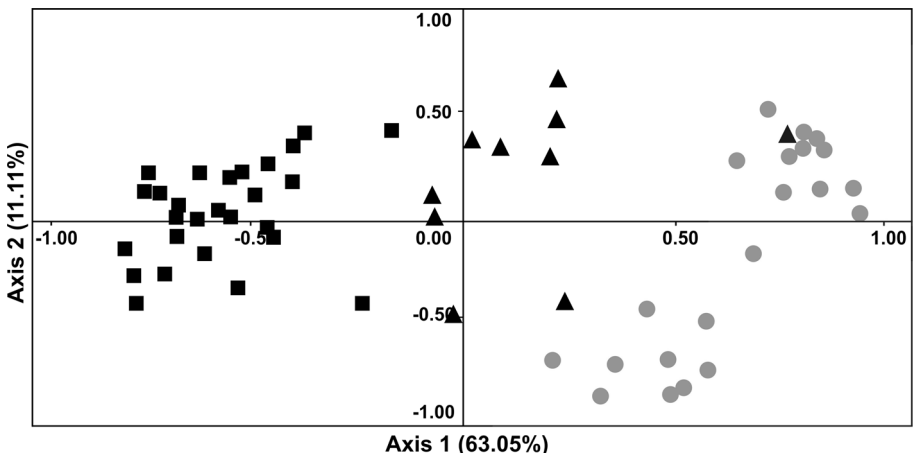

Fig. 5 Ordination plot of the sample points in the plane of the first two principal components (PCoA) based on RAPDs data of plants of *Turnera sidoides* from the contact zone. *Circles* represent individuals assigned as *T. sidoides* subsp. *pinnatifida*, *squares* represent those from *T. sidoides* subsp. *sidoides*, and *triangles* represent putative hybrids between both subspecies

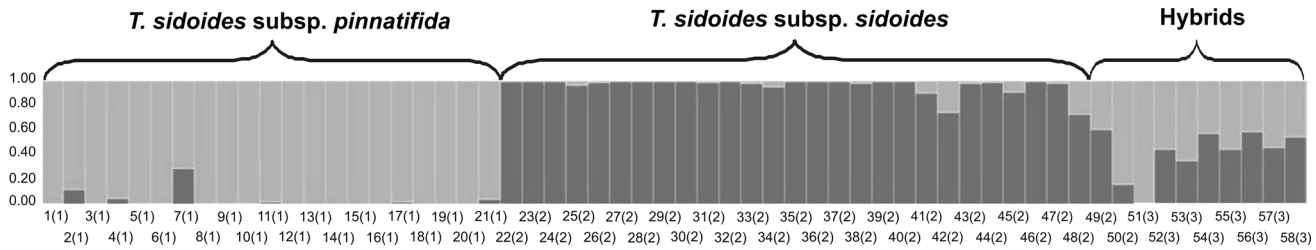

Fig. 6 Population structure inferred by the program STRUCTURE on the basis RAPD markers. Each individual is represented by a *single vertical bar*, which is partitioned into two $K = 2$ *colored components* that represent each individual's proportional assignment to one of the genetic clusters. The *numbers outside the parentheses* correspond to the order of each individual in the input matrix. *Between parentheses* is given the name of the group to which each individual was assigned (1 = *T. sidoides* subsp. *pinnatifida*, 2 = *T. sidoides* subsp. *sidoides*, 3 = putative hybrids)

subspecies likely acted as the maternal parent more frequently than subspecies *pinnatifida*. In hybrid zones, a trend to a unidirectional gene flow can result from partial reproductive barriers among species and, the major abundance and/or viability of one of the parental species (Nason et al. 1992). In Aguas Blancas, both subspecies exhibited high pollen viability, although owing to the higher abundance of subspecies *sidoides* in the study area, the asymmetrical gene flow is probably explained by differences in population density.

Difference in ploidy level is an additional factor that may contribute to asymmetrical gene flow among subspecies (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998). The presence of triploids in contact zones between diploids and tetraploids, and in the progeny of experimental crosses in some species (Zohary and Nur 1959; Felber and Bever 1997), including *T. sidoides* (Elías et al. 2011; Kovalsky and Solís Neffa 2012), shows that the triploid block is often overcome. Failure of a triploid block to cause triploid seed abortion frequently occurs when the plants with higher ploidy levels were used as the maternal parent (Shore and Barrett 1985; Fernández and Solís Neffa 2004; Fernández et al. 2010). Indeed, in the studied contact zone, the higher frequency of hybrids with the cpDNA haplotype of the tetraploid subspecies *sidoides* suggests that the triploid block is overcome more frequently when this subspecies, which has a higher ploidy level, acts as the maternal parent. The fact that in the experimental crosses between subspecies, fruits were only produced when the tetraploid subspecies *sidoides* was used as maternal parent supports this hypothesis.

Our analysis of the spatial arrangement of cpDNA haplotypes in the contact zone indicates that haplotype distribution is not random, but hybrids with different haplotypes occur near individuals of the subspecies that acted as the maternal parent. Given the maternal inheritance of chloroplasts in *T. sidoides* (Speranza et al. 2007), only seeds may disperse chloroplast markers. Since seeds are dispersed principally by gravity and tend to concentrate near the mother plant (Solís Neffa 2000), the genetic structure observed in the hybrid zone most likely results from short-distance seed dispersal.

Evolutionary consequences of the hybrid zone

Current biogeographical patterns of the *T. sidoides* complex have been explained in terms of historical events (Solís Neffa and Fernández 2001; Solís Neffa et al. 2004; Speranza et al. 2007; Solís Neffa 2010). It has been proposed that fragmentation of the flora, as a result of geomorphologic and climatic changes, has led to the genetic differentiation of *T. sidoides* populations in isolation at diploid level. The subsequent evolutionary history of the complex apparently mainly involved the range expansion of the differentiated populations to their current limits through polyploids (Solís Neffa and Fernández 2001; Solís Neffa et al. 2004). Ultimately, tetraploids from different genetic origins may have established secondary contacts between subspecies.

The presence of hybrid swarms in areas where diploids occur in sympatry with tetraploids is also suggestive of the potential role of gene flow among ploidy levels as a source of genetic variation in *T. sidoides*. Even though the potential for gene exchange across ploidy levels has long been recognized (Stebbins 1971; Levin 1975; Stift et al. 2010), knowledge about rates of gene flow among different cytotypes in *T. sidoides* remains limited. The production of unreduced gametes by diploids (Solís Neffa 2000; Panseri et al. 2008; Kovalsky and Solís Neffa 2012) is a mechanism enabling gene flow from these diploids to polyploid cytotypes in *T. sidoides*. The reverse gene flow direction, from a higher to a lower ploidy level, has been proposed for some other polyploid complexes (Savidan and Pernès 1981), but there is still no evidence for it in *T. sidoides*.

Triploids may have played an important role as a bridge to the gene flow between diploid and tetraploid cytotypes. Since triploids of Aguas Blancas are not completely sterile and produce some viable seeds, some gene exchange among hybrids and their parental subspecies may actually

occur. Previous experimental crosses carried out between triploids and diploids of the *T. sidoides* complex yielded diploid and triploid progeny, indicating that triploids produce *n* and 2*n* gametes (Elías 2010; Kovalski et al. 2011; Kovalski 2013). Considering that and taking into account that the experimental crosses here performed verified the crossability of hybrids with both subspecies, in Aguas Blancas, the likelihood of triploid hybrids backcrossing with both parental species would be similar. In both backcrosses, it is expected that progeny exhibit different proportions of their genome assigned to both parental subspecies, as it was found in triploid hybrids from Aguas Blancas.

Introgression of cpDNA haplotypes across subspecific boundaries was suggested to provide one possible explanation for the subspecies-independent cpDNA variation in *T. sidoides* (Speranza et al. 2007). In this sense, even though in Aguas Blancas the diagnostic haplotype of one subspecies was not found in the other one, the asymmetrical gene flow from diploid (paternal parent) to tetraploid (maternal parent) subspecies provides evidence that suggests that intersubspecific hybridization could also account for the introgression of cytoplasmic haplotypes among the subspecies.

Conclusions and prospects

The analysis of the morphological and genetic variation performed in a contact zone between subspecies *pinnatifida* and *sidoides* suggests that, although hybridization may not have been of significance in the early phase of the *T. sidoides* differentiation, it is ongoing enhancing the current morphological and genetic variability of the complex. The question that arises now is whether, in the hybrid zone, parental individuals will persist without losing their identity as subspecies, or introgression will homogenize the two subspecies. Experimental and field studies of the progeny of the hybrids from Aguas Blancas should allow us to analyze, more accurately, the dynamics and maintenance of the hybrid zone between subspecies of *T. sidoides*.

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