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RESEARCH ARTICLE

Exploratory karyological and genome size studies in Chilean *Sophora* species

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ABSTRACT

The genome of *Sophora toromiro* (Phil.) Skottsb. (*Papilionaceae*) is characterised by means of chromosome counts of accessions called Viña del Mar Botanical Garden (JBV), Göteborg (Got) and Titze. Karyotypes were obtained and genome size quantified using flow cytometry and compared to the closely related species *Sophora macrocarpa* J.E.Sm. and *Sophora cassioides* (Phil.) Sparre. No differences were detected in chromosome number of *Sophora* at intra-specific or inter-specific levels with $2n = 2x = 18$. Analyses on the symmetry coefficients CV_{cl} and M_{ca} shows affinity between the analysed material of the Titze line. The graph clearly indicates differences between *S. toromiro* lines if compared with *S. cassioides* and *S. macrocarpa*. Genome size range from 788 Mbp for *S. cassioides,* 795 Mbp *for S. macrocarpa* and a range of 796–808 Mbp in *S. toromiro*. This is the first report of chromosome number and genome size for *S. toromiro, S. macrocarpa* and *S. cassioides*. This is of particular importance in *S. toromiro*, as it is has been considered extinct in its natural habitat for the past 50 years.

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Chile; chromosome number; conservation; DNA; Easter Island; *Edwardsia*; genome size; *Sophora*

Introduction

The genus *Sophora* has an extensive distribution covering both northern and southern hemispheres (Peña et al. 2000). Genomic studies on several species of *Sophora*, including *S. microphylla* and *S. macrocarpa*, using sequences of intergenic regions of the chloroplast atpB-rbcL and transcribed spacers (ITS) revealed small genetic differences, indicative of a recent speciation of the genus (Hurr et al. 1999; Mitchell & Heenan 2002). In Chile, 60% of species are catalogued with conservation problems and these lie in island locations.

The species *S. toromiro* (Phil.) Skottsb., *S. cassioides* (Phil.) Sparre and *S. macrocarpa* J. E.Sm. belong to the genus *Sophora* and, in turn, are grouped in the section *Edwardsia*. This section is represented mainly in the South Pacific: the following species are all taxonomically related such as: *S. fernandeziana* (Phil.) Skottsb., *S. fernandeziana var. radeana* (Juan Fernández), *S. masafuerana* Skottsb. (A.Selkirk) all in Chile island area, *S. microphylla*

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Ait., *S. tetraptera* J.S.Muell., *S. chatamica* Cockayne, *S. fulvida* (Allan) Heenan and de Lange, *S. godleyi* Heenan and de Lange, *S. longicarinata* G.Simpson and J.S.Thomson, *S. molloyi* Heenan and de Lange (New Zealand), *S. chrysophylla* (Salisb.) Seem (Hawaii), *S. howinsula* (W.R.B.Oliver) P.S.Green (Australia), *S. denudata* Bory (Reunión Island), *S. rapaensis* H.St.John (Rapa Island), *S. raivavaeensis* H.St.John (Raivavae Island) and *S. mangarevaensis* H.St.John (Mangareva Island), (Mackinder & Staniforth 1997; Hurr et al. 1999; Peña et al. 2000; Mitchell & Heenan, 2002; Heenan et al. 2004).

In *Sophora* spp. section *Edwardsia*, chromosome counts and karyotype studies have been reported for *S. microphylla* Aiton (Rattenbury 1957), *S. fernandeziana* (Phil.) Skottsb. (Stiefkens et al. 2001) and *S. tetraptera* Mill. (Stiefkens et al. 2003) with until now only 30 counts reported for the entire genus (Stiefkens et al. 2003).

Sophora cassioides has a wide distribution in central Chile from Constitucion (35°31S, 72°41′ W) to south Puyuhuapi (44°19′ S, 72°33′ W). With the common name Pelu or Pilo it is a tree that reaches 10 m high, trunk 10–40 cm in diameter, branched, dark coffee–slightly rough bark. It is evergreen with imparipinnate rachis 8–12 cm long, glabrous or barely pubescent, 13 to 37 leaflets that may be ovate, oblong, elliptical or obovate 7–10 mm long and 4–6 mm wide. Its ecological distribution ranges between 5–900 m mainly in estuaries, rivers or ponds preferably in the shade (Rodríguez et al. 1983). *Sophora macrocarpa* is known as Mayo or Mayu. Growing in central Chile, from Coquimbo (29°57′ S, 71°20′ W) to Caburgua (39°12 S, 71°46′ W) (Martinez 1991), it is defined as a shrub or small tree up to 3 m tall with a thin stem, flexible branches and densely tomentose. It is perennial, with compound, imparipinnate leaves 10–15 cm long with 10–20 pairs of elliptical or oval leaflets. It grows in open, sunny places. A natural hybrid (Donoso 1975), it is apparently quite common in areas where populations or individuals grow sympatrically, further favoured by vectors such as *Bombus dalhbomii* (bumble bee) and *Sephanoides sephanoides* (hummingbird).

Sophora toromiro (Phil.) Skottsb. is a small tree approximately 2 m tall, with a stem diameter between 10 cm and 15 cm, sinuous trunk and branches that appear from the base. Branches are short, crooked with perennial compound leaves rachis pubescent. Leaflets 7–21 ovate elliptic, 9–12 mm long and 4–7 mm wide (Rodríguez et al. 1983). Considered extinct in its natural habitat, this species has great cultural value for the people of Rapa Nui (Espejo & Rodriguez 2013). Its ex situ survival is due to the collection of seeds in 1953 and 1956 from the last specimen growing in Easter Island (Ricci & Eaton 1997; Maunder et al. 1999). It has been reported that progenies from these collections have contributed with seeds to the present day (Espejo et al. 2008). The conservation of this species represents a major challenge with attempts to reintroduce it to its native habitat. From a biological point of view it is a species of interest, due to it is role in the speciation of the genus in the South Pacific (Skottsberg 1956; Sykes 1967; Hoeneisen et al. 1993; Peña et al. 1993; Peña & Cassels 1996; Ruiz et al. 1999).

The present study provides karyotype and genome size information on the currently recognised lines of *S. toromiro* (i.e. Viña del Mar Botanical Garden [JBV], Göteborg [Got], Titze), in comparison to *S. macrocarpa* and *S. cassioides*. Currently there are no published values for *S. toromiro* lines that could allow establishing a proposal for reintroducing this species into its natural habitat with certified authenticity of the plant material, considering its cytological variability.

Materials and methods

Seeds of two lines of *S. toromiro* (JBV and Got) were collected from open-pollinated cultivated material (Table 1). For the Titze line, seeds were obtained from two private collections (Tit, Tit*). For *S. macrocarpa* and *S. cassioides* seeds were collected from natural populations on the Rucamanqui farm, Biobío Region (37°11′ S, 71°43′ W). All seeds were germinated in the Forestal Mininco S.A. nursery, Los Angeles, Biobío Region, Chile.

Karyotypes

For karyotype studies, seeds were germinated by pretreatment with sulphuric acid, following the method of González et al. (2008). Roots was collected when radicles were 2 mm to 3 mm in length. The germination time was approximately 2.5 weeks post-sowing in Petri dishes lined with moistened paper at room ambient conditions.

These roots were immersed in a 0.002 M solution of 8-hydroxyquinoline for 24 h at 5 °C, using the method of Baeza et al. (2000). Tips were fixed in an ethanol:acetic acid (3:1 v/v) solution for a minimum of 30 min, followed by an acid hydrolysis with 0.5 N HCl for 20 min at 45 °C, and further washed with distilled water. Samples were mounted on a slide with a drop of 1% acetic orcein and crushed by applying pressure to the coverslip for later selection of intact cells with visible chromosomes. Observation, interpretation and analysis of chromosomes was made using a Zeiss Axioscope microscope coupled with a monochromatic camera. Measurements for karyotyping were made with MicroMeasure software (Reeves 2001). The resulting values were used to obtain indices of intra-chromosomal asymmetry:

$$A1 = 1 - \Sigma\left((b/B)n\right)$$

where b is the mean length of short and B the long arms in every homologous chromosome pair and n is the number of homologous chromosome pairs. A2 = (s/x) is the ratio between the standard deviation (s) and the mean chromosome length (x) (Romero Zarco 1986). The value for the intra-chromosomal index was $CV_{cl} = A2 \times 100$ (Paszko 2006). Similarly, the value for the index of mean centromeric asymmetry was M_{ca}= A $\times$ 100 (Peruzzi & Eroglu 2013).

Genome size

Genome size of the *Sophora* species under study was estimated using flow cytometry following the method of Galbraith et al. (1983). During preliminary tests fresh leaves were

Table 1. Description of the material of *Sophora toromiro* used in this study.

Provenance	Line	Collection	Observation (material)
Viña del Mar Botanical Garden (Chile)	JBV	Efrain Volosky (1953)	Seedlings from season 2004.
Göteborg (Sweden)	Got	T. Heyerdhal (1956)	Specimen delivered to Universidad Austral de Chile (1990) and propagated by grafting, 2008.
Santa Amara (Chile)	Tit	–	Seedlings obtained from Las Brujas Nursery 2002.
Sergio de la Cuadra (Chile)	Tit*	–	Material from seedlings established in 2005 at Valparaiso Region.

shown to be inappropriate for analysis due to the interference of a large amount of cytosolic compounds released upon chopping (as revealed by Loureiro 2007), and a portion of the whole stem was used instead. Therefore, approximately 50 mg of stem tissue of the sample species and 50 mg of leaves of the internal reference standard (*Glycine max* L., 2C = 2.5 pg DNA; Doležel et al. 1992) were co-chopped with a razor blade in a glass Petri dish containing 1 ml of WPB nuclear isolation buffer (0.2 M Tris.HCl, 4 mM $MgCl_2 \cdot 6H_2O$, 2 mM EDTA $Na_2 \cdot 2H_2O$, 86 mMNaCl,10 mM of sodium bisulphite, 1% [m/v] PVP-10, 1% [v/v] Triton X-100, pH 7.5 and stored at 4 °C; Loureiro 2007). The released nuclei were then filtered through a 50 µm nylon filter and 50 $\mu g.mL^{-1}$of propidium iodide (PI, Fluka) and 50 $\mu g.mL^{-1}$of RNase (Fluka) were added to sample tubes to stain the DNA and prevent staining of double stranded RNA, respectively. The sample was incubated for 5 min at room temperature and analysed in a Beckman Coulter EPICS-XL flow cytometer, equipped with an air-cooled argon laser operating at 488 nm. For each species, the instrument settings were established at the beginning of the analyses and kept constant throughout the whole experiment. Relative fluorescence intensity (FL) histograms were obtained and evaluated using the System II software (v3.0, Beckman Coulter). Also, FL vs. time and FL vs. side light scatter in logarithmic scale cytograms were obtained. In the latter graphic, a region of interest comprising mostly the isolated nuclei was defined. The FL histogram in linear scale was gated around this region. At least 1300 nuclei in both sample's and standard's G1 peaks were analysed per sample (Suda et al. 2007). For each taxon, five to six individuals were assessed. The holoploid genome size in pg (2C; sensu Greilhuber et al. 2005) of each individual was estimated using the following formula:

$$GS_{sample} = (GS_{1sample}/G_{1rs})^{*}\_GS_{rs}$$

where GS_{sample} and GS_{rs} are the genome size of sample and reference nuclei, respectively, and $G_{1sample}$ and G_{1rs} are the mean G_1 fluorescence of sample and reference nuclei, respectively. The obtained values were expressed in picograms (pg) and in mega base pairs (Mbp) using the formula by Dolezel et al. (2003) (1 pg = 978 Mbp).

Results

Karyotypes

Chromosome counts obtained from a total of 56 metaphase plates (Table 2) for all *Sophora* plants studied corresponded in all cases to $2n = 2x = 18$. These numbers

Table 2. Summary of metaphase plates studied for *Sophora* spp. and the studied coefficients.

Line/species	*S. toromiro* (JBV)	*S. toromiro* (Got)	*S. toromiro* (Tit)	*S. toromiro* (Tit*)	*S. cassioides*	*S. macrocarpa*
Metaphase plates studied	5	13	9	10	13	6
Chromosomes (2n)	18	18	18	18	18	18
M_{ca}	15.1 ± 2.2	14.8 ± 7.1	14.9 ± 3.9	15.2 ± 4.2	14.4 ± 2.5	13.7 ± 2.8
CV_{cl}	18.5 ± 5.4	14.7 ± 2.7	20.2 ± 3.76	20.2 ± 4.4	17.6 ± 3.9	17.0 ± 3.1
A1	0.26 ± 0.01	0.22 ± 0.02	0.30 ± 0.02	0.26 ± 0.05	0.30 ± 0.06	0.30 ± 0.17
A2	0.18 ± 0.05	0.15 ± 0.02	0.20 ± 0.03	0.21 ± 0.04	0.18 ± 0.04	0.17 ± 0.03

Mca, mean centromeric asymmetry coefficient (Paszko 2006); CVcl, variation coefficient of the centromeric index of chromosomes (Peruzzi & Eroglu 2013); A1, intra-chromosomal asymmetry index; A2, inter-chromosomal asymmetry index (Romero Zarco 1986).

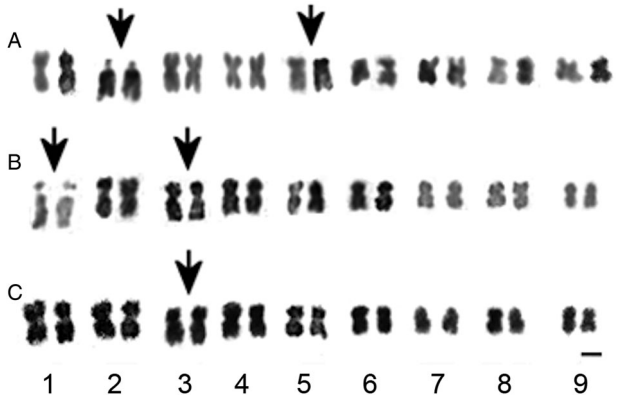

Figure 1. Karyotypes of **A,** *Sophora toromiro* (JBV); **B,** *S. cassioides*; **C,** *S. macrocarpa* (scale bar = 1 µm). Arrows indicate for *S. toromiro* (A) pair number 2 is subtelocentric and pair 5 is submetacentric; for *S. cassioides* (B) pair 1 with satellites and pair 3 is submetacentric; for *S. macrocarpa* (C) pair 3 is submetacentric.

were expected based on counts for other species of the *Edwardsia* section. The karyotype formula for *S. toromiro* is 7 metacentric (m) + 1 submetacentric (sm) + 1 telocentric (t); for *S. cassioides* it is 7 metacentric (m) + 1 satellite (sat) + 1 submetacentric (sm), and in the case of *S. macrocarpa* it is 8 metacentric (m) + 1 submetacentric (sm). (Figures 1–2).

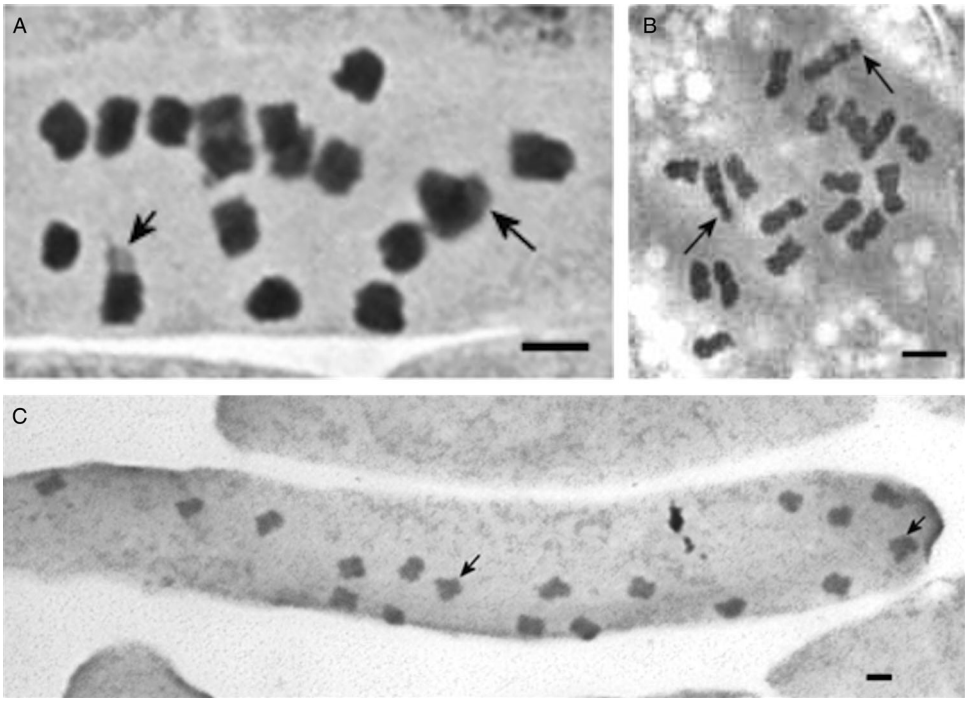

Figure 2. Metaphase plates showing: **A**, *Sophora toromiro* (Got line) pair 2 with satellites (scale bar = 2 µm); **B,** *S. cassioides* with satellites (scale bar = 2 µm); **C,** *S. macrocarpa* with submetacentric chromosomes (scale bar = 2 µm).

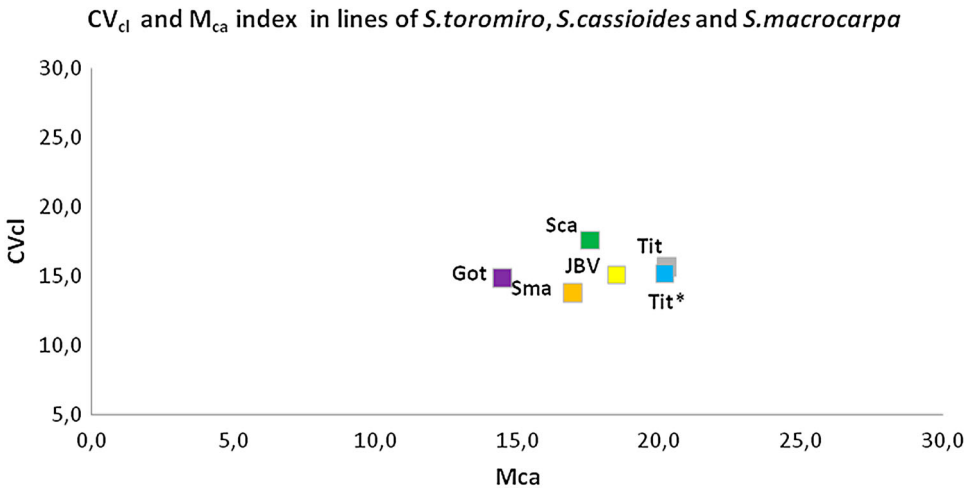

Figure 3. Mean dispersion of dots in axis of the indices CVcl, Mca belonging to the three lines of *Sophora toromiro* Goteborg (Got), Titze (Tit and Tit*), Viña del Mar Botanical Garden (JBV), *S. macrocarpa* (Sma) and *S. cassioides* (Sca).

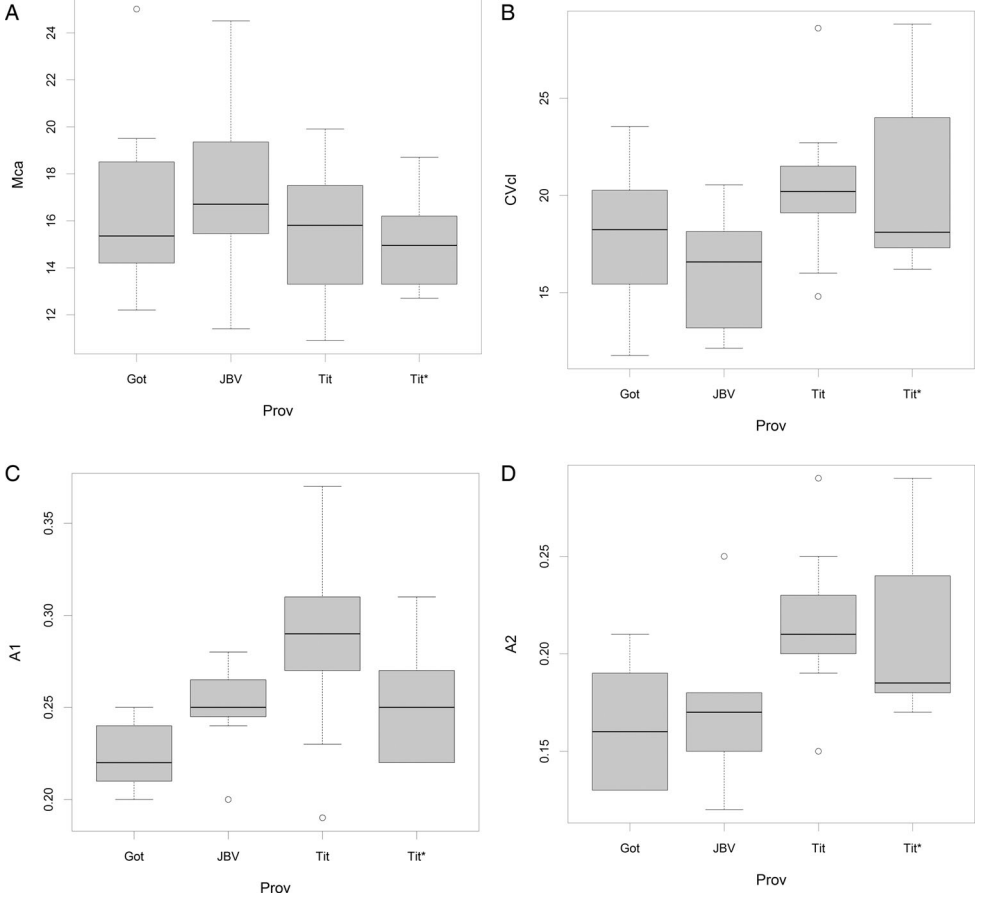

Figure 4. Box plots of *S. toromiro* lines for indices of intra-chromosomal asymmetry. **A–B,** For index Mca, CVcl; **C–D,** A1, A2.

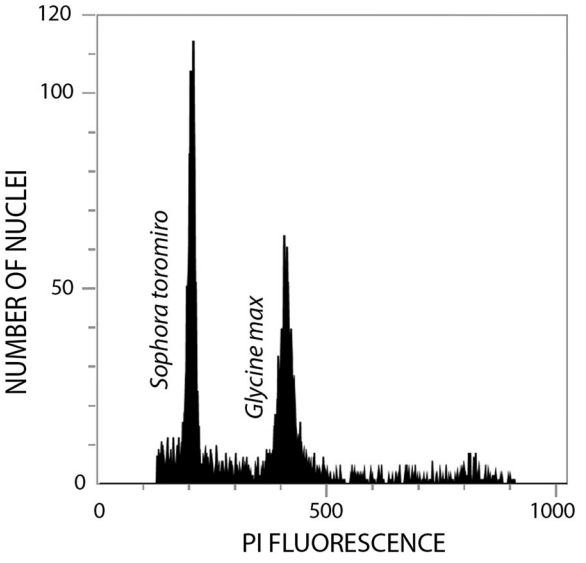

Figure 5. Histogram showing fluorescence of isolated nuclei areas in *Sophora toromiro* and *Glycine max*.

Data of mean coefficients of centromeric asymmetry (M_{ca}) and the variation of centromeric index (CV_{cl}) for the studied species of *Sophora* indicate only slight differences. Scatter plots of M_{ca} against CV_{cl}, demonstrate high similarity of progenies from the private collection called Titze (Figure 3).

The indices, M_{ca}, CV_{cl}, A1 and A2, were analysed statistically in order to verify similarities between them and comparison made using box plots (Figure 4). Only indices A1 and A2 showed significant differences between the studied lines of *S. toromiro*.

Flow cytometry

Considering the recalcitrant nature of the plant material of the three species of *Sophora*, an alternative plant tissue had to be used (i.e. the stems). The use of stems for the estimation of genome size has already been suggested as a valid alternative tissue in tree species (Anamthawat-Jónsson et al. 2010), and in our specific case it enabled us to obtain FL histograms with CV values always below 5% (mean CV value = 3.22%) (Figure 5).

The values of the genome size in *S. toromiro*, *S. cassioides* and *S. macrocarpa* expressed in megabase pairs (Mbp) ranged from 788 Mbp to 808 Mbp (Table 3), confirming the

Table 3. Genome size (2C in pg; 1C, in Mpb) of the species of *Sophora* from *Edwardsia* section analysed in this work.

		Genome size (2C/pg)			Genome size (2C/Mbp)
Species/line	Individuals (*n*)	Mean ± SD	Min.	Max.	Mean
S. toromiro	6	1.64 ± 0.02	1.61	1.68	804
JBV	1	1.65	–	–	808
Got	2	1.63 ± 0.02	1.61	1.64	796
Tit	3	1.65 ± 0.03	1.63	1.68	808
S. macrocarpa	5	1.63 ± 0.01	1.61	1.65	795
S. cassioides	5	1.61 ± 0.03	1.58	1.65	788

similarity of the genome size of the three species. These values are higher than those reported for *S. japonica* with 657 Mbp (Barow & Meister 2003) and much lower than those of *S. mollis* with 1999 Mbp (Ohri et al. 2004), a possible tetraploid with $2n = 4x = 36$ chromosomes.

Discussion

This work establishes that the number of chromosomes for *S. toromiro*, *S. cassioides* and *S. macrocarpa* is $2n = 2x = 18$. This result corroborates the degree of ploidy (i.e. diploid) found in other species belonging to the tribe *Sophorae* (Atchinson 1949, 1951, Rattenbury 1957; Hair & Beuzenberg 1966; Palomino et al. 1993; Stiefkens et al. 2003). The basic number of chromosomes places *Sophorae* in the same clade, together with the tribes *Euchresteae* and *Thermopsidae* (Lewis et al. 2005; Poggio et al. 2008).

There is a high morphological similarity in the karyotypes within the lines of *S. toromiro*. When compared to *S. cassioides* and *S. macrocarpa*, there are significant differences mainly in the first pair of chromosomes for *S. cassioides*, in which satellites can be observed, whereas in *S. macrocarpa* most chromosomes are metacentric with the exception of pair 3 which is submetacentric. In *S. toromiro* chromosome 2 is subtelocentric, whereas pair 5 is submetacentric. It is interesting to note that studies on the species *S. fernandeziana*, related to *S. toromiro*, present a complement of chromosomes that are mainly metacentric and submetacentric (Stiefkens et al. 2001). Similar results have been confirmed by Stiefkens et al. (2003) when the karyotype of *S. tetraptera* was described.

The chromosomes in the species in this study are very small (< 3 μm), typical of woody species (Stebbins 1971; Levin 2002; Sharma & Sen 2002). Regarding their symmetry it can be noted that the chromosomes of *S. macrocarpa* are more symmetrical than those of *S. cassioides* and *S. toromiro*. Again, it can be hypothesised that *S. macrocarpa* is the most distantly related of the *Edwardsia* section.

We found evidence of satellites in chromosomes of *S. cassioides* and, probably, *S. toromiro*. Interestingly, chromosome studies presented by Atchison (1949) indicated satellites in *S. tetraptera*, *S. microphylla* and *S. secundifolia* with no metacentric chromosomes.

The means of the indices (CV_{cl}/M_{ca}) for the studied species support the affinity of these taxa that comprise the *Edwardsia* section. In the case of the lines of *S. toromiro*, trends or groupings in the set of analysed individuals can be found, as is the case with the Titze line. For JBV and Got lines we found little differences. These registered collections come from the last specimen of *S. toromiro* in Easter Island, although the seeds were harvested and cultured in nurseries in different years (Ibañez et al. 2001). For samples catalogued as Titze, a very high similarity was found among the material from different localities. In both samples there were only slight intraspecific variations between individuals that comprise this population.

According to Romero Zarco (1986) the chromosomal asymmetry expressed in indices A1 and A2 are good parameters to differentiate the morphology of plant karyotypes due to evolutionary processes. The inter-chromosomal index A2 for JBV and Got with values 0.18 and 0.15, respectively, shows high similarity. In turn, Tit and Tit* lines present values of the order 0.20 and 0.21. In general the values are very similar but it is possible

that the differences may be due to hybridisation. According to Ricci & Eaton (1997), this suggests the possibility that the progenies of Titze origin are a product of hybridisations and introgression of cultured material with related species growing nearby. Indeed, hybridisation or introgression within the *Edwardsia* section is common and has been reported in both New Zealand and Chile (Donoso 1975; Heenan et al. 2001; Donoso 2004).

The small genome size for the studied species is in the range already observed in angiosperms (Greilhuber et al. 2006; Pellicer et al. 2010). Results from flow cytometry show that the values are very close between the studied species and it confirms the similarity of the genomes in these species already reported in the literature. Molecular studies using DNA sequences from *atpB-rbcL,* nrDNA and ITS (Hurr et al. 1999; Mitchell & Heenan 2002; Heenan et al. 2004), suggest a close affinity and evidence of recent speciation. Finally, because there is little information on comparative genome size studies of the genus *Sophora*, this work provides valuable new information to enable further studies related to speciation phenomena in the *Edwardsia* section.

The results from this study support Levin's (2002) suggestion that woody congeners show small differences in genome size compared with herbaceous plants. As there is little information on comparative genome size within the genus *Sophora*, this study provides valuable new information to aid future research related to both speciation and conservation in the *Edwardsia* section, where understanding relationships between species is of importance. Movement of species can lead to loss of rare species through interspecific hybridisation and subsequent swamping (e.g. *Leucaena* taxa; Hughes et al. 2007). However, the converse may also be true with such hybridisation/introgression providing vital new genetic diversity for a species which has been reduced to one or two individuals. Such is the case with *Trochetiopsis erythroxylon* (Rowe 1995; Rowe & Cronk 1995), a tree which was once common on the island of St Helena, but suffered drastic rapid decline due to over exploition for its timber and bark. Similar to *S. toromiro*, the last wild tree was the ultimate source of the few cultivated individuals, with inbreeding depression and a depauperate gene pool leading to poor growth and high mortality of cultivated specimens. Hybrids between cultivated trees of this species and *T. ebenus*, by contrast, were extremely vigorous and show a possible option for the survival for this part of the gene pool.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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