



ECOSYSTEMS

Species diversification in the lowlands of mid-latitude South America: *Turnera sidoides* subsp. *carnea* as a case study

ERCILIA M.S. MORENO, GISELA M. VIA DO PICO, IVANA E. KOVALSKY, JUAN M. ROGGERO LUQUE, JOSÉ G. SEIJO & VIVIANA G. SOLÍS NEFFA

Abstract: The lowlands of mid-latitude South America comprise complex temperate ecoregions characterized by a unique biodiversity. However, the processes responsible for shaping its species diversity are still largely unknown. *Turnera sidoides* subsp. *carnea* is a variable subspecies occurring in the lowlands of northeastern Argentina and Uruguay, extending to southern Paraguay and Brazil. It constitutes a good model to perform evolutionary studies. Here we used an integrative approach to understand the process of diversification within this subspecies and to increase the knowledge concerning patterns and processes responsible for shaping the species diversity in the temperate lowlands of South America. The results provided strong evidences that this subspecies is an autopolyploid complex *per se*, being in an active process of intrasubspecific diversification. Morphological and genetic data show that the diversity of *T. sidoides* subsp. *carnea* is in congruence with the great past and present abiotic and biotic variability of the mid-latitude South American lowlands. The evolutionary history of this subspecies is consistent with past fragmentation and allopatric differentiation at diploid level. Geographic isolation and local adaptation would have promoted strong morphological, ecological, and genetic differentiation, resulting in two morphotypes and different genetic groups indicative of incipient speciation.

Key words: biodiversity, ecological niche modeling, genetic diversity, morphotypes, South American lowlands, *Turnera sidoides* subsp. *carnea*.

INTRODUCTION

South America is composed of different biogeographical regions (Morrone 2000, 2006) and harbors the highest biodiversity in the world (Myers et al. 2000). Its evolutionary history was linked to a succession of climate changes and major geological events from Miocene onwards that have modified both the continent and oceans (Clapperton 1993, Iriondo 1993, 1999, Graham 2009, Aragon et al. 2011, Lavina & Fauth 2011). Such climatic and geological events were critical in determining current patterns of species distribution (Ab'Sáber 1977, Burnham &

Graham 1999) and creating complex scenarios for species diversification (Colinvaux et al. 1996a, b, Behling 2002, Antonelli & Sanmartin 2011, Aragon et al. 2011). The lowlands of mid-latitude South America comprise complex temperate ecoregions, including the Pampa and the Campos of Uruguay, northeastern Argentina and southern Brazil, characterized by a unique biodiversity (Miñarro & Bilencia 2008). However, these ecoregions have been overlooked in evolutionary studies, being the patterns and the processes responsible for shaping its species diversity still largely unknown (Speranza et al. 2007, Antonelli & Sanmartin 2011, Turchetto-Zolet et al. 2013).

Integrative approaches combining morphological, biogeographic, environmental together with genetic data in species complexes provide information about the first steps in species radiation, the colonization of new habitats and, the selective pressures in different environments (Mäder et al. 2013, Turchetto et al. 2014, Andre et al. 2015, Lagomarsino et al. 2016, Xu et al. 2017). Such analyses improved the knowledge about the evolutionary history of species as well as the potential processes underlying the diversification and speciation events. Thus, in the last decade many studies adopted species complexes to test different evolutionary hypotheses (He et al. 2013, Longo et al. 2014, Zamudio et al. 2016, Pinheiro et al. 2018).

The South American flora is rich in species complexes composed of recently diverged lineages like *Calceolaria* spp., *Eugenia* spp., *Petunia* spp. and *Solanum* spp. (Alvarez et al. 2008, Mäder et al. 2013, Longo et al. 2014, Turchetto et al. 2014, Brünger et al. 2015, Maubecin et al. 2016, Pinheiro et al. 2018). *Turnera sidoides* L. (Passifloraceae, Turneroideae) is among these species complexes and, constitutes an informative biogeographic and evolutionary model system (Speranza et al. 2007, Solís Neffa 2010, Moreno et al. 2018). This autopolyploid complex of outcrossing, perennial, rhizomatous herbs, covers a large extension of the South America lowlands and, ranges naturally from the southern regions of Bolivia and Brazil, through Paraguay to Uruguay and Argentina, reaching 39° S. Biogeographically, the area of *T. sidoides* is included in the Chacoan subregion proposed by Morrone (2000, 2006, 2014), while, from the phytogeographical point of view, most of this area is included in the Pampeana, Espinal, Chaqueña and Pre-Puneña provinces (Chaqueño Domain), extending into the Paranaense province

(Amazónico Domain) proposed by Cabrera (1971) and Cabrera & Willink (1973, 1980).

Turnera sidoides presents a huge morphological and geographically structured variability, suggesting that an active process of diversification is happening in the complex (Solís Neffa 2010). Based on the leaf shape and indumentum variability as well as on their geographical distribution, five subspecies were recognized in *T. sidoides*: *T. sidoides* subsp. *carnea* (Cambess.) Arbo, *T. sidoides* subsp. *holosericea* (Urb.) Arbo, *T. sidoides* subsp. *integrifolia* (Griseb.) Arbo, *T. sidoides* subsp. *pinatifida* (Juss. ex Poir.) Arbo, and *T. sidoides* subsp. *sidoides* (Arbo 1985, 2008). Different ploidy levels based on $x=7$, from $2n=2x=14$ to $2n=8x=56$, have been reported within the subspecies (Fernández 1987, Solís Neffa & Fernández 2001, Solís Neffa et al. 2004, Speranza et al. 2007, Elías et al. 2011, Kovalsky & Solís Neffa 2012). Based on the geographic distribution of the morphological and genetic variation, two main centres of the species diversification were identified, one in the west (northwestern Argentina and Bolivia) and, the other in the east (northeastern Argentina, Uruguay and Southern Brazil) of the *T. sidoides* geographical range (Solís Neffa et al. 2004, Speranza et al. 2007, Moreno et al. 2018). The later centre is of interest in this report.

Turnera sidoides subsp. *carnea* is a variable subspecies occurring in the east of the *T. sidoides* area. It mainly grows in the lowlands of northeastern Argentina and Uruguay, extending to southern Paraguay and Brazil (Fig. 1). This widespread range is geomorphologically diverse, comprises a great diversity of climates and different phytogeographical provinces. Moreover, the area is divided in a NE-SW direction by the large Uruguay river. As expected from its distribution, populations of *T. sidoides* subsp. *carnea* present a striking variation in

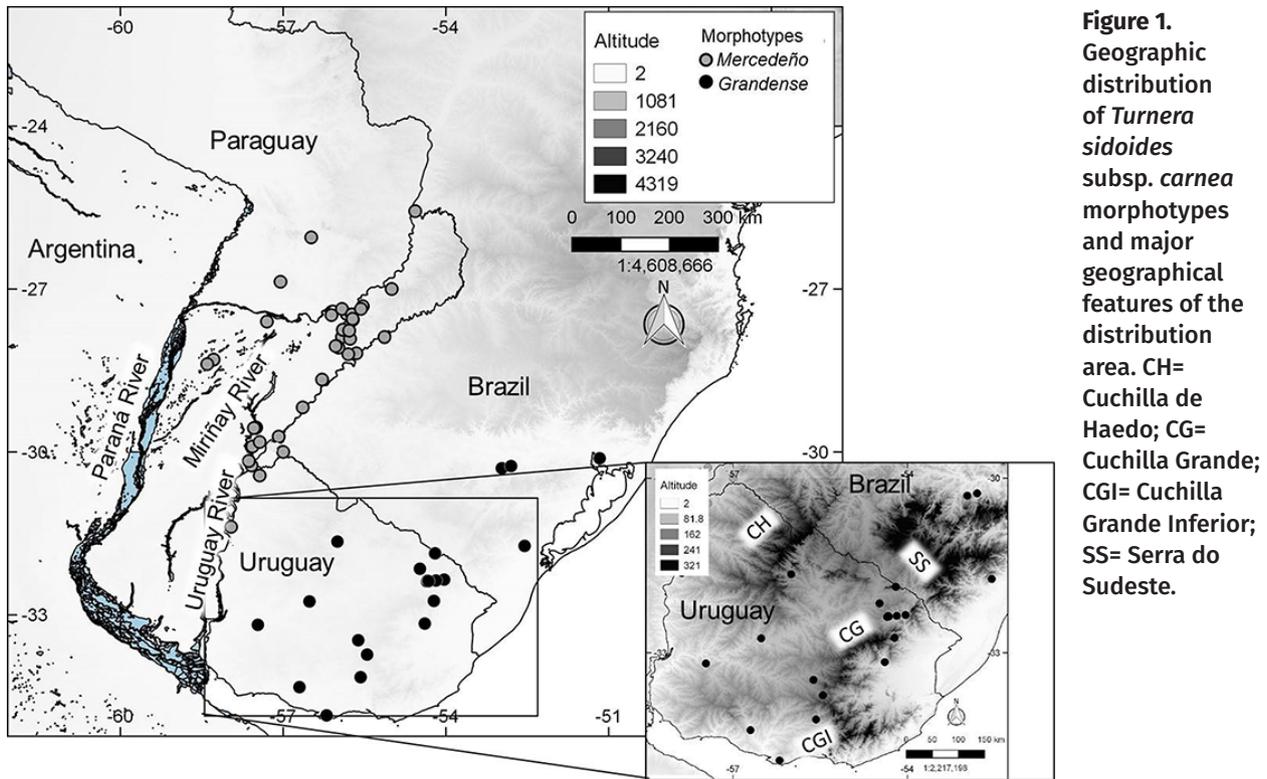


Figure 1. Geographic distribution of *Turnera sidoides* subsp. *carnea* morphotypes and major geographical features of the distribution area. CH= Cuchilla de Haedo; CG= Cuchilla Grande; CGI= Cuchilla Grande Inferior; SS= Serra do Sudeste.

different traits along its area of occurrence, among them, the flower color (light or dark pink) and the leaf consistency (membranous or coriaceous). Therefore, *T. sidoides* subsp. *carnea*, constitutes a good model to detect early stages of divergence, and to identify the potential mechanisms underlying the diversification and speciation processes within the eastern centre of variation of *T. sidoides* complex.

Thus, in this study an integrative approach combining morphological data, ecological niche modeling and population genetic analysis was done to understand the process of diversification within *T. sidoides* subsp. *carnea* and to increase the knowledge concerning the patterns and the processes responsible for shaping the species diversity in the temperate lowlands of South America.

MATERIALS AND METHODS

Study area

It is a large extension of lowlands that comprises the northeastern Argentina, Uruguay and, southern Paraguay and Brazil, divided in a north–south direction by the Uruguay River. Due to its geographic position at the limit for tropical vegetation types (Cabrera & Willink 1980), the study area occupies a transitional zone between subtropical and temperate climates. Annual rainfall northwards increases from 600 mm to 1,800 mm, while mean temperature increases in the same direction from 13 °C to 22 °C (Van Der Sluijs 1971, Carnevalli 1994). Phytogeographically, the study area is in the Neotropical Region and are part of two biogeographical domains, Amazonian and Chacoan, represented by the Paranaense province as well as the Pampean and Espinal provinces (Cabrera 1971, Cabrera & Willink 1980), respectively, with large ecotones

in their contact areas, resulting in very different floristic patterns (Carnevali 1994).

Northwest of the Uruguay River, in the northeastern Argentina and southern Paraguay, is the oldest geological formation which consists of basic volcanic rock of Triassic age and hard, layered, often silicified red sandstone of aeolian origin. The sandstone forms the parent material from which red lateritic clays, common to the northeast of this area, have developed. Outcrops of the same sandstone and of basalt are found in the Mercedes plateau (Popolizio 1999, Iriondo & Paira 2007). The major vegetation types are medium-tall grass savannas with remnants of depauperated subtropical forest on the better-drained lateritic soils, humid and wet tussock grasslands found on poorly drained soils or permanently covered by a high water table, forests along the main river banks, and a mosaic of sclerophyll woodlands, palm savannas and flood lowlands towards the south of the Mercedes plateau (Van Der Sluijs 1971, Carnevali 1994, Oyarzabal et al. 2018).

Southeast to the Uruguay River, in the Uruguayan and Brazilian portions of the study area, a diverse array of rocks, such as Precambrian granite, Carboniferous sandstone, and Jurassic basalt, is exposed to surface and soil-forming processes (Paruelo et al. 2007, Bizzi et al. 2001). The landscape is an undulated peneplain in which the most important orographic systems are the Cuchillas de Haedo and Grande (in Uruguay) and the Serra do Sudeste (in Brazil), the principal dividers of the hydrographic basins (Fig. 1). Lower forms of relief are located on the periphery of the peneplain, formed from sedimentary rocks, arranged horizontally with gentle slopes and poor drainage, where marshlands are frequently found. Variation in geological substrate and altitude further contribute to the diversity of vegetation types (Waechter 2002). Although the grasslands are

the main vegetation in the entire region, two subregions are clearly differentiated, the Campos in the north and the Pampas in the south. The northern area not only differs in temperature and humidity with respect to the southern one, but also in the flora, which has subtropical elements intermingled with temperate flora. The hilly areas are covered by woody vegetation that was considered as southward extensions of the Paranense forest formations (Grela 2004, Paruelo et al. 2007). In the westernmost limit, an *Acacia-Prosopis* parkland vegetation provides a transition to the Chaco and Espinal formations further to the west (Waechter 2002).

Sampling

The material analyzed was obtained from field collections during the years 1995-2009 (Appendix S1). Overall, 42 populations were sampled along the geographical distribution of *T. sidoides* subsp. *carnea*. Whenever possible, the plants were randomly sampled in each locality and some of them were transferred and cultivated in a greenhouse in similar soil and culture conditions at the Instituto de Botánica del Nordeste (IBONE) in Corrientes, Argentina. Representative vouchers for each population analyzed are deposited at the herbarium of IBONE (CTES) and at the herbarium from Instituto de Biociências, Universidade Federal do Rio Grande do Sul (ICN). A second set of material was composed of 30 herbarium specimens from the herbaria CTES, G, LIL, and MVFA (citation follows Holmgren et al. 1990). Details of the material studied are given in the Supplementary Material - Appendix S1.

Morphological analyses

Aiming to explore the morphological variation among populations of *T. sidoides* subsp. *carnea* a two steps analysis was conducted. The first step comprised the morphological inspection

of living material from 42 populations in the field and cultivated in greenhouse for several generations. Several reproductive traits (pollen size and number, number of ovules, pollen / ovule ratio, pollen viability, diameter of the corolla, petals length and width, style length, ovary length, anthers length, number of stigma branches, anther length / style and, length ratio) as well as fruit set varied among and within populations in relation to ploidy level or distily, respectively (Solís Neffa 2000, Panseri 2011). However, only flower color (dark or light pink) and leaf consistency (membranous or coriaceous leaves) varied consistently. Plants with dark pink flowers always have coriaceous leaves while plants with light pink flowers have membranous leaves. Since the variation in these traits was associated to each other, they were used to define two morphotypes (see morphological analysis in results) and to identify 30 historical herbarium materials in the second step of the morphological analysis. The consistency of the leaves was visually surveyed in the herbarium vouchers while the color of the flowers was obtained from the data recorded in the labels.

Ploidy level estimations

Ploidy level of some populations was estimated by meiotic chromosome counting and flow cytometry according to the methods described in Kovalsky & Solís Neffa (2012), while ploidy level data of other populations were recorded from previous contributions of our research team (Fernández 1987, Solís Neffa & Fernández 2001, Speranza et al. 2007, Kovalsky & Solís Neffa 2012, Kovalsky et al. 2017).

Geographic distribution and ecological preferences

The geographic distribution of 72 populations was analyzed based on geo-referenced points

obtained in the field trips and from herbarium specimens. The distribution map was made using the software QGIS 3.4.2-Madeira (QGIS Development Team 2018). The ecological preferences of each population were determined from observations in the field during collection trips and by analyzing the information provided in the labels of herbarium specimens.

Ecological niche modeling (ENM)

The current potential distribution of *T. sidoides* subsp. *carnea* was modeled using point locality information and environmental data in QGIS 3.4.2-Madeira (QGIS Development Team 2018) and MaxEnt 3.4.1 (Phillips et al. 2017). To detect spatial autocorrelation and prevent the spatial dependence, the response and the predictor variables, as well as the residuals of each model were tested by building Moran's correlograms. As a result, 20 geo-referenced points corresponding to the morphotype with dark pink flowers, and membranous leaves and, 35 geo-referenced points corresponding to the morphotype with light pink flowers and coriaceous leaves were used for the analysis (Appendix S1).

For the modeling, 19 bioclimatic variables were extracted from the WorldClim database (<<http://www.worldclim.org/>>) (Hijmans et al. 2005) and were used at a resolution of 2.5 min. The mean average, standard deviation, and range of variation of the bioclimatic variables were calculated for each morphotypes. To evaluate the existence of significant differences for each variable between morphotypes, one-way ANOVA at a significance level of 5 % ($\alpha= 0.05$) after Bartlett's test of homogeneity was made. Moreover, a Principal Component Analysis (PCA) based on a correlation matrix was performed to minimize multicollinearity and to reduce the number of predictors' variables to four axes explaining most of the climatic variance (76.35 %). After this analysis, we select the following

four uncorrelated variables ($r \geq 0.80$) to perform ENM: BIO 4 (Temperature Seasonality [standard deviation $\times 100$]), BIO 6 (Minimum Temperature of Coldest Month), BIO 14 (Precipitation of Driest Month) and BIO 18 (Precipitation of Warmest Quarter).

An ENM analysis was made for each morphotype of *T. sidoides* subsp. *carnea* to evaluate their climatic identity and to determine how current climatic conditions affected their distributions. For the morphotype with dark pink flowers and membranous leaves, MaxEnt was run using the following settings: feature classes: LQH, Cloglog output format, replicate run type= crossvalidate, regularization multiplier= 2, maximum iterations= 500, ten replicates; while for the morphotype with light pink flowers and coriaceous leaves the settings used were: feature classes: LQ, Cloglog output format, replicate run type= crossvalidate, regularization multiplier= 1, maximum iterations= 500, ten replicates. Binary maps of all models were obtained using the smallest suitability value from the species presence points as the threshold value. The importance of each variable was determined comparing percent contribution values and Jackknife plots.

To evaluate statistically the model performance, the area under the curve (AUC) of the receiver operating characteristic (ROC) plot (Phillips et al. 2006) was used, and True Skill Statistic (TSS) (Allouche et al. 2006) was estimated. The AUC is a threshold-independent measure of model performance and varies from 0 to 1; 0.5 means no predictive ability or randomness and 1.0 shows perfect predictive ability (Fielding & Bell 1997). TSS is defined as sensitivity (correctly classified presences) + specificity (correctly classified absences) - 1. TSS values range from -1 to 1; when the values are negative or close to zero, the models are

not different of a randomly generated model; models with values close to 1 are considered excellent. Acceptable models present TSS values over 0.5 (Allouche et al. 2006). Also, the Akaike Information Criterion corrected for small sample sizes (AICc; Johnson & Omland 2004) was calculated. The AICc is a balanced statistic between the goodness of fit and the number of parameters of the model (Johnson & Omland 2004), which allows selecting models with optimal complexity (Warren & Seifert 2011). All statistical analyzes were conducted with the RStudio (ver. 3.3.1; R Core Team 2014) except for Moran's I correlation which was performed with the software PAST (Hammer et al. 2001), and the AICc analysis, which was run with the modular R-based platform Wallace (Kass et al. 2018).

The niche overlap was tested with ENMTools v 1.4.4 (Warren et al. 2010) to examine niche divergence between morphotypes. Schoener's D (Warren et al. 2008) and Hellinger's-based I (Schoener 1968) indexes were calculated based on the habitat suitability comparison from ENM. Both indexes range from 0 (complete divergence/no overlap) to 1 (high similarity/complete overlap, niche being equally suitable for both species) (Warren et al. 2008, Broennimann et al. 2012). Then, the identity test was executed to validate whether the obtained niche-overlap scores exhibited statistically significant values (Warren et al., 2008). A pseudoreplicated null distribution was generated with this test with 100 replicates. Finally, Schoener's D and Hellinger's I were compared with the generated null distribution. The null hypothesis of this test (the observed values of niche overlap do not differ from random values) is rejected when the estimated value for D and/or I is significantly different from the null distribution.

Table I. Populations of *Turnera sidoides* subsp. *carnea* analyzed with molecular markers. ID= population identity, g= *grandense*; m= *mercedense*. ¹EMBL GenBank accession numbers for cpDNA intergenic spacer sequences. ²New ploidy level estimations. ³Speranza et al. (2007). ⁴Kovalsky et al. (2017). ⁵Solis Neffa & Fernández (2001).

ID	Morphotype	RAPD	cpDNA	Location	Lat	Long	GenBank accession number ¹	Ploidy level
1	m	†	†	Argentina, Misiones	-27.46	-55.83	MH190473-MH190635	
2	m	†		Argentina, Corrientes	-27.48	-56.11		
3	m	†	†	Argentina, Misiones	-27.70	-55.80	MH190470-MH190632	
4	m	†	†	Argentina, Corrientes	-28.08	-55.98	MH190462-MH190624	
5	m		†	Argentina, Corrientes	-27	-55	MH190463-MH190625	6x ³
6	m	†	†	Argentina, Misiones	-27.55	-55.72	MH190471-MH190633 MH190472-MH190634	
7	m	†	†	Argentina, Corrientes	-29.55	-57.50	MH190464-MH190626 MH190465-MH190627 MH190466-MH190628	2x ³
8	m	†		Argentina, Corrientes	-29.55	-57.50		2x ⁴
9	m	†	†	Argentina, Corrientes	-29.56	-57.51	MH190467-MH190629	4x ⁴
10	m		†	Argentina, Corrientes	-29.56	-57.54	MH190468-MH190630	2x, 3x ⁴
11	m	†	†	Argentina, Corrientes	-29.18	-56.64	MH190469-MH190631	2x ²
12	g	†	†	Brazil, Rio Grande do Sul	-31.73	-52.55	MH190476-MH190638 MH190477-MH190639	4x ²
13	g	†	†	Brazil, Rio Grande do Sul	-30.3	-52.97	MH190474-MH190636 MH190475-MH190637	4x ²
14	g	†		Brazil, Rio Grande do Sul	-30.26	-52.80		4x ²
15	g		†	Uruguay, Treinta y Tres	-33.16	-54.39	MH190485-MH190647	
16	g		†	Uruguay, Cerro Largo	-32.37	-54.33	MH190478-MH190640	4x ⁵
17	g		†	Uruguay, Cerro Largo	-32.35	54.03	MH190479-MH190641	4x ⁵
18	g		†	Uruguay, Cerro Largo	-32.37	-54.33	MH190480-MH190642	4x ⁵
19	g		†	Uruguay, Tacuarembó	-32.75	-56.52	MH190483-MH190645 MH190484-MH190646	2x ²
20	g	†	†	Uruguay, San José	-34.33	-56.70	MH190481-MH190643 MH190482-MH190644	

Genetic analyses

To evaluate the existence of genetic divergence between populations and morphotypes, a subset of individuals was analyzed genetically with nuclear markers and non-coding chloroplast region sequences (Table I).

Nuclear genotyping

Total DNA was extracted from dry leaves using the protocol of Speranza et al. (2007). The genetic variability at nuclear level of thirteen populations (62 individuals) was analyzed using RAPD (Random Amplified Polymorphism DNA) markers. Four arbitrary RAPD primers of *Operon Technologies* (OPO2-OPO4-OPO10-OPO20) that produce polymorphic amplicons in *T. sidoides* (Panseri 2011) were chosen. The PCR mix contained 37.5 ng DNA approximately, 0.75 mM MgCl₂, 0.1 mM dNTP, 0.31 U Taq polymerase in 1× reaction buffer and, 0.2 μM primer in total volume of 8 μL. PCR amplification consisted of one cycle of 94 °C for 2 min, 35 cycles of 94 °C for 30 sec, 37 °C for 1 min, 72 °C for 2 min and, a final extension step at 72 °C for 5 min. PCR products were separated by electrophoresis on 2 % agarose gel at 2.3 V/cm for 160 min, which then were stained with ethidium bromide (10mg/ ml) and photographed under UV light. Bands of identical size were considered homologous across the individuals, while those having different sizes were assumed to belong to different *loci*. Bands resulting from the PCR amplifications were scored for each locus based on their presence (1) or absence (0), and a binary data matrix was constructed.

From the presence/absence matrix, the total number of bands, the number of exclusive bands, the percentage of polymorphic *loci*, and the unbiased expected heterozygosity with the correction factor for little samples according to Lynch & Milligan (1994) for dominant markers

were calculated. Shannon's diversity index (Shannon & Weaver 1949) was calculated to provide a relative estimate of the degree of variation within each population.

The degree of genetic structuring in *T. sidoides* subsp. *carnea* was evaluated by an analysis of molecular variance (AMOVA, Weir & Cockerham 1984, Excoffier et al. 1992). Also, the genetic divergence among populations and morphotypes was estimated using the Φ_{ST} index (Excoffier et al. 1992). A Bayesian clustering method implemented in the software STRUCTURE 2.3.3 (Pritchard et al. 2000) was used to assess population structure of *T. sidoides* subsp. *carnea* without any *a priori* information about morphotypes. Two to fourteen possible groups (k) were analyzed, with three independent repetitions for each k. The admixture model and the option of correlated allele frequencies between populations were chosen, and the degree of admixture alpha was inferred from the data. 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. The best Δk value was estimated by means of Evanno et al. (2005) method with STRUCTURE HARVESTER 6.94 software (Dent & Von Holdt 2012). Finally, a Principal Coordinate Analysis (PCoA) based on Nei's genetic distances (Nei 1972) between each pair of individuals was performed using the software GenALEX 6.5 (Peakall & Smouse 2012).

The correlation of genetic (Φ_{ST}) and geographic distances was performed by a Mantel test (Mantel 1967), as implemented in Alleles in Space 1.0 (AIS, Miller 2005). The significance of this correlation was assessed by a probability distribution with 10,000 random permutations.

Additionally, a Neighbour-joining tree (NJ) (Saitou & Nei 1987) based on Nei and Dice's genetic distances between individuals was

constructed with FreeTree (Pavlicek et al. 1999) and edited with FigTree 1.4 (Rambaut 2009).

Chloroplast genotyping

Sequences of the intergenic cpDNA spacers *trnL-trnF* and *matk5`R-matk6* obtained previously (Moreno et al. 2018) and deposited in the GenBank (accession numbers detailed in Table 1) were concatenated for all analyses. Sequences were read and manually edited with Chromas 2.33 (<http://www.technelysium.com.au>), and aligned with the Clustal W method implemented in MEGA 5 (Tamura et al. 2011). All of the polymorphic positions detected in the alignment were corroborated in the original chromatograms. All poly-T/A stretches were eliminated from the analyses since the mutational dynamics that rules the insertion/deletion events (indels) involved in poly-T/A stretches cannot be assessed (Aldrich et al. 1988, Kelchner 2000). In addition, all the contiguous indels were treated as one mutational event (Simmons & Ochoterena 2000).

Haplotypes were inferred with DnaSP 5.0 (Librado & Rozas 2009). The standard diversity indices, including the number of segregating sites (S), nucleotide diversity (π) and haplotype diversity (h) were calculated. The genetic structure among and within morphotypes was inferred with AMOVA (Excoffier et al. 1992). All calculations were accomplished with Arlequin 3.5.1.2 (Excoffier & Lischer 2010).

Signatures of demographic expansion were inferred computing Tajima's D and Fu's F statistics (Tajima 1989, Fu 1997) and conducting a mismatch distribution analysis for exponential/decline population size change models (Rogers & Harpending 1992, Schneider & Excoffier 1999), with DnaSP v.5 (Librado & Rozas 2009). The goodness of fit between the observed mismatch distributions to that expected under a sudden expansion model was tested with

the sum of squared deviations (SSD) using parametric bootstrapping (1000 replicates) and the raggedness index (HRag) (Harpending 1994).

Phylogenetic relationships between haplotypes were estimated using the Bayesian inference method implemented in BEAST 1.8.0 (Drummond et al. 2012). Two independent runs of 1×10^8 Markov chain Monte Carlo (MCMC) iterations were performed, sampling every 1000 generations. The initial 10 % of the iterations were removed as burn-in. The HKY substitution model was selected using the Corrected Akaike information criterion (AICc, Burnham & Anderson 2004) with jModeltest 2.1.4 (Guindon & Gascuel 2003, Darriba et al. 2012), using the Yule tree prior and a lognormal molecular clock. Tracer 1.5 (Rambaut & Drummond 2009) was used to check for convergence of the Markov chains and adequate effective sample sizes (ESS > 200). All trees generated were summarized in a maximum clade credibility tree using TreeAnnotator 1.7.1 (Drummond et al. 2012), which was edited in FigTree 1.3.1 (Rambaut 2009). Statistical support was determined by assessing the Bayesian posterior probabilities (Rannala & Yang 1996). In the absence of a fossil record for the estimation of divergence times in *T. sidoides*, we used the data obtained by Ramos-Fregonezi et al. (2015), which compiles rates calculated for plastid markers for herbs with similar life traits to the *T. sidoides* complex. We used a gamma distribution prior, with a shape parameter 1.0, a scale parameter 1.6×10^{-9} as prior and we assumed an offset value of 1×10^{-9} s/s/y.

RESULTS

Morphological analyses

An initial morphological survey of the 42 field collected populations, evidenced that those that presented dark pink flowers consistently had coriaceous leaves; while populations with

light pink flowers consistently had membranous leaves. These traits persisted in cultivated plants after several years of growing in similar soil composition, under the same greenhouse conditions. More relevant is that the traits remained constant in the offspring of plants collected in the field and cultivated in the greenhouse. Thus, based on flower color and leaf traits two different morphological groups of populations (morphotypes) were established. Moreover, the morphotypes were named as *grandense* (plants with coriaceous leaves and dark pink flowers), and *mercedense* (plants with membranaceous leaves and light pink flowers) in allusion to the different regions where they tend to occur (see below). As a result, from the 72 populations of *T. sidoides* subsp. *carnea* analysed, 30 were assigned to the *grandense* morphotype and, 48 to the *mercedense* one (Fig. 2, Supplementary Material - Appendix S1).

Ploidy level estimations

New ploidy levels determinations were done from meiotic chromosome counts and flow cytometry estimations of five populations (Table I, Appendix S1). Two populations were diploids ($2n=2x=14$) and three were tetraploids ($2n=4x=28$). Additionally, the ploidy level for other eight populations were recovered from previous works and compiled in Table I. From the 13 populations with information of ploidy levels, five were diploid, seven tetraploid and one hexaploid. One of the population (ID 10) registered diploid and triploid individuals. The diploid and tetraploid ploidy levels were detected in both morphotypes, while the hexaploid was only found in the *mercedense* morphotype.

Geographic distribution and ecological preferences

The analysis of the geographic distribution of populations showed that those belonging to

different morphotypes tend to live in disjunct areas (Fig. 1). Populations of the *grandense* morphotype occurred in the southeastern portion of the subspecies range in Uruguay and south Brazil. They grow in stony soils with grasslands composed of many herb, shrub and treelet species co-occurring within the grass matrix (Pampa province). By contrast, populations of the *mercedense* morphotype occurred mainly in the northwest portion of the species range in the undulating landscape of northeastern Argentina and southern Paraguay. This area is covered by subtropical grasslands in the north (Paranaense province) and outcrops of Triassic sandstones with low xerophitic woodlands characterized by species of *Prosopis* and *Acacia* towards the south (Espinal province). Some few populations extend eastwards the Uruguay River.

Ecological niche modeling

Most of the bioclimatic variables differed significantly between morphotypes (Supplementary Material - Table S1). The climatic niches inferred using the 4 non-correlated bioclimatic variables and the 55 filtered occurrence dataset are shown in Figure 3. Niche models generated by MaxEnt analysis had AUC 0.95 and 0.98 for *grandense* and *mercedense* morphotypes, respectively, and together with the AICc, indicates that the models performed well at predicting current species distribution.

ENMs under current climatic conditions predicted different suitable areas for each of the two morphotypes, with an overlapping zone in northern Uruguay and southwestern Brazil. The predicted areas were consistent with the respective points of occurrence of the two morphotypes and extend somewhat the potential ranges (Fig. 3). The bioclimatic variable that mostly contributed to the models of both morphotypes was the Precipitation of Driest Month (BIO14) (Table II).



Figure 2. Blooming individuals of *Turnera sidoides* subsp. *carnea* showing the most remarkable morphological traits of the *mercedeño* (a) and *grandense* (b) morphotypes. Note the light pink petal and membranaceous leaves in (a) and the dark pink petal and coriaceous leaves in (b).

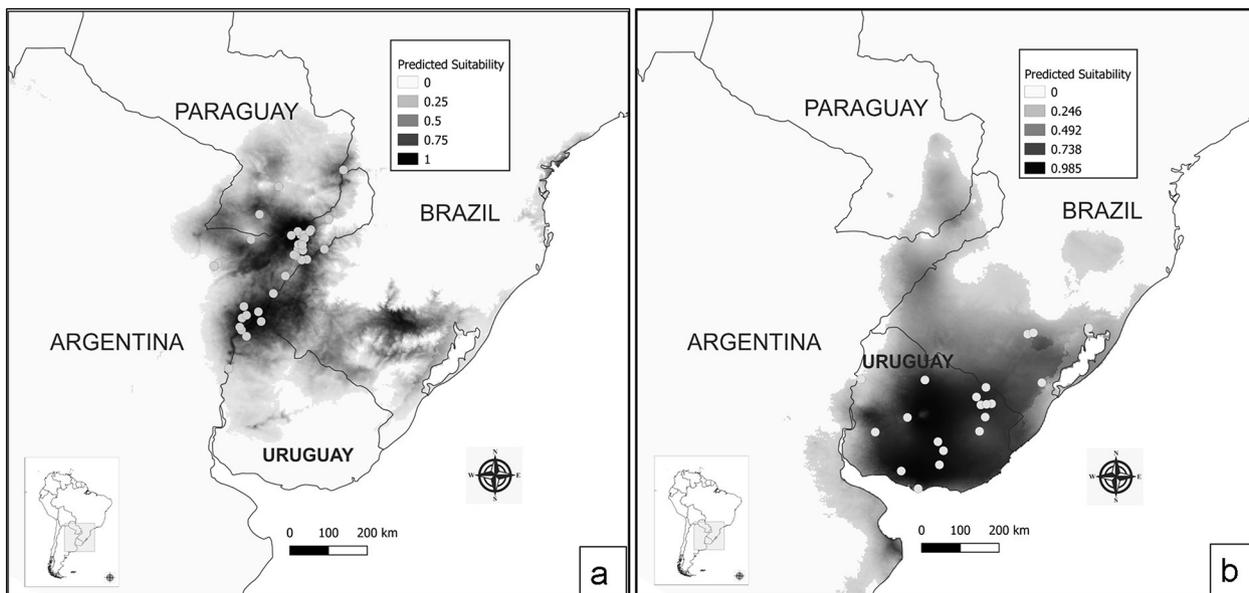


Figure 3. Potential geographic distributions of the *Turnera sidoides* subsp. *carnea* morphotypes based upon maximum entropy niche models using current environmental data and point occurrences. Dots represent the occurrence of *mercedeño* (a) and *grandense* (b) morphotypes.

Table II. Contribution and permutation values for the four variables used in current ENMs for the morphotypes of *Turnera sidoides* subsp. *carnea*. Bio 4= Temperature Seasonality (standard deviation × 100), Bio 6= Minimum Temperature of Coldest Month, Bio 14= Precipitation of Driest Month, Bio 18= Precipitation of Warmest Quarter.

Variable	<i>mercedeño</i> morphotype		<i>grandense</i> morphotype	
	Contribution (%)	Permutation importance	Contribution (%)	Permutation importance
Bio 4	24.60	41.80	25.70	6.50
Bio 6	7.90	35.90	0.50	0.00
Bio 14	55.30	16.10	69.00	77.60
Bio 18	12.10	6.30	4.70	15.90

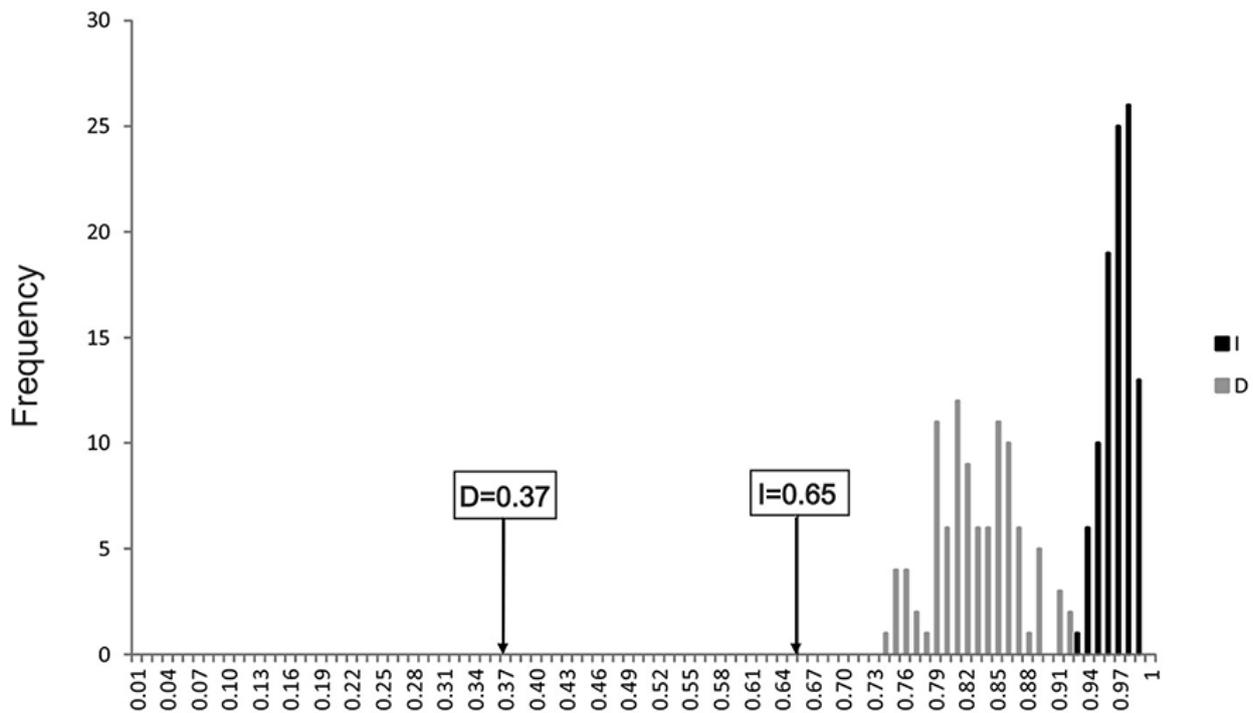


Figure 4. Niche overlap evaluated by the identity test. Arrows refer to the actual niche overlap (D and I) as calculated by ENMTools. The dark and light gray columns are calculated by replicates with identity test models.

The niche overlap of the *grandense* and *mercedeño* morphotypes within environmental space (Schoener’s $D = 0.37$, Hellinger’s-based $I = 0.65$) was significantly lower than the null hypothesis of niche equivalency (Fig. 4; $p < 0.05$), indicating that the bioclimatic niches differ more between each morphotype than expected by chance.

Nuclear genotyping

The statistics calculated to estimate the genetic diversity for 13 populations of *T. sidoides* subsp. *carnea*, eight of the *mercedeño* and five of the *grandense* morphotypes, are summarized in Table III. Fifteen (23 %) out of the sixty-six loci detected were polymorphic. The total expected heterozygosity was 0.09 and the total Shannon’s index was 0.21. Populations presented one to eight exclusive bands, except populations

Table III. Summary statistics of genetic diversity base on 66 RAPD loci for the 13 populations of *Turnera sidoides* subsp. *carnea* analyzed. N = simple size; TNB= total number of bands; NEB= number of exclusive bands; PPL= percentage of polymorphic loci; IHe= impartial expected heterozygosity with Lynch & Milligan correction factor; Sh= Shannon´s index; SD= standard deviation.

Pop ID	mercedeño morphotype									grandense morphotype					
	1	2	3	4	6	7	8	9	Total	11	12	13	14	20	Total
N	3	4	3	2	2	17	4	6	46	5	5	5	3	3	16
TNB	12	24	16	15	13	31	16	20	57	20	12	20	12	17	31
NEB	2	4	2	2	1	8	0	2	33	1	0	1	1	3	7
PPL	7.58	36.36	22.73	22.73	15.15	46.97	16.67	30.30	86.36	28.79	10.61	28.79	7.58	25.76	46.97
IHe (SD)	0.04 (0.02)	0.14 (0.03)	0.11 (0.03)	0.13 (0.03)	0.08 (0.03)	0.12 (0.02)	0.07 (0.02)	0.09 (0.02)	0.12 (0.02)	0.10 (0.02)	0.05 (0.02)	0.11 (0.02)	0.03 (0.01)	0.12 (0.03)	0.11 (0.20)
Sh (SD)	0.05 (0.02)	0.19 (0.03)	0.13 (0.03)	0.14 (0.03)	0.09 (0.03)	0.18 (0.03)	0.09 (0.03)	0.13 (0.03)	0.21 (0.03)	0.14 (0.03)	0.06 (0.02)	0.15 (0.03)	0.04 (0.02)	0.15 (0.03)	0.17 (0.03)

ID 8 and ID 12 in which no exclusive bands were recorded. Concerning morphotypes, the *mercedeño* showed higher values for all the diversity statistics than the *grandense* morphotype. Also, the exclusive bands were more frequent in the *mercedeño* (33) than in the *grandense* (8) morphotype. Despite the greatest genetic variation was observed within morphotypes (Table IV); the structure index was significant and evidenced a moderate genetic differentiation between them ($\Phi_{ST} = 0.17$).

The existence of genetic divergence between morphotypes was supported by the results of STRUCTURE analysis (Fig. 5). The optimal partitioning of the nuclear genetic diversity inferred by the Bayesian clustering was obtained with $K=3$. Thus, three well-defined genetic clusters were recognized. Cluster I included almost all individuals belonging to the *grandense* morphotype (except population

ID 20), and it was genetically homogeneous. The other two clusters (II and III) included all individuals of the *mercedeño* morphotype (plus population ID 20 of the *grandense* one) and showed more mixture. Cluster II included only populations of the *mercedeño* morphotype which mostly occur in the Paranaense province, while cluster III include populations that grow in the Espinal province. The PCoA analysis of the genetic data (Fig. 6) showed that the first two coordinates accounted for 51.81 % of variation and clearly separated populations of both morphotypes as different groups. Moreover, within the *mercedeño* morphotype, populations were arranged in two different genetic groups in consistence with the results of the Bayesian analysis. No significant association between genetic and geographical distances for the subspecies as a whole was shown by the Mantel test ($r= -0.03$; $p= 0.48$).

Table IV. Hierarchical distribution analysis of genetic variation estimated by AMOVA between and within morphotypes of *Turnera sidoides* subsp. *carnea* with RAPD markers and cpDNA sequences. df= Degrees of freedom, SS= sum of squares, VC= variation coefficient, %= percentage of the total variance.

Source	df	SS	CV	%
RAPD				
Between morphotypes	1	157.75	1.96	31
Within morphotypes	60	209.25	4.27	69
Total	61	367	6.23	100
$\Phi_{st} = 0.17^*$				
cpDNA				
Between morphotypes	1	11.58	0.72	19.68
Within morphotypes	22	64.67	2.94	80.32
Total	23	76.25	3.66	100
$\Phi_{ST} = 0.20^*$				

*Significant $p < 0.05$.

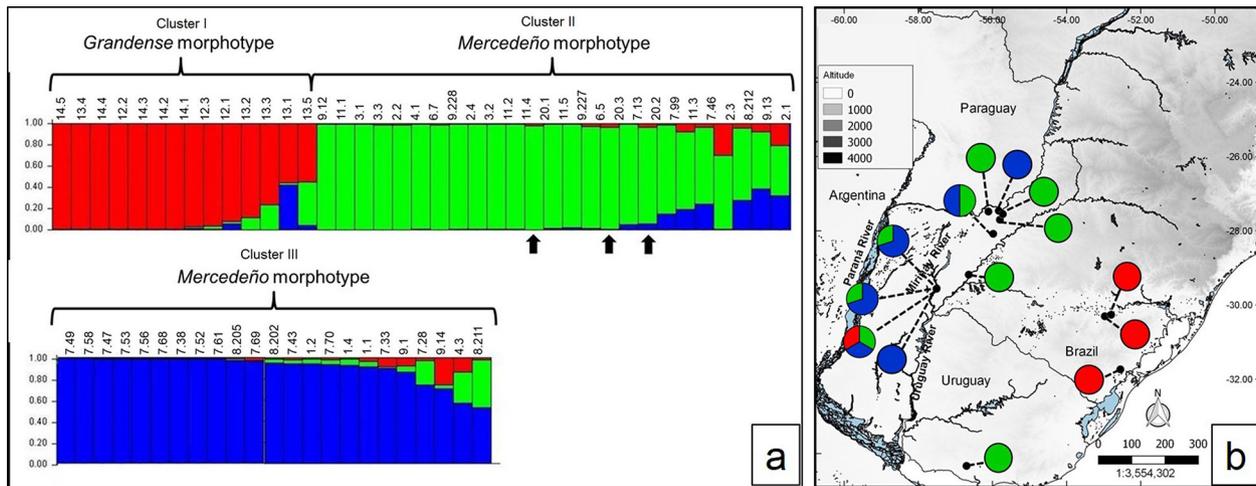


Figure 5. Population structure of *Turnera sidoides* subsp. *carnea* inferred by the STRUCTURE software on the basis RAPD markers. a. Each individual is represented by a single vertical bar, which is partitioned into $K=3$ colored components that represent each individual's proportional assignment to one of the genetic clusters. The population ID and the number of each individual analyzed are indicated above its corresponding bar. Arrows mark those individuals of the *grandense* morphotype which were grouped with *mercedeño* morphotype. b. Geographic distribution of each genetic cluster inferred in populations analyzed.

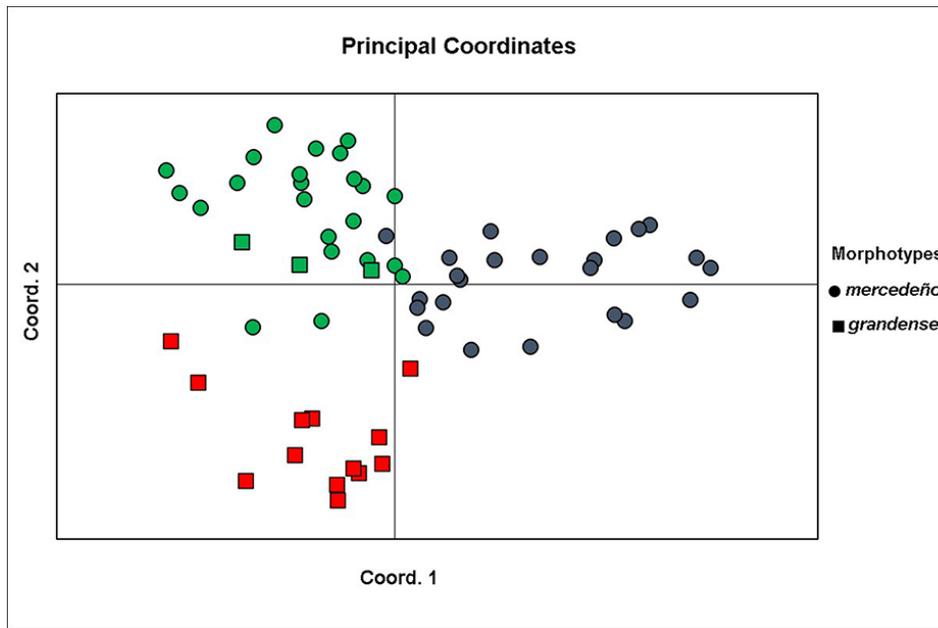


Figure 6. Principal coordinates analysis ordination plot of the sample points in the plane of the first two principal axes based on RAPD data of plants of *Turnera sidoides* subsp. *carnea*. Colors indicate Bayesian groups resulted from STRUCTURE analysis. Red symbols: Bayesian group I; green symbols: Bayesian group II, and blue symbols: Bayesian group III.

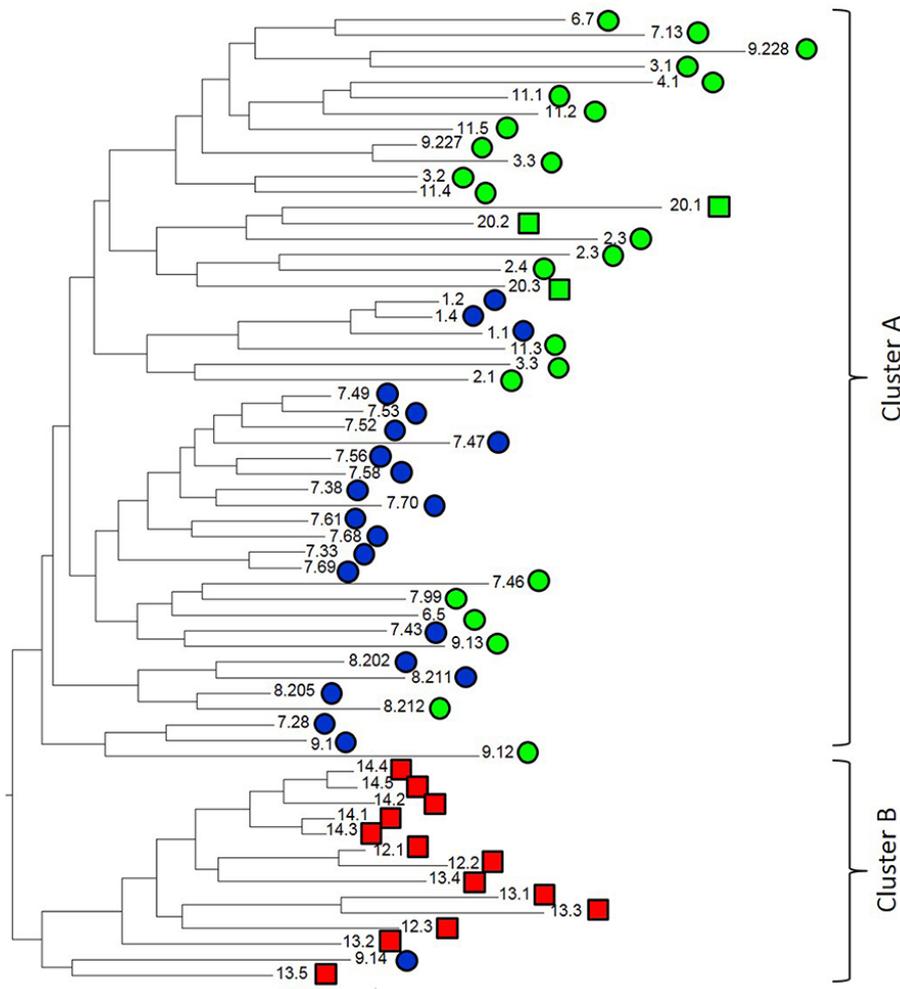


Figure 7. Neighbor-Joining tree constructed with RAPD markers based on Nei and Dice's genetic distances between individuals from *Turnera sidoides* subsp. *carnea*. Dark and grey circles represent individuals assigned to the *grandense* and the *mercedefno* morphotypes, respectively. Numbers refer to population and individual's ID.

Neighbor-Joining tree (Fig. 7) grouped individuals in two big clusters, Cluster A was formed by almost all individuals belonging to the *mercedense* morphotype, plus population ID 20 from the *grandense* one; while Cluster B was constituted by individuals of the *grandense* morphotype plus one individual (ID 9.14) of the *mercedense* one.

Chloroplast genotyping

The concatenated alignment of chloroplast intergenic spacers for the 24 samples (17 populations) resulted in a sequence of 1006 bp in size. From the twenty polymorphic sites retrieved, sixteen corresponded to substitutions and four were indels. Six different haplotypes were recovered, two of them (H5 - H6) were exclusive of the *grandense* morphotype, and three (H1 - H2- H4) were of the *mercedense*

morphotype, while the H3 was the only one shared by both morphotypes (Fig. 8, Table V). Nucleotide and haplotype diversity indexes were high for the subspecies and the morphotypes (Table V). AMOVA revealed that 80.32 % of the variation was explained by differences within morphotypes (Table IV); notwithstanding, the fixation index evidenced a great and significant genetic differentiation between morphotypes ($\Phi_{st} = 0.20$).

Pairwise mismatch distribution was multimodal, with significant SSD and Raggedness index in both morphotypes. In addition, the results of both neutrality tests were not significantly different from zero, neither in the subspecies as a whole, nor within each morphotype (Table VI).

Genealogical relationships and the geographical distribution of cpDNA haplotypes

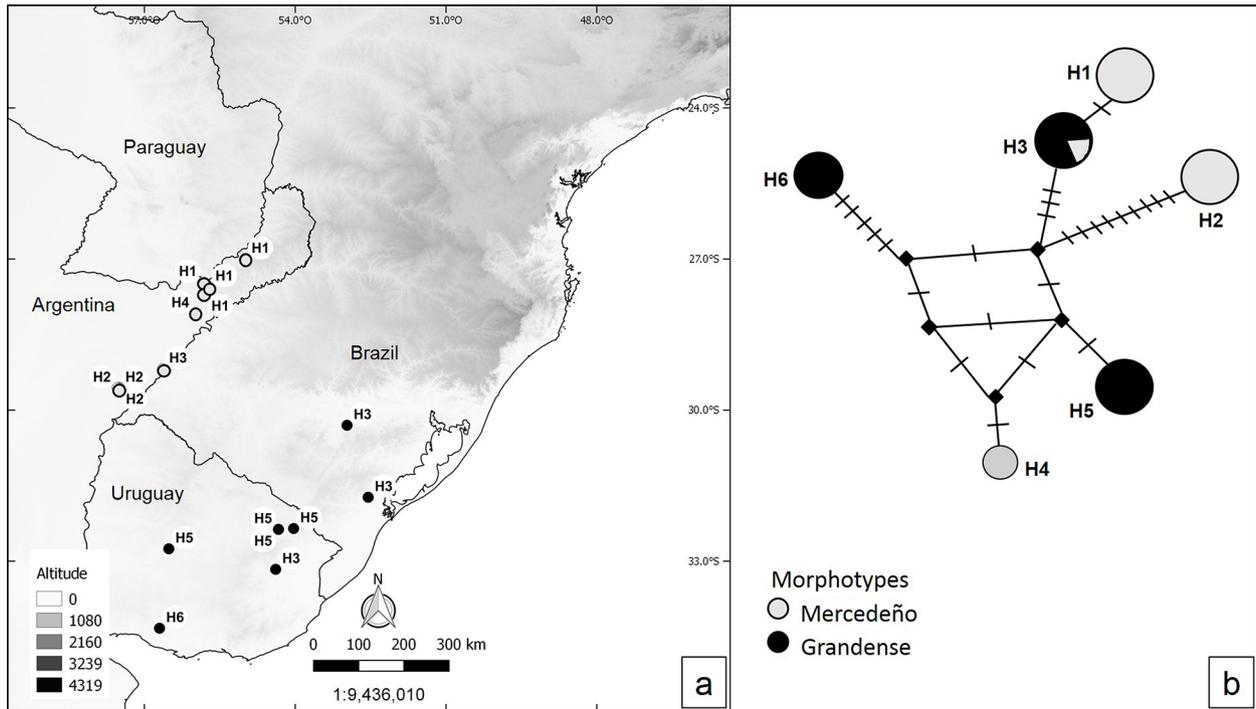


Figure 8. Geographic distribution of cpDNA haplotypes (a) and evolutionary relationships among cpDNA haplotypes using a median-joining network approach (b) of the 17 populations studied of *Turnera sidoides* subsp. *carnea*. Circle sizes in the network are proportional to the haplotype frequency, crossed lines are substitutions inferred in the branches and diamonds are median vectors placed by the software.

are shown in Figure 8. The resolution of the network was not sufficient to indicate ancestor-descendant relationships, since five median vectors were required to join the haplotypes. Haplotypes were differentiated each other by several mutational steps, except for H1 which derived from H3 by only one mutation. Despite of the low ancestry-descent information provided by the network, the haplotypes were differentially distributed between morphotypes and, thus, in the geographic range of the subspecies (Fig. 8)

The Bayesian tree constructed from the cpDNA clearly reflected two different clusters (Fig. 9). In the first one (cluster I), two subclusters were identified. A larger one (cluster I.a) that included the populations with H1 of the *mercedense* morphotype living in the Paranaense province (largely coincident with cluster II of nuclear markers from structure analysis) together with those populations of the *grandense* morphotype with H3 distributed in southern Brazil and northern Uruguay. A smaller subcluster (cluster I.b) grouped the individuals of northern Uruguay with H5 with the only one of the *mercedense* morphotype with H4. The second cluster (cluster II) grouped all the individuals of the *mercedense* morphotype with H2 that live in the Espinal province and,

it was coincident with cluster III from structure analysis based on nuclear markers. This cluster was distantly associated as a brother group with the southernmost population of the *grandense* morphotype with H6 haplotype. Thus, the cpDNA reveals three different groups geographically arranged in a north-south direction (Fig. 9).

DISCUSSION

The evolutionary patterns that shaped the unique biodiversity of the complex temperate eco-regions of the Pampa and Campos in the mid-latitude lowlands of South America (Miñarro & Bilenca 2008) are still largely unknown (Speranza et al. 2007, Antonelli & Sanmartin 2011, Turchetto-Zolet 2013). In the present study we analyzed the diverse *T. sidoides* subsp. *carnea* to investigate to what extent the morphological variation is accompanied by genetic differentiation and to make inferences on the processes that may have determined such diversification. The results provided strong evidences that this subspecies is an autopolyploid complex *per se*, being in an active process of intrasubspecific diversification. The patterns of morphological and genetic differentiation in this subspecies may be partially explained by current climatic

Table V. Summary statistics and haplotypes obtained from intergenic cpDNA sequences analyzed in morphotypes of *Turnera sidoides* subsp. *carnea*. S= polymorphic sites, π= nucleotide diversity, h= haplotype diversity, SD= standard deviation.

	S	π (SD)	h (SD)	Haplotypes
<i>Turnera sidoides</i> subsp. <i>carnea</i>	20	0.35 (0.19)	0.83 (0.03)	H1 - H2 - H3 -H4 - H5- H6
<i>mercedense</i> morphotype	15	0.38 (0.22)	0.70 (0.09)	H1 - H2 - H3 - H4
<i>grandense</i> morphotype	11	0.24 (0.14)	0.68 (0.08)	H3 - H5 - H6

and environmental differences, but also by past fragmentation and allopatric differentiation.

The morphological analysis done in *T. sidoides* subsp. *carnea* evidenced two discrete morphotypes, named *grandense* and *mercedeño*, that could be recognized based on the flower color and the consistency of leaves. The identification of diploid and polyploid populations within each morphotype suggests that the phenotypic diversification was independent of the ploidy level, as was observed at the subspecies level in *T. sidoides* (Solís Neffa 2000). However, the fact that these traits persisted in plants cultivated under similar greenhouse conditions for several generations evidenced that morphological differences between both morphotypes of *T. sidoides* subsp. *carnea* have a high genetic component, as it was previously demonstrated for the morphotypes of other subspecies of the *T. sidoides* complex (Solís Neffa 2010).

The geographic segregation along the subspecies range observed for individuals of the *grandense* and *mercedeño* morphotypes, were concordant with the different habitat preferences resulted from the niche modeling analysis, with a small potential overlapped area (with significant values) in an ecological transition identified between the Paranaense forest and the Campos biome in southern Brazil (Pasos Cordeiro & Hasenack 2009). Field observations

confirmed the results of modeling since the *grandense* morphotype was found in an area with diverse array of Precambrian, Carboniferous and Jurassic rocky soils in the Pampa province, where the main vegetation type is a treeless grasslands dominated by tussock grasses. By contrast, the *mercedeño* morphotype grows in the interfluvial region between the Paraná and Uruguay Rivers and adjacent regions, on Mesozoic basalt elements and red lateritic clays characterized by medium-tall grass savannas with remnants of subtropical forest of the Paranaense province. Some populations of this morphotype were also found in the southwest of the subspecies range, into the Espinal province in parklands dominated by mimosoid species. The ENM based on bioclimatic variables effectively showed that the *grandense* and *mercedeño* morphotypes inhabit regions with significant different climatic regimes. However, other variables (such as soil conditions and species community interactions) may be also contributing to the niche delimitation of both morphotypes that deserves further studies.

Ecological divergence due to habitat differences plays an important role in population differentiation (Foster et al. 2007, Lowry et al. 2008, Zheng & Ge 2010). The environmental differences between the areas of *T. sidoides* subsp. *carnea* morphotypes would be critical

Table VI. Results of the mismatch distribution analysis and neutrality tests of *T. sidoides* subsp. *carnea* and their morphotypes, obtained from intergenic cpDNA sequences.

	SSD (P-value)	Raggedness index (P-value)	Tajima's D (P-value)	Fu's Fs (P-value)
<i>Turnera sidoides</i> subsp. <i>carnea</i>	0.05 (0.07)	0.08 (0.12)	0.71 (0.74)	4.79 (0.99)
<i>mercedeño</i> morphotype	0.19 (0.00)	0.29 (0.03)	1.72 (0.96)	5.21 (0.99)
<i>grandense</i> morphotype	0.19 (0.01)	0.43 (0.01)	1.06 (0.88)	5.10 (0.99)

factors in determining their current spatial distribution at regional scale. Moreover, these factors may probably have determined the differentiation of many other components of the biota. Few species that covered the area of *T. sidoides* subsp. *carnea* were deeply studied so far to test this hypothesis. *Petunia axillaris* (Lam.) Britton, Sterns & Poggenb (Solanaceae) is one of them, and the geographic boundary between the areas of two of their subspecies, *P. axillaris* subsp. *axillaris* and *P. axillaris* subsp. *parodii* (Turchetto et al. 2014) is consistent with the boundary here detected for the morphotypes of *T. sidoides* subsp. *carnea*. The coincidence of morphological discontinuities supports that past and present environmental factors may have been involved in the divergence of many species of the biota at both sides of a NE – SW axis that coincides with the transition of Paranaense forest and the Campos to the north and, the transition between the savanna and the

grasslands toward the south along the eastern shore of the Uruguay River.

The correspondence of phenotypic differentiation with genetic differentiation in *T. sidoides* subsp. *carnea* was evaluated both with nuclear and chloroplast markers. The results showed that the highest genetic diversity was within populations and morphotypes in the AMOVA results, as expected for obligate outbreeders (Loveless & Hamrick 1984, Tsukamoto et al. 2002). Nevertheless, the significant Φ_{ST} values evidenced that the 17 % and 20 % of the total nuclear and cpDNA genetic variation, respectively, attributed to differences between morphotypes may be interpreted as the existence of a moderate to high genetic differentiation among the *grandense* and *mercedeño* morphotypes. The almost lack of admixture and population clustering in the Bayesian analysis based on nuclear genetic data supports the genetic differentiation

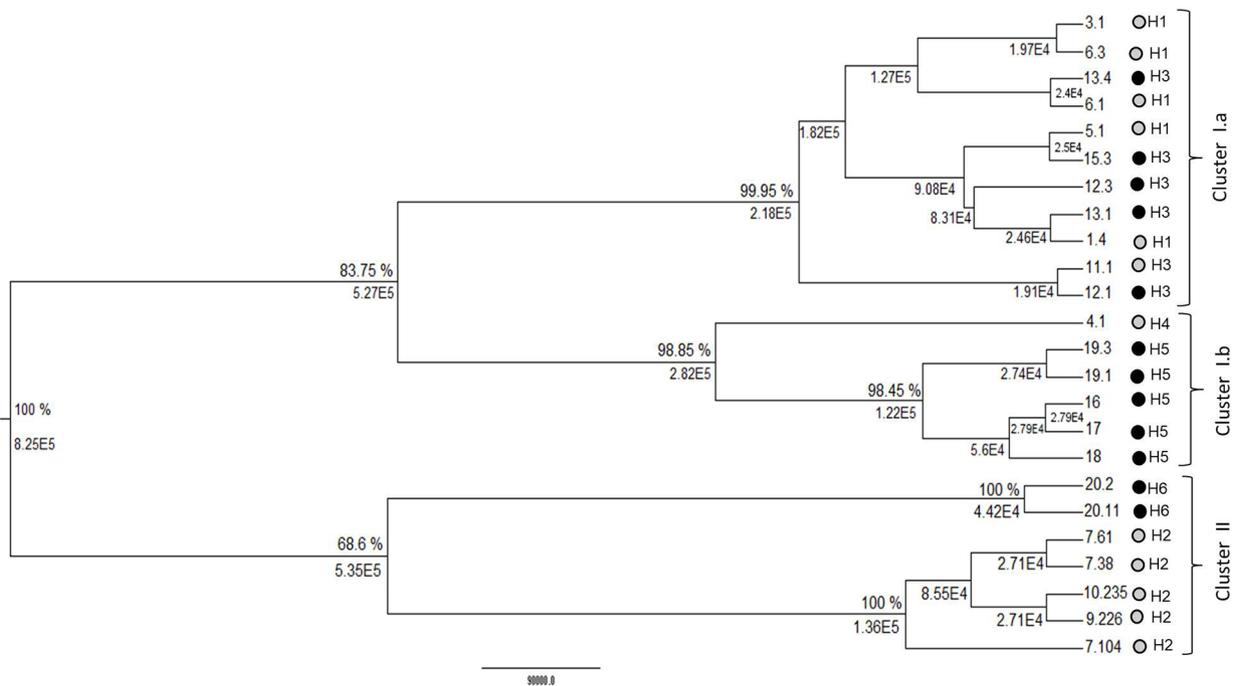


Figure 9. Bayesian inference tree of *Turnera sidoides* subsp. *carnea* based on two plastid DNA intergenic sequences. Posterior probabilities > 50 % are indicated at the beginning of each node. Numbers forward each node show estimates for the ages. Dark and grey circles represent individuals assigned to the *grandense* and *mercedeño* morphotypes, respectively. The haplotype found in each individual is pointed to the left.

between morphotypes. In agreement with the morphotypes geographic range and the major groups delimited by nuclear markers, the cpDNA haplotypes were differentially distributed, that is, H3, H5, and H6 in the *grandense* morphotype and, H1, H2, H3 and H4 in the *mercedense* one. The presence of H3 mostly in the *grandense*, but also in one population of the *mercedense* morphotype, can be more parsimoniously attributed to a common ancestral polymorphism than to a recent hybridization of the morphotypes, considering the current vicariant distribution of the morphotypes and the restrictions for dispersion exhibited by *T. sidoides* (Solís Neffa 2000). Thus, the results revealed that the phenotypic variation in *T. sidoides* subsp. *carnea* is consistent with the genetic differentiation of the morphotypes from a common ancestor.

Interestingly, the molecular data showed further differentiation within the morphotypes. The Bayesian and the PCoA analysis using nuclear markers showed a clear genetic differentiation among populations of the *mercedense* morphotype that grow in the Paranaense subtropical grassland from those that live in the Espinal Province. The chloroplast supports the differentiation of populations of the *mercedense* morphotype occurring in the Paranaense (with H1, H3 and H4) from those of the Espinal (with H2) provinces, but also clearly segregates populations of the *grandense* morphotype of southern Brazil (H3) from those of northern (H5) and southern (H6) Uruguay. The genetic basis of the morphological traits distinguishing the *grandense* and *mercedense* morphotypes and their habitat preferences, in combination with their genetic distinction, suggest that the divergent phenotypes in *T. sidoides* subsp. *carnea* may be the result of the adaptive response of populations to succeed under different climatic and edaphic regimes.

Moreover, the two clusters and three subclusters revealed by the Bayesian analysis based on chloroplast DNA sequences suggest a latitudinal pattern of population differentiation aside of the main longitudinal pattern observed for nuclear markers and phenotypic traits. This pattern of chloroplast relationship is telling another story in the evolution of *T. sidoides* subsp. *carnea*, probably related to the climatic and landscape changes that took place during the Pleistocene (ca. 2.6 – 0.0085 Ma.), as was proposed for *T. sidoides* complex (Moreno et al. 2018) and other South American plant species (Mäder et al. 2013, Longo et al. 2014, Turchetto et al. 2014).

Evolutionary model

The morphological and genetic data clearly show a structured pattern of variation in *T. sidoides* subsp. *carnea*. Although still insufficient to arrive to a conclusive model of diversification of *T. sidoides* complex and the factors that may have been associated to that process, the data here provided allowed the proposal of hypothetical model to be tested in future studies including additional phenotypic and molecular data.

The results here obtained suggest that present-day patterns of morphological and genetic distribution and diversity in *T. sidoides* subsp. *carnea* can be the result of ancient vicariant events. Previous studies in *T. sidoides* proposed the refuge model to explain this mechanism. Consistently with earlier interpretations that the hilly areas of Argentina and Uruguay, as well as some lowland areas may have served as refuges for the flora during the Quaternary cold-dry phases (Ab'Sáber 1977, Haffer 1982), refugial centres for *T. sidoides* were hypothesized in the Cuchilla de Haedo, the Cuchilla Grande, Eastern Serranías systems, and in northeastern Argentina (Speranza et al. 2007,

Moreno et al. 2018), where *T. sidoides* subsp. *carnea* occurs.

During the Pleistocene, in the east of the *T. sidoides* subsp. *carnea* area, intervals of drier climates caused a retraction and fragmentation of the Atlantic Forest (Rambo 1954) as well as the expansion of tropical and subtropical grasslands and savannas (Behling & Lichte 1997). These events could promote the isolation of populations of *T. sidoides* subsp. *carnea* and the allopatric differentiation of the *grandense* morphotype. In northeastern Argentina, where the *mercedeño* morphotype occurs, owing to the rise of the Andes, tectonic movements and active faulting processes, which continued into the Quaternary, have played an important role in the shaping of the present landscape. Just after the tectonic uplift of the Mercedes plateau (140 m. a. s. l.), the Uruguay and Paraná Rivers took their present courses (Popolizio 1999). Also, the Quaternary climate oscillations favored changes in the relief and vegetation of the region (Carnevali 1994). The climate and vegetation of this area varied from dry conditions with xeric vegetation and mega / mesothermic grasses to more humid conditions with the presence of wetlands, forests, and palm groves. During the Quaternary, the xerophytic and the tropical and subtropical vegetation types would have suffered, alternatively, displacements following a NE-SW direction. During the last interglacial, more humid warm-temperate palm and mimosoid forests would have developed in the area (Barreda et al. 2007, Contreras et al. 2019). Such geomorphological and environmental events could have significant impacts on the distribution and abundance of populations of *T. sidoides* subsp. *carnea*, disrupting the subspecies range and confining some populations to a number of isolated regions with particular selective pressures, leading to the differentiation of the *mercedeño*

morphotype and both genetic groups by divergent evolution from an ancestral form. The presence of diploids, both in the morphotypes and the genetic groups of *T. sidoides* subsp. *carnea*, supports the hypothesis that the initial morphological and genetic differentiation of the complex occurred at diploid level (Solís Neffa 2000, Roggero Luque et al. 2015).

CONCLUSIONS

The present study provides strong evidence on the autopolyploid nature of *T. sidoides* subsp. *carnea*, which is in an active process of intrasubspecific diversification. Morphological and genetic data showed that the diversity of *T. sidoides* subsp. *carnea* is in congruence with the great past and present abiotic and biotic variability of the mid-latitude South American lowlands. The evolutionary history of this subspecies is consistent with past fragmentation and allopatric differentiation. Geographic isolation and local adaptation would have promoted strong morphological, ecological, and genetic differentiation, resulting in two morphotypes and different genetic groups indicative of incipient speciation.

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SUPPLEMENTARY MATERIAL

Appendix S1. Detailed information about the material of *Turnera sidoides* subsp. *carnea* examined in this study. *New ploidy level estimations.

Table S1. Altitude and bioclimatic variables analyzed in the geographical area of each morphotype of *Turnera sidoides* subsp. *carnea*. Basic statistics: average, standard deviation and range of variation of each trait. The variables selected for ENMs analyses are highlighted in bold letters.

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ERCILIA M.S. MORENO^{1,2}

<https://orcid.org/0000-0003-0517-2947>

GISELA M. VIA DO PICO¹

<https://orcid.org/0000-0003-4004-561X>

IVANA E. KOVALSKY^{1,2}

<https://orcid.org/0000-0002-2368-6135>

JUAN M. ROGGERO LUQUE¹

<https://orcid.org/0000-0002-9953-7937>

JOSÉ G. SEIJO^{1,2}

<https://orcid.org/0000-0001-8699-2044>

VIVIANA G. SOLÍS NEFFA^{1,2}

<https://orcid.org/0000-0002-7657-0806>

¹Instituto de Botánica del Nordeste (UNNE-CONICET), Sargento Cabral 2131, 3400, Corrientes, Argentina

²Universidad Nacional del Nordeste, Facultad de Ciencias Exactas y Naturales y Agrimensura, Libertad 5460, 3400, Corrientes, Argentina

Correspondence to: **Ercilia María Sara Moreno**
E-mail: emsaramoreno@gmail.com

Author contributions

E.M.S. Moreno conducted the study collecting materials in the field and making substantial contributions to acquisition, analysis and interpretation of molecular data as well as intellectual contribution to manuscript drafting. Gisela Via do Pico guide the ENM analysis and made contributions to the draft. I.E. Kovalsky participate of the samplings in the field and cytogenetic analysis. JM Roggero Luque participate in cytogenetic analysis and final manuscript revision. JG Seijo and VG Solís Neffa participate actively of the sampling and data acquisition in the field, in the final edition of the text, and reviewed critically the final version of the text adding a relevant intellectual contribution to the draft. VG Solís Neffa also participate of data acquisition in herbaria and was responsible for the settlement research projects, funded by ANPCyT-FONCyT, CONICET and SGCyT-UNNE, and which gave rise to the research.

