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Citogenética Vegetal y Animal en América Latina

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Pedro E. Jara Seguel

Nota del Editor General

La Citogenética -definida por Walter Sutton en los inicios del siglo XX- se desarrolló a partir de la convergencia de dos disciplinas originalmente separadas de las Ciencias Biológicas: Genética y Citología. Rápidamente se constituyó en una poderosa herramienta para resolver problemas biológicos sobre la base de la relación existente entre características genéticas y citológicas, principalmente cromosómicas. A través de los años, esta disciplina se fue enriqueciendo con los avances de las Ciencias Químicas y la Biología Molecular, hasta llegar a la actual citogenética molecular

Los cromosomas contienen la información genética y proveen los mecanismos para liberarla y regular la transmisión de la misma de acuerdo a un preciso programa o secuencia de eventos durante el desarrollo. Estas complejas actividades de los cromosomas han evolucionado a través de procesos naturales de mutación y selección debido al valor adaptativo que confieren a los organismos o poblaciones que los contienen. En este fascículo temático se presentan algunos de los problemas en plantas y animales que han sido abordados desde la citogenética por grupos de investigación de América Latina, con una diversidad de enfoques que van desde la citogenética clásica a la citogenética molecular.

Este fascículo fue co-editado por la Dra. Liliana Mola y el Dr. Julio Daviña, a quienes quiero manifestar mi más sincero agradecimiento por el trabajo realizado, que ha sido mucho y de excelencia.

Elsa L. Camadro

Julio de 2022

Note from the General Editor

Cytogenetics- defined by Walter Sutton at the beginning of the XX century- developed from the convergence of two originally separated disciplines within the Biological Sciences: Genetics and Cytology. Very rapidly, it became a powerful tool to solve biological problemas based on the existing relationship between genetic and cytological, principally chromosomal, features. Throughout the years, this discipline was enriched with the advancementes of the Chemical Sciences and Molecular Biology, until arriving at the actual molecular cytogenetics.

Chromosomes contain genetic information and provide mechanisms for its release and the regulation of its transmission according to a precise program or sequence of events. These complex activities of the chromosomes have evolved through natural processes of mutation and selection for the adaptive value that they confer to the organisms or populations that contain them. In this thematic issue, some of the problems in plants and animals that have been addressed from Cytogenetics by research groups of Latin America are presented, with a variety of approaches that range from classical to molecular cytogenetics.

This issue was co-edited by Dr. Liliana Mola and Dr. Julio Daviña, to whom I want to express my most sincere acknowledgement for the performed work, which has been much and of excellence.

Elsa L. Camadro

July 2022

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CYTOGENETICS OF CHILEAN LAND PLANTS (EMBRYOPHYTA): STATE-OF-THE-ART AND PROSPECTS

CITOGENÉTICA DE PLANTAS TERRESTRES CHILENAS (EMBRYOPHYTA): ESTADO DEL ARTE Y PERSPECTIVAS

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ABSTRACT

Chile is located in the south-western region of South America along the Pacific Ocean and contributes to the worldwide flora with *ca.* 6,120 species of Bryophyta, Pteridophyta, Pinophyta, Gnetophyta, and Magnoliophyta (1.9% of worldwide total species), exhibiting high endemism across all plant divisions. Little is known about the genetic diversity of Chilean land plants worldwide, including their cytogenetic and molecular characteristics. In 2012 we published the first state-of-the-art review in Cytogenetics of Chilean Angiosperms. The article gathered 78 publications from 1924 to 2010 accounting for approximately 139 species (2.8% of total Chilean species). The aim of this paper was to review the advances in cytogenetic studies of Chilean land plants, reporting additional cytogenetic data for species of four botanical divisions until 2020. Cytogenetic data were searched in the CPCD (Chilean Plants Cytogenetic Database). In total, we found 180 publications from both Chilean and foreign researchers. To date, cytogenetic data have been reported for 499 Chilean land plant species (8.2% of total) belonging to 244 genera and 117 families. In this context, the 2001-2020 period has been among the most productive regarding publications, with 74 available reports that include 163 additional species. Based on chromosome numbers, angiosperms and bryophytes registered the greatest diversity with 55 and 29 different $2n$, respectively; both divisions having the greatest number of studied species. Given the importance of increasing information on Chilean land plants, it is expected that more publications will contribute to the knowledge of their cytogenetic diversity in the near future.

Key words: chromosome banding, chromosome number, DNA C-value, karyotype morphology, polyploidy.

RESUMEN

Chile está ubicado en la región suroeste de América del Sur a lo largo del Océano Pacífico y contribuye a la flora mundial con aproximadamente 6.120 especies de Bryophyta, Pteridophyta, Pinophyta, Gnetophyta y Magnoliophyta (1,9% del total de especies en todo el mundo), que presentan un alto endemismo en todas las divisiones de plantas. Poco se conoce sobre la diversidad genética de las plantas terrestres chilenas en todo el mundo, incluidas sus características citogenéticas y moleculares. En 2012 publicamos la primera revisión sobre el estado del arte en Citogenética de Angiospermas Chilenas. El artículo reunió 78 publicaciones desde 1924 hasta 2010, que representan aproximadamente 139 especies (2,8% del total de especies chilenas). El objetivo de este trabajo fue revisar los avances en estudios citogenéticos de plantas terrestres chilenas, reportando datos citogenéticos adicionales para especies de cuatro divisiones botánicas hasta el 2020. Los datos citogenéticos se buscaron en el CPCD (Base de Datos Citogenéticos de Plantas Chilenas). En total, encontramos 180 publicaciones sobre citogenética de plantas terrestres chilenas, con datos citogenéticos para 499 especies (8,2% del total) pertenecientes a 244 géneros y 117 familias. En este contexto, el período 2001-2020 ha sido uno de los más productivos en cuanto a publicaciones, con 74 artículos disponibles que incluyen 163 especies adicionales. Basado en los números cromosómicos, angiospermas y briófitos registran la mayor diversidad, con 55 y 29 $2n$ diferentes, respectivamente; ambas divisiones tienen también el mayor número de especies estudiadas. Dada la importancia de incrementar la información sobre plantas terrestres chilenas, se espera que más publicaciones contribuyan al conocimiento de su diversidad citogenética en un futuro próximo.

Palabras clave: bandeo cromosómico, número cromosómico, valor C de ADN, morfología del cariotipo, poliploidía.

INTRODUCCIÓN

Continental Chile is located in the south-western region of South America (between 17°30'-56°30'S and 66°-75°W), but with additional insular territories along its coast such as islands and archipelagos distributed in the Pacific Ocean, among them Isla de Pascua, Archipiélago de Juan Fernández, Isla Mocha, and Isla Grande de Chiloé are the most representative. Geographically, continental Chile spans the dry desert to the north, the Andes mountain range to the east, the Pacific Ocean to the west, and Antarctica to the south (Moreira Muñoz, 2011). Along its latitudinal configuration Chile displays a mosaic of environments extending along the length of more than 4,329 km, with marked climatic north-to-south gradients. In addition, the longitudinal gradient of Chile is marked by differences in altitude (up to 6,000 m.a.s.l.) which give rise to microclimate variations and thus to environments that are favorable for plant growth. In fact, 55% of its territory is covered by vegetation of which 45% corresponds to floristic endemism. In addition, insular Chile is an interesting mosaic covering a surface area of 374 km^2, with an endemism that varies between 64% and 87%, depending on the island (Villagrán and Hinojosa, 2005; CONAF, 2013; Urbina Casanova *et al.*, 2015; Jara Seguel and Urrutia, 2018).

The total richness of land plants for continental and insular Chile has been estimated at *ca.* 6,120 species belonging to Bryophyta (1,457 species), Pteridophyta (167 species), Pinophyta (nine species), Gnetophyta (seven species) and Magnoliophyta (4,480 species) (Teillier, 2006; Hässel de Menéndez and Rubies, 2009; Müller, 2009; Stuessy and Baeza, 2017; Rodriguez *et al.*, 2018), thus representing *ca.* 1.9% of the worldwide flora which has been estimated at 330,000 species (Christenhusz and Byng, 2016; Villagrán, 2020).

Methods and techniques for the analysis of plant genome diversity have emerged over the decades, particularly the estimation of nuclear DNA content or C-value, a feature that has also been related to chromosome number and ploidy level (Bennet and Leitch, 2005a; 2005b; Leitch and Leitch, 2012; Pellicer *et al.*, 2018). Today, cytogenetics is recognized as a powerful tool to understand genetic variation processes, genome structure and dynamics, as well as evolution and speciation (Leitch and Leitch, 2012). Cytogenetics has also supported taxonomic circumscriptions in various plant groups (Widham and Yatskievych, 2003; Jara Seguel *et al.*, 2010; Jara Seguel and Urrutia, 2012; Guerra, 2012) and in several cases has related extant species with extinct species found in fossil records (Bonde *et al.*, 2004).

The first review reporting the state-of-the-art on cytogenetics of Chilean flora was carried out in angiosperms and it gathered information for approximately 139 continental and insular species (2.8% of total angiosperms) across 78 publications from years 1924 to 2010 (Jara Seguel and Urrutia, 2012). Recently, the flora of some protected wild areas in the southern zone has also been studied based on the diversity of chromosome numbers (Jara Seguel *et al.*, 2020). In addition, the flora of insular Chile has also been a focus of interest from a cytogenetic point of view, with a number of reviews reporting valuable information on chromosome number. The mechanisms behind cytological evolution have also given rise to various hypotheses in regard to the islands (*e.g.*, Juan Fernández Archipelago, Stuessy and Baeza, 2017). However, a large part of continental and insular Chilean flora has not been studied, while cytoevolutionary processes based on genome structure and dynamics are still poorly understood (Jara Seguel and Palma Rojas, 2021).

In this review we document the advances in cytogenetic studies of Chilean land plants, focusing our analysis on the number of publications on this subject, their taxonomic representation and geographical range, as well as the cytogenetic markers that were analyzed and their resolution to determine the genomic characters of the species. In this context, the diversity of Chilean land plants and its contribution to worldwide diversity is reported in this review.

Number of publications

One hundred eighty articles on cytogenetics of Chilean land plants have been published from 1924 to 2020 (Figure 1). The literature on cytogenetics has increased significantly in the last two decades, with 74 articles published between 2001 and 2020. This reflects not only the growing interest of Chilean researchers in the study of cytogenetic characters of the native flora, but also that of foreign researchers in studying chromosome variation in intercontinental and insular floras (Sanders *et al.*, 1983; Spooner *et al.*, 1987; Sun *et al.*, 1990; Lammers and Hensold, 1992; Rahman *et al.*, 2001; Hanson *et al.*, 2003; Kiehn *et al.*, 2005; Talluri and Murray, 2009; Zonneveld, 2012; Hizume and Kan, 2015; Souza *et al.*, 2015; Lujea and Chiarini, 2017; Sassone *et al.*, 2018). Notwithstanding, growth in cytogenetic contributions depends in part on the interest of specialized botanical journals in publishing the data. Thus, only 43 articles have been published in five Chilean journals since 1954, 19 of which correspond to the last decade (2011 to 2020), whereas most of the reports on the cytogenetics of Chilean land plants since 1924 have been published in foreign journals, in many cases authored by foreign cytogeneticists.

Taxonomic representation and geographical range

In our revised literature, listed in the version 4.0 of Chilean Plants Cytogenetic Database (Jara Seguel and

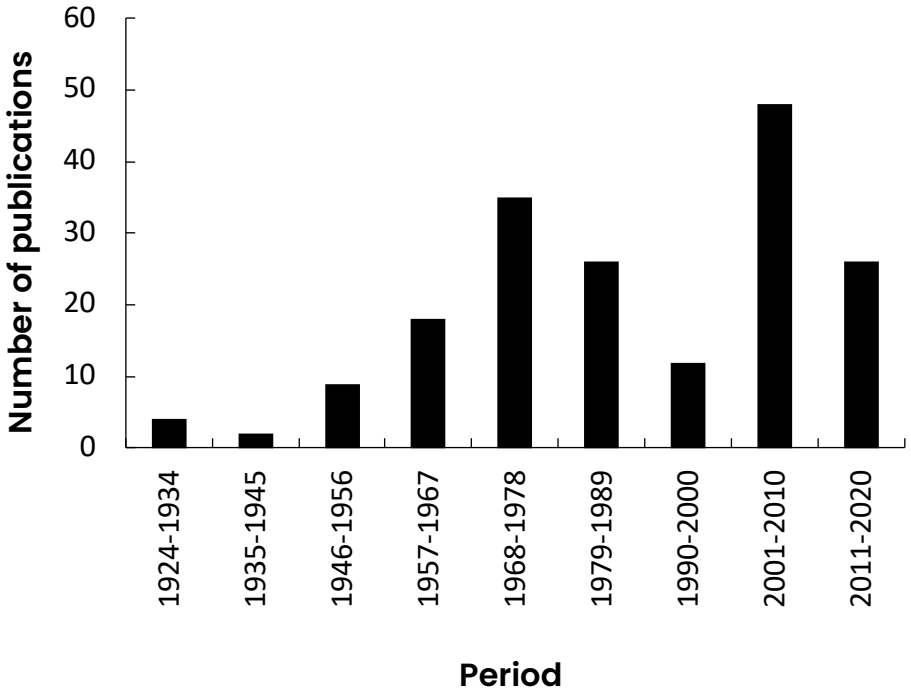

Figure 1. Number of publications on cytogenetics of Chilean land plants since 1924.

Urrutia Estrada, 2021), we found cytogenetic data for 499 Chilean species belonging to Bryophyta, Pteridophyta, Pinophyta and Magnoliophyta, which represent 244 genera and 117 families accounting for *ca.* 8.2% of total Chilean land plant species (Table 1). However, the number of studied species is likely to be higher than that reported here, particularly with regard to chromosome number data, due to the difficulty in compiling information from old sources of publication. A particular case is Bryophyta, for which little knowledge exists in Chile due to the scarcity of taxonomy specialists for this plant group. A similar situation has been observed in Pteridophytes although there is evidence of incipient advances in knowledge regarding chromosome number and polyploidy (Jara Seguel *et al.*, 2006; Morero *et al.*, 2015). With this in mind, reviews such as "Flora de Chile" (Rodríguez and Quezada, 1995; Rodriguez *et al.*, 2018), "Libro Rojo de la Flora Nativa" (Squeo *et al.*, 2001; 2008), "Plant Geography of Chile" (Moreira Muñoz, 2011), and contributions to the identification and biogeography of Chilean bryophytes (Müller, 2009; Larraín, 2007; 2010a; 2010b; Villagrán, 2020), may play a fundamental role in updating the taxonomic knowledge, in addition to increasing data on the geographic distribution, biogeography, endemism and conservation status of Chilean land plants.

The largest number of continental Chilean species that have been cytogenetically studied is present in a long latitudinal gradient from 17°30' to 56°S, with many of the taxa located within the continental biodiversity hotspots (Arroyo *et al.*, 2006). In addition, cytogenetic data of native flora from Juan Fernández Archipelago are also available and are included within insular hotspots (Stuessy and Baeza, 2017). There is data for only one species from Easter Island (Baeza, 1996) and for several Chilean species shared with the Falkland Islands in the southern tip of Patagonia (Moore, 1967). Currently, two frost-resistant angiosperm species have been described for Chilean Antarctica (at 62°S), *Colobanthus quitensis* (Kunth) Bartl. and *Deschampsia antarctica* E. Desv. (Molina Montenegro *et al.*, 2012), with karyotype and cytomolecular data reported for populations of *D. antarctica* included in the Argentinian flora (Cardone *et al.*, 2008; Amosova *et al.*, 2015).

An important number of taxa are undersampled in certain geographical extensions (*ca.* 91.8% of the total species), such as those from southern Chile along South Patagonia (>53° S) including continental and insular lands, as well as those from high altitudes in both the Nahuelbuta coastal mountains and the Andean range. Efforts should be focused on the cytogenetic study of these plant groups, paying special attention to either local endemic taxa or to highly specialized species adapted to their environment (*e.g.*, parasites, xerophytes, hydrophytes, halophytes, carnivorous). In the case of hydrophytes and marsh plants, a number of advances exist with regard to chromosome numbers described for species of nine genera, among

them, *Pinguicula* (2*n*=16, 26; Lentibulariaceae), and *Potamogeton* (2*n*=*ca.* 78, Potamogetonaceae) (Casper and Stimper, 2009; Chepinoga *et al.*, 2012). To date, *ca.* 455 species of hydrophytes and marsh plants have been described for the Chilean flora (Hauenstein, 2006).

Major cytogenetic characteristics that were studied here for Chilean land plants are summarized in Table 2. Chilean plants contribute to the diversity of the worldwide flora with an endemism that reaches between 45% on the continent and 64-87% in the islands. Along continental territories many species or genera are ancient, a large part of them proceeding from Gondwanan forests with a long evolutionary history, such as the austral Antarctic floristic element (*ca.* 20-55 Mya), or others proceeding from Neogene subtropical paleoflora (*ca.* 15 Mya). In the northern zone, modern floral elements are more recent in origin (*ca.* 4-15 Mya) (Scherson *et al.*, 2017). Various genera and families are currently shared with other geographical regions such as part of North America, Central America, and Australasia. In this way, Chilean flora acquires relevant value as a natural heritage that must be recognized and protected by the worldwide community in its different biological levels. Proof of their value is the designation of extensive Chilean areas as hotspots for worldwide biodiversity (Arroyo *et al.*, 2006).

Table 1. Divisions of land plants present in Chile with available cytogenetic data

	Number of families		Number of genera		Number of species	
Division	Total	Studied	Total	Studied	Total	Studied
Bryophyta[1,2]	119	41	366	63	1,457	95
Pteridophyta[4,3]	22	8	52	10	167	25
Pinophyta[4,3]	3	2	8	4	9	4
Gnetophyta[4,3]	1	0	1	0	7	0
Magnoliophyta[4,3]	160	67	970	167	4,480	375
Total	305	118	1,397	244	6,120	499

Number of families, genera and species present in Chile were taken from: [1]Hässel de Menéndez and Rubies (2009), [2]Müller F (2009), [3]Teillier (2006), [4]Rodríguez *et al.* (2018).

Table 2. Major cytogenetic characteristics studied in continental and insular Chilean land plants. Range, mean and standard deviation (SD) of chromosome number; ploidy; karyotype characteristics present within each division and DNA C-value in picograms (pg).

Division	Chromosome number (*2n*)		Ploidy	Karyotype characteristic		C-value (pg)
	Range	Mean (SD)		Symmetry classification	Organization	Range
Bryophyta	8-96	28.0 (14.6)	2, 4, 6, 8	--	--	--
Pteridophyta	66-480	110.8 (100.3)	2, 4, 8	--	--	--
Pinophyta	22-44	28.5 (10.5)	2, 4	symmetrical	bimodal	10.2-25.8
Magnoliophyta	8-114	28.4 (20.1)	2, 4, 6, 8	symmetrical, asymmetrical	unimodal, bimodal	0.3-34.7

Chromosome number and polyploidy

As a genome feature, the chromosome number includes the so-called A-chromosomes and B-chromosomes or supernumerary elements. The A-chromosome number is the most studied cytogenetic feature within Chilean land plants, while B-chromosomes have been found in three Chilean angiosperm species. In this work all 499 gathered land plant species have data on chromosome number, in many cases including subspecies, varieties or natural hybrids.

Chilean angiosperm species cytologically studied to date amount to 375 including 67 families and 167 genera, which represent 6.1% of total land plants and 8.4% of total angiosperms. The mean somatic chromosome number ($2n$) estimated here for Chilean angiosperms was 28.4±20.1 (n~14.2) ranging from 8 in some *Hypochaeris* species (Asteraceae), which are diploids, to 114 in the possible polyploid *Libertia chilensis* (Molina) Gunckel (Iridaceae) (Jara Seguel and Urrutia Estrada, 2021). This mean chromosome number is lower than the 32 previously documented including only 139 species (Jara-Seguel and Urrutia, 2012), therefore new data have made it possible to update the previously reported statistical records. In addition, this mean $2n$ number was similar to that described for plants from the Cerrado phytogeographic domain in Brazil (*ca.* $2n$ = 28, n~14; Roa and Telles, 2017) with 699 studied species. Both n numbers were lower than the gametic number (n=16) estimated for worldwide angiosperms, with a range that varied from 4 to *ca.* 640 (Soltis and Soltis, 2000; Leitch *et al.*, 2010). Several Chilean families show relatively high levels of cytogenetic variation with diverse basic chromosome numbers. In this context, cytoevolutionary mechanisms that modify the chromosome number have been described, such as, end-to-end fusions, Robertsonian traslocations, and polyploidization in families such as Alstroemeriaceae, Amaryllidaceae and Asteraceae, all studied on the basis of chromosome counts and karyotype morphology (Buitendijk and Ramanna, 1996; Weiss Schneeweiss *et al.*, 2003; Baeza and Schrader, 2005a; Jara Seguel *et al.*, 2006; 2010; Baeza *et al.*, 2007a; Palma Rojas *et al.*, 2007; Chacón *et al.*, 2012a).

B-chromosomes have been reported for three monocot species: *Alstroemeria angustifolia* Herb. subsp. *angustifolia, Alstroemeria hookeri* subsp. *recumbens* (Herb.) Ehr.Bayer (Alstroemeriaceae) both located between 32°S and 33°S in Central Chile (Buitendijk and Ramanna, 1996; Sanso, 2002), and *Lapageria rosea* Ruiz & Pav. (Philesiaceae) (Hanson *et al.*, 2003; Jara-Seguel and Zúñiga, 2004). *L. rosea* inhabits two distinct zones, at 30°S in northern Chile (Fray Jorge National Park) and from 33°S to 40°S in the forests of southern Chile. B-chromosomes are supernumerary elements (additional to the standard complement, or A-genome) that are present in *ca.* 1,300 plant species worldwide, and are mostly distributed in monocots or in plants with large genomes but low chromosome numbers (Camacho *et al.*, 2000; Houben, 2017). It is likely that B-chromosomes may have an adaptive role on a geographic scale (Peruzzi *et al.*, 2011) but they do not appear to have had a relevant role in the adaptation and evolution of Chilean angiosperms due to their low presence in the species studied to date (less than 1% of the species).

The fossil record suggests that angiosperms appeared *ca.* 145-125 Mya, with an increment in diversity between 112-96.6 Mya (De Bodt *et al.*, 2005; Leitch and Leitch, 2012). The origin of polyploidy in angiosperms has also been widely debated. The ancestral number may be x=7 and x=8, and various numbers higher than x=9 (*e.g.*, x=10, 12, 14, 19, 21) are suggested to be of ancient polyploid origin (Stebbins, 1971; Goldblatt, 1980; Soltis and Soltis, 2000; Soltis *et al.*, 2003; De Bodt *et al.*, 2005; Leitch *et al.*, 2010; Leitch and Leitch, 2012; Carta *et al.*, 2020). However, no evidence exists of a clear link between ancestral chromosome numbers and ancient polyploidization events, suggesting that further insights are needed to elucidate the organization of genome packaging into chromosomes (Carta *et al.*, 2020). In fact, several estimations suggest a wide variation in the percentage of polyploid angiosperms, ranging from 30% to 80% of the species studied worldwide, and molecular evidence points to ancient genome duplication at the base of monocots and dicots (Soltis *et al.*, 2003; Bennett, 2004; Leitch *et al.*, 2010). Bennett (2004) had already questioned this wide range, remarking that a difference of 40–50% represents over 100,000 species, which he considers an unacceptably high error rate for this key element of plant evolution. Nevertheless, these percentages may be still higher because a representative number of plant species have not been studied in various regions around the world according to current data, among them, flora from Chile (91.8%), New Zealand (20%), Italy (65%), Slovenia (40%), Poland (60%) and other countries (Peruzzi *et al.*, 2011; 2012).

Within the Chilean angiosperms, *ca.* 70 continental species were found to be polyploid (*ca.* 21.5% of the continental studied species), whereas for the endemic taxa of Juan Fernández archipelago the level of polyploidization is estimated to be *ca.* 66% (Sanders *et al.*, 1983). These data suggest that polyploidy has been a relevant evolutionary mechanism in Chilean oceanic islands, possibly providing greater adaptive capacity to survive in changing environments (Sanders *et al.*, 1983; Stuessy and Baeza, 2017). As an example of polyploidy within continental and insular Chilean angiosperm taxa, mention can be made of the tetraploidy present within the families Lamiaceae ($2n$=44, x=11), Onagraceae ($2n$=44, x=11), Asteraceae ($2n$=44, 80; x=11, 20; $2n$=*ca.* 94), Amaryllidaceae ($2n$=32, x=8), and the hexaploidy

present within Apiaceae (2*n*=48, *x*=8) (Covas and Schnack, 1946; Sanders *et al.*, 1983; Grau, 1987; Kiehn *et al.*, 2005; Talluri and Murray, 2009; Palma Rojas *et al.*, 2012) and Campanulaceae (2*n*=42; *x*=7) (Lammers and Hensold, 1992). However, the highest variation in ploidy levels is found within the Poaceae family with tetra, hexa, hepta, and octoploid species (2*n*=24, 36, 42, and 48, *x*=6) (Baeza, 1996). The polyploidy described for Chilean Poaceae is consistent with the estimation that 80% of all Poaceae described worldwide are polyploid, with events of whole genome duplication dating back 50-70 million years, close to the origin of the family (*ca.* 89 Mya) (Leitch *et al.*, 2010). Another particular case of polyploidy is the endemic genus *Leucocoryne* (Amaryllidaceae), where the tetraploid species 2*n*=18 and an exceptional case of 2*n*=19 have derived from cytotypes 2*n*=10 (*x*=5) by Robertsonian translocation and chromosome duplication (Crosa, 1988; Jara Arancio *et al.*, 2012; Souza *et al.*, 2015). To date different ploidy levels have been reported in various families of Chilean plants, but evolutionary implications of the polyploidy have not been studied in detail using cytogenetic and molecular methods. According to our dataset we estimated that *ca.* 21.5% of the continental species and *ca.* 63.1% of insular species studied so far are polyploids. This suggests that polyploidy has been a more important mechanism in the diversification of insular angiosperms than of continental ones, as described by Peruzzi *et al.* (2011) when comparing floras from a continental country (Italy) and an insular country (New Zealand). Up to now, only the *Leucocoryne* genus has been subject to estimations of polyploidy (Jara Arancio *et al.*, 2012; Souza *et al.*, 2015) and DNA C-value (Sassone *et al.*, 2018). This has provided an interesting framework to understand evolutionary trends, strengthened by phylogenetic studies of their chromosome and molecular evolution (Souza *et al.* 2015) and also by analyzing biome shifts in relation to climatic niche evolution along their distributional area (Jara Arancio *et al.*, 2013). We think that *Leucocoryne* may be good as a model plant to study evolutionary aspects regarding the origin of polyploidy due to the interesting genome data that has been documented so far.

In contrast to the quantity of cytogenetic data available for Chilean angiosperms, chromosome number information in gymnosperms is scarce, with only four Chilean species described to date. Three species are diploids such as *Austrocedrus chilensis* (D. Don) Pic. Serm. & Bizzarri, *Pilgerodendron uviferum* Florin (both 2*n*=22; Cupressaceae), and *Araucaria araucana* (Molina) K. Koch (2*n*=26; Araucariaceae) whereas *Fitzroya cupressoides* (Molina) I.M. Johnst. is tetraploid (2*n*=44; Cupressaceae) (Price *et al.*, 1973). *F. cupressoides* is a long-lived tree, dating back at least 4,000 years in some individuals, and inhabits from 39° to 43°S at an altitude of between 100 and 1,200 m.a.s.l. Currently the species inhabits areas which were glacial refuges during Quaternary glaciations and are a relic of continuous forests of that period. Besides, this species reaches great height and because of its slow growth requires optimal conditions for germination (Premoli *et al.*, 2000). In this regard, some hypotheses suggest that *F. cupressoides* may be allotetraploid, having originated by hybridization between the sympatric *A. chilensis* and *P. uviferum* (both 2*n*=22) (De Azkue, 1982). Unfortunately, no cytomolecular studies have been carried out to gather evidence to prove hybridization between *A. chilensis* and *P. uviferum* or/neither to corroborate genome duplication in *F. cupressoides*. Moreover, the findings regarding polyploidy in *F. cupressoides* support previous assumptions maintaining that Cupressaceae is one of the few conifer families having polyploid species (Leitch and Leitch, 2012). Despite the few species studied we estimated a mean chromosome number 2*n*=28.5±10.5 (*n*~14.2) for Chilean gymnosperms in a range between 2*n*=22 and 2*n*=44. The remaining five species have not yet been studied. Chilean gymnosperms date from Gondwana times, being related to species from the Australasia Region and in a number of cases with species from parts of Africa (Araucariaceae, Podocarpaceae) (Setogushi *et al.*, 1998; Scapa and Catalano, 2013), whereas Cupressaceae is the only family with a virtually worldwide distribution and is represented in all continents except Antarctica (Yang *et al.*, 2012). Gymnosperms had their origin *ca.* 290 Mya in the Carboniferous period with greater species diversity during the Mesozoic Era (Leitch and Leitch, 2012). To date, chromosome numbers for gymnosperms worldwide have been reported by various authors (Khoshoo, 1961; Marchant, 1968; Delevoryas, 1980; Hanson, 2001; Leitch *et al.*, 2001; Sedel'nikova *et al.*, 2011; Murray and De Lange, 2011), although the number of species has not been accurately determined. With this information in mind, we counted almost 150 species available in cytogenetic cadasters and estimated a mean chromosome number 2*n*=25.05±4.35. This estimation is consistent with the data previously reported by Murray (2013), who described a narrow range of chromosome numbers within the division (2*n*=14-66, with a predominant 2*n*=24). Chromosome numbers of the gymnosperms from New Zealand range between 2*n*=18 and 2*n*=38, and are represented by five families, three of which are shared with Chile (Araucariaceae, Cupressaceae, and Podocarpaceae) (Murray and De Lange, 2011) thus presenting greater variation and a higher number of studied species compared to Chile. Nonetheless, polyploidy is rare within the worldwide gymnosperm genera, with tetraploidy constituting the most evident level (36, 44, and 48 chromosomes) and octoploidy an extreme case reported in *Ephedra* (2*n*=56, *x*=7) (Sedel'nikova *et al.*, 2011; Leitch and Leitch, 2012). In addition, B-chromosomes have been reported for almost 11 species around the world (Sedel'nikova *et al.*, 2011) although not for the Chilean taxa.

Pteridophytes show notable cytological differences compared to both angiosperms and gymnosperms and are known to date from as far back as 400-380 Mya. Fossil records for the division have been obtained from rock strata belonging to the Silurian and Devonian periods of the Paleozoic Era. Currently, *ca.* 12,000 species are recognized worldwide (Pellicer *et al.*, 2018) of which *ca.* 167 species from various families and genera (the major part of Gondwanan distribution) are present in Chile (Rodríguez, 1995; Teillier, 2006; Rodríguez *et al.*, 2018). Chromosome number has only been determined for 25 Chilean species, across eight families and ten genera representing 14.5% of the total. We estimated a mean chromosome number $2n$=110.8±100.3 (n~55.4) for Chilean pteridophytes in a range between $2n$=22 (diploids) and *ca.* $2n$=480 (polyploids) (Jara Seguel and Urrutia Estrada, 2021). The n value is close to the gametic chromosome number described for homosporous ferns distributed around the world which is n=57 (Soltis and Soltis, 2000).

Many fern species from various families worldwide have been described as polyploids (Brownlie, 1958; Löve *et al.*, 1977; Roux, 1997; Bennett and Leitch, 2001; Obermayer *et al.*, 2001; Perrie *et al.*, 2003; Jara Seguel *et al.*, 2006; Hennequin *et al.*, 2010; Dubuisson and Schneider, 2010; Morero *et al.*, 2015). Ploidy levels might be higher in pteridophytes compared to angiosperms and gymnosperms, with an estimated percentage of polyploids up to 95% (Soltis and Soltis, 2000). In the case of Chilean ferns, some species show high ploidy levels, such as the octoploid *Polystichum subintegerrimum* (Hook. & Arn.) R.A. Rodr. ($2n=8x=328$, $x=41$; Jara Seguel *et al.*, 2006; Morero *et al.*, 2015), and the tetraploids *Polystichum plicatum* (Poepp. ex Kze.) Hicken ex Hosseus, *P. andinum* Phil., *P. chilense* (H. Christ) Diels, *P. multifidum* (Mett.) T. Moore, and *P. tetragonum* Fée ($2n=4x=164$; Morero *et al.*, 2015) all belonging to the Dryopteridaceae family. The high variation in ploidy levels described for Dryopteridaceae is striking and their phenotypic effects have been studied evaluating the so-called gigas effect on morphological features such as spore size (among other characteristics) which is larger in octoploid species from New Zealand than in similar tetraploids [*P. oculatum* (Hook.) J.B. Armstr. and *P. wawranum* (Szyszyl.) Perrie both tetraploid, and *P. neozelandicum* Fée an octoploid] (Perrie *et al.*, 2003). However, adaptive advantages of the morphological differences have not been explained although they may be related to the geographical distribution and climates in which each species inhabits. Tetraploidy has been described in *Asplenium dareoides* Desv. ($2n=4x=144$; Aspleniaceae), *Adiantum chilense* Kaulf. ($2n=4x=116$; Adiantaceae), *Hymenophyllum cruentum* Cav. [Syn. *Hymenoglossum cruentum* (Cav.) C. Presl], *H. caudiculatum* Mart., *H. fuciforme* Sw., and *H. ferrugineum* Colla (all $2n=4x=72$, $x=18$; Hymenophyllaceae) (Jara Seguel *et al.*, 2006; Jara Seguel and Urrutia Estrada, 2021). A particular case is the chromosome number of *Ophioglossum vulgatum* L. (Ophioglossaceae) with a meiotic n=240 (Verma, 1958), which coincides with a high somatic $2n$=480 (Krivenko *et al.*, 2017). Notably, a number of diploid species have strikingly high chromosome numbers, as is the case of *Equisetum bogotense* Kunth. (Equisetaceae) which is widely distributed in Southern Chile, with $2n$=216 (Jara-Seguel *et al.*, 2006), whereas other genera include species with a lower $2n$ number, among them *Hymenophyllum* ($2n$=22, 24, 26, 28), *Blechnum* ($2n$=66) (Blechnaceae) and *Megalastrum* ($2n$=82) (Jara Seguel and Urrutia Estrada, 2021). However, $2n$=66 and $2n$=82 are high diploid numbers in comparison with some Chilean angiosperms (up to $2n$=114) and gymnosperms ($2n$=22). Several of these high chromosome numbers present in the homosporous families Dryopteridaceae and Equisetaceae, $2n$=82 and $2n$=216 respectively, have been considered ancient polyploids, although studies based on isozyme analysis have shown that they are diploid, which has given rise to interesting debates among cytogeneticists and molecular biologists (Haufler and Soltis, 1986).

Bryophytes is a very diverse plant group with a total of 17,250 species recognized worldwide (Pellicer *et al.*, 2018) and their origin has been dated back to *ca.* 470-407 Mya since the Ordovician period according to fossil records (Cox, 2018). Chilean bryophytes are extremely diverse with *ca.* 1,457 species being recognized (Müller, 2009), many of which have been related to taxa from New Zealand (*ca.* 181 species) as well as to neotropical flora (Blöcher and Frahm 2002). Data on chromosome number are scarce for Chilean bryophytes, with only 95 studied species including liverworts and mosses (6.5% of the total, according to a list published by Fritsch, 1991) belonging to 41 families and 63 genera. The mean somatic chromosome number estimated here for Chilean bryophytes in general was 28.0±14.6 (n~14) with a range from $2n$=8 in the liverwort *Ricciocarpos natans* (L.) Corda (Ricciaceae) to $2n$=96 (possible octoploid) in the moss *Tortula muralis* Hedw. (Pottiaceae). Fritsch (1991) gathered data on chromosome number for 1,550 taxa of bryophytes distributed around the world, but did not provide an estimated mean chromosome number. Przywara and Kuta (1995) estimated in *ca.* 2,242 the species of bryophytes with known chromosome number worldwide, ranging from n=4 to n=10 in Anthocerotae, n=3 or 8 to n=48 in Hepaticae, and n=4 to n=96 in Musci. In the case of New Zealand flora, a mean somatic chromosome number of 19.4±9.04 was reported for 63 species of mosses (Peruzzi *et al.* 2014).

Based on our estimation, which is still preliminary, it is remarkable that the mean chromosome number of Chilean bryophytes (mostly mosses) is higher than that estimated for New Zealand species, despite the substantial bryophytic similarity between both floras.

Thus, the high richness of bryophyte species so far described for Chile may be correlated with a high diversity in chromosome number, although future studies covering a more extensive geographic span would be helpful to corroborate this assumption.

Karyotypes

Karyotype morphology has primarily been studied for angiosperms and only one gymnosperm species. In the case of pteridophytes and bryophytes, karyotype studies are still a pending task and the scarce studies available to date could be a consequence of the presence of large chromosome numbers and, in many cases, the existence of small chromosomes which make measurements difficult. Nevertheless, the use of computerized methods for image analysis can potentially facilitate studies in the near future such as those performed in various plant groups many years ago (Bauchan and Hossain, 2001; Munot *et al.*, 2011).

The first techniques used for obtaining the first karyotypes reported for Chilean plants, including the first known chromosome numbers, used histological sections of somatic tissues and male gametophytes (Whyte, 1929; Titov de Tschischow, 1954; Cave, 1966). Later, squash techniques were performed on root tip meristems treated with different antimitotic reagents, followed by fixation and stain procedures, all accepted within standard methods (Singh, 2003). Nomenclature for the description of chromosome morphology mainly follows Levan *et al.* (1964), which in many studies is combined with other methods to determine karyotype asymmetry (Stebbins, 1971; Arano and Saito, 1980; Romero Zarco, 1986; Paszko, 2006; Peruzzi and Eroğlu, 2013, Eroğlu 2015). In addition, interchromosomal relationships based on the ratio between the largest and shortest pair of chromosomes has provided valuable information on karyotype unimodality or bimodality.

Karyotype data have been collected for 84 angiosperm species belonging to ten families (Alstroemeriaceae, Amaryllidaceae, Asteraceae, Fabaceae, Krameriaceae, Luzuriagaceae, Myrtaceae, Philesiaceae, Poaceae and Solanaceae) all encompassed within the orders Asparagales, Asterales, Fabales, Liliales, Myrtales, Poales, Solanales and Zygophyllales. In several genera of Liliales –where the karyotypes are asymmetric and bimodal–, the largest chromosome pair is three to seven times longer than the shortest pair (*e.g.*, *Alstroemeria*, *Lapageria*, *Luzuriaga*) (Jara Seguel *et al.*, 2004; Jara Seguel and Zúñiga, 2004; Baeza *et al.*, 2010a; Jara Seguel *et al.*, 2010; 2021). The karyotype morphology for species of the families Alstroemeriaceae, Asteraceae, and Amaryllidaceae has been the most intensively researched in Chile, with various species and subspecies restudied using fluorescent methods (FISH, DAPI, CMA_3).

Alstroemeriaceae harbors *ca.* 204 species distributed in Central and South America, as well as in Oceania. In Chile, this family comprises 41 species within the genera *Alstroemeria, Bomarea* and *Luzuriaga*. Karyotype morphology has been described for 18 Chilean species (Jara Seguel and Urrutia Estrada, 2021; Jara Seguel *et. al.*, 2021). Alstroemeriaceae are known worldwide for their ornamental appeal due to their fleshy flowers and fruits, among which *Alstroemeria* is the most valued genus for which artificial interspecific hybrids have been produced.

Species identification in the *Alstroemeria* genus has been controversial and has been based mainly on morphological characteristics (Bayer, 1987; Muñoz Schick and Moreira Muñoz, 2003; Finot *et al.*, 2018a; 2018b). Karyotype studies have served to elucidate the taxonomy of the genus in addition to molecular and morphological analyses. For instance, thanks to karyotype morphology it was possible to confirm *Alstroemeria graminea* Phil. within *Alstroemeria* (Jara Seguel *et al.*, 2004), thus rejecting its inclusion within the monotypic genus *Taltalia* as proposed by Bayer (1998). A similar situation was described in the case of *Bomarea ovallei* (Phil.) Ravenna, for which karyotype morphology supplied additional cytogenetic data to morphology that allowed it to be classified within *Bomarea*, thus deleting the monotypic *Leontochir* (Hofreiter, 2006; Palma Rojas *et al.*, 2007). In addition, all *Alstroemeria* species are characterized by the presence of an asymmetrical and bimodal karyotype $2n$=16. Almost five species that inhabit arid and semiarid zones of Chile have the most asymmetrical and bimodal karyotype within the genus, whereas *A. ligtu* and *A. presliana* inhabiting humid zones in southern Chile, has more uniform chromosome sizes (Jara Seguel *et al.*, 2021). Intraspecific variation in karyotype morphology has also been found within the complex *A. hookeri* Sweet subsp. *hookeri* which has been studied in two localities, one inhabiting the coast near the Nahuelbuta mountain range and the other the Central Valley (Baeza *et al.*, 2010b). A particular situation has been recently published comparing *A. pulchra* Sims. subsp. *pulchra* and *A. pulchra* subsp. *lavandulacea* Ehr. Bayer chromosomes (Baeza *et al.*, 2018). In these subspecies, both components of the homologous chromosome pair 1 –a large metacentric typical of the *Alstroemeria* genus– showed a notable heteromorphism in size. However, the cytological explanation given for that chromosome heteromorphism is preliminary and its frequency in the populations of both subspecies was not mentioned. It would be useful to carry out meiotic studies analyzing bivalent configuration and to conduct sequential FISH banding or chromosome painting on these subspecies in order to elucidate mechanisms of chromosome change, and more specifically on those containing pair 1. In the case of the *Bomarea* genus the karyotypes are less asymmetrical and uniform in chromosome length than in *Alstroemeria* and *Luzuriaga*

(Palma Rojas *et al.*, 2007; Jara Seguel *et al.*, 2010; 2021). *Alstroemeria* and *Bomarea* grow in a wide range of environments along the length of Chile, ranging from arid and semiarid zones in the north to humid zones in the south (Muñoz Schick and Moreira Muñoz, 2003). *Luzuriaga* shows asymmetric and bimodal karyotypes, all $2n$=20, for three intercontinental species (Chilean and New Zealand species; Jara Seguel *et al.*, 2010). The three Chilean *Luzuriaga* species inhabit humid environments in the forest community of southern Chile, extending from 34°S to 53°S in Patagonia.

In the case of the cosmopolitan family Asteraceae, 927 species are found in the Chilean flora (Marticorena, 1990) and 18 species have been described in terms of karyotype morphology. To date, karyotype evolutionary trends among intracontinental and/or intercontinental taxa have been interpreted for the genera *Chaetanthera, Haplopappus*, *Hypochaeris*, *Grindelia*, and *Taraxacum* using different methods (Weiss Schneeweiss *et al.*, 2003; Baeza and Schrader, 2005a; 2005b; 2005c; Baeza *et al.*, 2006; Baeza and Torres Díaz, 2006, Baeza *et al.*, 2013). For example, for New World members of *Grindelia* and *Haplopappus* their evolution has not been accompanied by large karyotype changes, although small chromosomal rearrangements have been described and differences are highlighted based on number and asymmetry level (Baeza and Schroder, 2005b). In the case of *Hypochaeris*, general uniformity of their karyotypes and a stable chromosome number $2n$=8 have been described for South American species including Chilean taxa, though differences in the location of secondary constriction and chromosome size have also been reported (Weiss Schneeweiss *et al.*, 2003). Secondary constrictions and Nucleolar Organizing Region (NOR) location are well differentiated characteristics among four groups of species within the *Hypochaeris* genus (Weiss Schneeweiss *et al.*, 2003). Another less studied genus is *Chaetanthera*, which is native to South America and for which eight Chilean diploid species ($2n$=20, 22, 24, and 28) have been studied. These showed symmetric karyotypes with predominantly metacentric and submetacentric chromosomes (Baeza and Schrader, 2005c; Baeza *et al.*, 2012), with one species [*Ch. renifolia* (J. Remy) Cabrera] being tetraploid ($2n$=44) presenting a highly asymmetric and bimodal karyotype with predominance of subtelocentric and telocentric chromosomes (Baeza *et al.*, 2010c). Amaryllidaceae is represented in Chile by 114 species and 18 genera. Up to now, karyotype morphology has only been described for 42 species belonging to the genera *Famatina*, *Gethyum*, *Gilliseia*, *Ipheion*, *Latace*, *Leucocoryne*, *Miersia*, *Miltinea, Myostema, Phycella*, *Rhodophiala*, *Placea*, *Rodholirium, Solaria, Speea, Traubia,* and *Tristagma*. Seven genera of Amaryllidaceae show asymmetric karyotypes and similar chromosome morphology among species with scant variations (Baeza *et al.*, 2004; Baeza and Schrader, 2004; 2005a; 2005c; Baeza and Torres Díaz, 2006; Baeza *et al.*, 2007b; 2008; 2012; Cisternas *et al.*, 2010; Sassone *et al.*, 2018). *Leucocoryne* species have symmetric karyotypes and predominantly metacentric chromosomes, although telo- or subtelocentric chromosomes, and exceptionally submetacentric chromosomes are also present. It is worth noting that the total haploid set length (THL) estimated for *Leucocoryne* taxa (ranging from 77.5 to 147.5 µm in diploids $2n$=10, and from 98.7 to 315.5 µm in tetraploids $2n$=18) is higher than the values previously documented for a number of *Alstroemeria* taxa (2n=16, THL ranging from 53.9 to 112 µm) which have been described as having the largest genome sizes (C-values) within monocots (Jara Arancio *et al.*, 2012).

A number of hypotheses have been proposed to explain the origin and adaptive significance of the karyotype asymmetry and bimodality (Stebbins, 1971; Vosa, 2005). Karyotype bimodality is a feature that has been related to a specialized type of nuclear architecture that can be independent of the genetic status (White, 1973). According to our dataset for Chilean plants, karyotype asymmetry with or without bimodality versus karyotype symmetry are present in species of various unrelated families inhabiting different environments, whether arid or humid. So it is possible that rather than representing an evolutionary adaptation to environmental conditions as described above, these karyotype structures could be reflecting a certain degree of karyotype conservation retained from their respective ancestors. Nevertheless, minor or major modifications of such structures are related to adaptive changes during their evolution. In *Alstroemeria* species, changes in asymmetry and bimodality are correlated with variations in total chromosome length and DNA C-values, each of them related to a type of environment in particular (Buitendijk and Ramanna, 1996; Buitendijk *et al.*, 1997; Jara Seguel *et al.*, 2004; 2021). Sanso (2002) offers a preliminary explanation to this question in his studies of *Alstroemeria* species, suggesting that these characteristics may be due to karyotypic orthoselection or karyotype conservation.

Within the Chilean gymnosperms only the karyotype morphology of *Araucaria araucana* has been described (Hodcent, 1968; Bandel, 1970; Cardemil *et al.*, 1984). In general, the authors agree on the presence of 26 chromosomes of metacentric and submetacentric morphologies, symmetric and with wide variations in size, with large and small chromosomes within bimodal karyotypes. In addition, these karyotype characteristics of *A. araucana* are similar to those described for 14 species of the family Araucariaceae found along a distributional range in the southern hemisphere (Hizume and Kan, 2015). Gymnosperms live principally in the Andean and Nahuelbuta mountain slopes at altitudes between 1,000 and 1,600 m.a.s.l., although some small populations of *F. cupressoides* inhabit the Central Valley of southern Chile.

Classic banding and fluorescent methods

Different banding methods have been performed in Chilean angiosperms (Jara Seguel and Urrutia 2012, Jara Seguel *et al.*, 2021). To date, ten species of the Alstroemeriaceae family belonging to *Alstroemeria* and *Bomarea* have been studied by means of classic C-banding. In *Alstroemeria* species, the haploid relative length values of the C-bands vary between 2.0 and 6.5% (Buitendijk and Ramanna, 1996; Jara Seguel *et al.*, 2004), while for *Bomarea ovallei* (Syn. *Leontochir ovallei*) this parameter reaches 20% (Jara Seguel *et al.*, 2005). Within the genus *Alstroemeria,* considerable intraspecific and interspecific variation in C-band relative length and chromosome location of constitutive heterochromatin have been observed (Jara Seguel *et al.*, 2021). These features have proved to be additional to heterozygosity in the size and location of C-bands among homologous chromosome pairs in a number of species (Buitendijk and Ramanna, 1996). The presence of large C-bands has been co-related with large chromosome size and high nuclear DNA content, and these features have been associated with geographical distribution and climate on a latitudinal gradient in line with increases in total chromosome length (Buitendijk and Ramanna, 1996; Buitendijk *et al.*, 1997; Jara Seguel *et al.*, 2004; 2021). C-banding has been an important tool to describe genome complexity in a number of Alstroemeriaceae species. For this reason, and due to the use of conventional microscopy and low-cost reagents, the C-band technique is affordable for any laboratory and could be performed in more angiosperm families, thus providing further valuable data on genome structure and dynamics. This information may be fundamental for the application of other modern molecular techniques such as cloning, sequencing and *in situ* hybridization of either C-heterochromatin regions or ribosomal cistrons, all focused on gaining more in-depth understanding of the phylogenetic relationships among species. In the case of the Ag-NOR method, only *Rhodophiala laeta* (Amaryllidaceae) has been studied, for which active rDNA sites have been located in chromosomes (Jara Seguel *et al.*, 2012).

The application of fluorescent banding such as DAPI, CMA_3 and FISH has been a key step in the study of genome characteristics in Chilean species belonging to the genera *Alstroemeria*, *Chaetanthera, Grindelia*, *Haplopappus, Hypochaeris*, *Leucocoryne, Nolana*, *Placea*, and *Rhodophiala* (Kamstra *et al.*, 1999; Weiss Schneeweiss *et al.*, 2003; Zhou *et al.*, 2003; Baeza *et al.*, 2004; Baeza and Schrader, 2004; 2005a; 2005b; 2005c; Baeza *et al.*, 2007a; Chacón, 2012a; Souza *et al.*, 2015; Lujea and Chiarini, 2017). To this end, the Antarctic species *Deschampsia antarctica* has also been studied, resulting in a total sum of 24 studied species. These modern methods have revealed interesting details on chromosome structure, supporting species relationships for the majority of the genera. Interestingly, mechanisms of duplication of 5S rDNA sites exhibited wide variability in the chromosome distribution of a number of *Leucocoryne* species (Souza *et al.*, 2015). Hypotheses on genome evolution within *Leucocoryne* and *Alstroemeria* have been proposed and have also extended to phylogenetic reconstructions (*e.g.*, *Alstroemeria*, Chacón *et al.* 2012; *Leucocoryne*, Souza *et al.*, 2015).

Chilean gymnosperms have been scantly studied using banding methods. In the sole case of *Araucaria araucana,* fluorescent banding using chromomycin A_3 (CMA) and 4′, 6-diamidino-2-phenylindole (DAPI) has been reported to date (Hizume and Kan, 2015). Large and thick CMA-bands at the proximal region or secondary constriction of two long metacentric chromosomes have been described as a remarkable genome characteristic in *A. araucana.* In addition, several weak DAPI-bands have been observed at the interstitial and/or centromeric regions of some chromosomes. For this reason, most chromosome pairs were identified by means of chromosome shape and fluorescent banding pattern. The application of the Ag-NOR technique in *Araucaria araucana* has been crucial for the identification of the chromosome location of active rDNA (Cardemil *et al.*, 1984).

In general, the scarce available data obtained for Chilean plants suggests that fluorescent chromosome banding is focused on physical chromosome gene mapping of specific sequences or DNA fragments. This opens the way to comprehensive studies on genome affinities and dynamics (*e.g.*, meiotic chromosome behavior, chromosome rearrangement, rDNA location), where promising advances in genome structure and functionality can be achieved. However, much remains to be done in the future for a large number of Chilean species, in particular for those with unknown karyotypes, *e.g.* the allotetraploid *Fitzroya cupressoides,* a long-lived species that can reach up to 4,000 years old.

Nuclear DNA content

Nuclear DNA content, C-value and genome size are recognized as a strong unifying element in biology, having practical and predictive applications across many fields of science such as ecology, biogeography, physiology and embryology (Bennett and Leitch,1997; 2005b; Gregory, 2005; Kraaijeveld, 2010; Greilhuber *et al.*, 2010; Grover and Wendel, 2010; Leitch and Leitch, 2012; Pellicer *et al.*, 2018). Many authors have documented data on C-values including local and global floras of different continents, with *ca.* 12,000 species described (Leitch *et al.*, 2017; Pellicer *et al.*, 2018). In addition, phylogenetic reconstructions have facilitated the understanding of trends on genome size evolution in land plants, suggesting that each major group has been subject to different evolutionary or selective forces

(Leitch *et al.*, 2005; Smarda *et al.*, 2014; Carta and Peruzzi 2016) resulting in a remarkable diversity (Pellicer *et al.*, 2018).

The C-values of only 22 Chilean angiosperm species have been studied to date, representing less than 0.5% of the total studied worldwide (*ca.* 10,768 species, Leitch *et al.*, 2017). Within Alstroemeriaceae, seven species belonging to the genus *Alstroemeria* have been among the most studied, for which C-values ranged between 19.9 pg in *A. pulchra* subsp. *pulchra* and 34.7 pg in *A. ligtu* L. subsp. *ligtu* (Buitendijk *et al.*, 1997), which is additional to data available for *Bomarea salsilla* Mirb. (C=10.3 pg) and *Luzuriaga radicans* Ruiz & Pav. (C=6.6 pg) (Smarda *et al.*, 2014). It is worth noting that C-values of *Alstroemeria* species fall within the largest genome sizes of the Plantae kingdom (Sanso and Hunziker, 1998), although they are lower than the maximum 1C=152.2 pg described for monocots (Leitch and Leitch, 2012). Other studied genera are *Krameria* (Krameriaceae, Palma Rojas *et al.*, 2017), *Leucocoryne* and *Tristagma* (Amaryllidaceae, Sassone *et al.*, 2018). *Leucocoryne* shows C-values around 28-30 pg, although somatic values vary; for example, in diploid species 2C-values are around 56-60 pg, in triploids they are close to 86 pg, whereas in tetraploids the range spans from 115.6 to 121.8pg. In the case of *Tristagma,* 2C-values range from 33 to 35.5 pg in diploid species and close to 66.48 pg in tetraploids (Sassone *et al.*, 2018). On the other hand, *Lapageria rosea* has an intermediate C-value of 6.8 pg (Bennett and Leitch, 2005a), whereas small C-values (*Prosopis* 1C=0.4 pg, *Berberidopsis* 1C=0.3 pg, and *Fuchsia* 1C=1.46 pg) (Bukhari, 1997; Bennett and Leitch, 2005a; Talluri and Murray, 2009) have been described for the remaining Chilean genera studied to date. These values are lower than the average 1C=5.9 pg estimated for angiosperms (Leitch *et al.*, 2005; Leitch and Leitch, 2012).

Studies on C-values for Chilean gymnosperms are scarce, with data only available for the diploids *Araucaria araucana* (C=22.7-25.8 pg, Price *et al.*, 1973; Zonneveld, 2012), and *Austrocedrus chilensis* (C=21.7 pg, Price *et al.*, 1973), in addition to the allotetraploid *Fitzroya cupressoides* (C=10.2 pg, Price *et al.*, 1973). Note that this C-value of *F. cupressoides* is inconsistent with the possible genome duplication regarding the C-value of their putative parents *A. chilensis* and *P. uvirerum*, which, although not yet studied, are estimated to have a C-value close to 21 pg with 22 chromosomes. This situation suggests that more complete studies are required to understand the polyploidization process in *F. cupressoides*. Worldwide, C-values are known for *ca.* 421 gymnosperm species (*ca.* 25% of the total) with a mean C-value of 18.8 pg ranging between 7.2 and 36 pg. In general, the C-value variation in gymnosperms is low, being of conserved lineages due to their relatively narrow range of chromosome numbers, constancy in chromosome numbers and karyotypes within genera and families, rare polyploidy, and displaying the lowest recombination rates reported so far in any eukaryotic lineage (Pellicer *et al.*, 2018).

C-values have been scarcely studied for Chilean pteridophytes and bryophytes. At present only the horsetail fern *Equisetum bogotense* has an estimated C-value of 21.3 pg (Christenhusz *et al.* 2021). Given the high incidence of polyploids (octoploids up to 2*n*=480) or the presence of diploid species with high chromosome number (2*n*=66, 82, 216) within pteridophytes, C-value estimation might elucidate interesting aspects on genome structure and complexity. A similar situation may occur within the bryophytes, with high polyploidy (up to octoploidy) being frequent within Chilean taxa, as has been described for species from the northern hemisphere (Kuta and Przywara, 1997). Therefore, efforts should be focused on the use of methods for estimating C-values via flow cytometry (Voglmayr, 2000; Bennet and Leitch, 2001; Obermayer *et al.*, 2002; Hanson and Leitch, 2002) or image microdensitometry (Voglmayr, 2000; Palma Rojas *et al.*, 2017), thus increasing the data sets available worldwide for these diverse plant groups (Leitch *et al.*, 2017). At a global level, C-values have been estimated for 303 species of pteridophytes and 309 species of bryophytes (Pellicer *et al.*, 2018). Mean C-values for pteridophytes vary between 5.15 pg (in diploids) and 4.59 pg (in polyploids), whereas the mean C-value in bryophytes has been estimated at 0.49 pg (Bennett and Leitch, 2001).

Prospects

The high floristic endemism recognized so far for continental and insular Chile (45% in the continent and 64-87% in insular areas) has a significant world biodiversity heritage value. This characteristic has been favorable for the inclusion of large extensions of the Chilean territory –continental and insular– within worldwide biodiversity hotspots. However, as shown in this review, the scarce knowledge on cytogenetic characteristics suggests that a series of aspects related to the genome structure, dynamics and evolution of Chilean land plants are unknown for *ca.* 91.8% of the species, including a basic feature such as chromosome number. The knowledge gap is alarmingly deeper for many species, as neither chromosome morphology and mapping nor nuclear DNA content have been studied to date. Nonetheless, these knowledge gaps are a challenge for the future of cytogenetics in Chile. It is important to note that many native plant species have been studied in parallel using modern molecular methods such as DNA fingerprinting and gene sequencing or DNA barcoding, although no reports have been made in terms of the determination of total number of species and their taxonomic representation to date. Currently, important advances in genome knowledge on land plants from other

geographical areas around the world have facilitated the understanding of different levels of genome structure, complexity and dynamics, even in the field of ecology. Many of the Chilean plants still not included in that valuable knowledge could be of relevant importance to assess their adaptive capacity prior to the climate crisis that has been affecting Chile and other regions of the continent over the present decade. For these reasons, a collaborative effort between cytogeneticists and, if possible, a peer undertaking with molecular geneticists and evolutionary ecologists could help to develop an integrated strategy to accelerate progress and evaluate plant response to different ecological settings. This endeavor would obviously require funding and the onus is on state institutions to channel the necessary financial resources to accomplish this key task. In addition, training of more specialists in cytogenetics is necessary to address as many taxonomic plant groups as possible. The cytogenetic information provided here for Chilean native plants –from continental, insular and Antarctic zones– constitutes an important contribution to knowledge of the South American and worldwide flora, given the high endemism that not only encompasses its own genomes, but also highlights their close relationship with other floras such as those in Oceania, part of North America, and Central America, bringing them together as part of a floral kingdom which has evolved for millions of years resisting dramatic geological and climate changes such as have occurred in the Southern cone.

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A BIRD´S-EYE VIEW OF CHROMOSOMES DURING MEIOTIC PROPHASE I

UNA VISTA PANORÁMICA DE LOS CROMOSOMAS EN LA PROFASE I DE LA MEIOSIS

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ABSTRACT

The present review aims to summarize the research carried out in relation to meiosis in birds, especially by observing the protein axes of the chromosomes in prophase I of meiosis. This line of research, initially developed in Argentina, has provided key data in the study of the evolution of sex chromosomes and the mechanisms involved in the frequency and distribution of crossing over in birds, among other topics. Some of these contributions, in addition to those made by other authors, are described also providing the general theoretical framework or the hypotheses that support them.

Key words: crossing over, meiosis, sex chromosomes, synaptonemal complex

RESUMEN

La presente revisión tiene por objetivo resumir las investigaciones realizadas en relación a la meiosis de las aves, especialmente mediante la observación de los ejes proteicos de los cromosomas en la profase I de la meiosis. Esta línea de investigación, desarrollada inicialmente en Argentina, ha aportado datos clave dentro del estudio de la evolución de los cromosomas sexuales y los mecanismos involucrados en la frecuencia y distribución del *crossing over* en las aves, entre otros temas. Algunas de estas contribuciones, además de las realizadas por otros autores, se describen proporcionando también el marco teórico general o las hipótesis que las sustentan.

Palabras clave: complejo sinaptonémico, cromosomas sexuales, entrecruzamiento, meiosis

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During meiosis, a single event of DNA replication is followed by two rounds of cell divisions; as a result, haploid gametes are originated. Besides the function of reducing the DNA content by a half, key events of meiosis are the occurrence of chromosome synapsis and homologous recombination during the prophase of the first division. During prophase I, chromosomes remain in an organized, individualized state for an extended period and the biochemical complexes responsible for recombination at the DNA level are physically associated with underlying chromosome protein axes. After synapsis, these proteinic axes become part of the synaptonemal complex (SC) that tethers the homologs together until recombination intermediates are solved as crossovers (COs) or non-crossovers (Zickler and Kleckner, 2015). Because the SC is almost universally present among eukaryotes, labeling its protein components provides a framework to investigate the molecular factors involved in synapsis and recombination by cytological methods in a large variety of organisms. Electron microscopy and the use of immunofluorescent techniques in nuclei with preserved meiotic axes are especially useful for cytogenetic studies of early meiosis in organisms as different as yeasts and higher plants and animals (Ashley and Plug, 1998; Zickler and Kleckner, 1999; Rockmill, 2009; Stack and Anderson, 2009; Thomas and McKee, 2009; Ribagorda *et al.*, 2019; Cuñado, 2020). The formation of double-strand breaks (DSBs) that initiate recombination interactions, the number and distribution of crossover events, synaptic abnormalities in the presence of chromosome rearrangements, sex-chromosome specific features, and the time course of meiotic proteins in wild-type *vs.* meiotic knock-out organisms, are some of the features that can be analyzed by looking at meiotic chromosome axes or the SCs and their associated proteins (Ashley *et al.*, 1995; Baudat *et al.*, 2000; Merico *et al.*, 2003; Garcia-Cruz *et al.*, 2009; Phillips *et al.*, 2013; Anderson *et al.*, 2014; Sciurano and Solari, 2014). In the present work, the contributions of SC analyses to the knowledge on meiosis and cytogenetics in birds are reviewed. First, the main features of the mitotic karyotype of birds are described to give a background of the contributions of prophase chromosome studies to avian cytogenetics. An outline of the prophase I is presented in the second section to introduce specific terminology related to the chromosome axes and some of the proteins involved in synapsis and recombination. The third section features the behavior of the sex chromosomes in avian oocytes and a comparison between them and the XY pair of mammals. The last part summarizes the current knowledge on crossover rates in birds inferred from immunocytological analysis of pachytene chromosomes.

1. Main features of the avian karyotype

Most avian karyotypes have diploid chromosome numbers between 78-82 (Figure 1), with the presence of numerous microchromosomes (~30 pairs) that are often undistinguishable even with a combination of cytogenetic and genomic methods (Damas *et al.*, 2018; Kretschmer *et al.*, 2018). As a consequence, the regularly sized chromosomes have been broadly analyzed but microchromosomes remain largely uncharacterized. In this context of apparent stasis, there are karyotype variations that, at least in some cases, correspond to birds of the same taxonomic group. For example, diploid numbers higher than 100 have been scored in several species of Piciformes, but they also occur in other orders (Degrandi *et al.*, 2020). Raptors (Accipitriformes and Falconiformes) tend to have smaller chromosome numbers, from 48 to 66, resulting from fusions of ancestral macro- and microchromosomes. Low diploid numbers are also present among Psittaciformes, but differently from birds of prey, there is a marked difference of size between macro- and microchromosomes (Kretschmer *et al.*, 2018).

Comparative FISH mapping using whole-chromosome painting probes and locus-specific probes, as well as genomic studies at the chromosome level ("chromonomics") show the existence of extensive chromosome conservation, with a comparatively small number of interchromosomal rearrangements in species with diploid numbers from 78 to 92 (Shetty *et al.*, 1999; Griffin *et al.*, 2007; Ellegren, 2010; Romanov *et al.*, 2014; Kretschmer *et al.*, 2018). Together with a conserved karyotypic structure, species of birds separated by more than 80 million years of evolution show a high degree of evolutionary stasis at the levels of nucleotide sequence and gene synteny (Zhang *et al.*, 2014a). The chicken karyotype has served as the model for comparative chromosome and genomic studies and it is considered to be close to the ancestral avian karyotype (Griffin *et al.*, 2007; Damas *et al.* 2018). Even though specific probes for each chicken microchromosome were designed (Masabanda et al., 2004), they are not stable and, as a consequence, it is not possible to identify all chicken chromosomes in mitotic metaphases or meiotic cell spreads. Moreover, in the last build of the chicken genome sequence, five linkage groups are still not assembled (Warren *et al.*, 2017), pointing out the difficulties that high chromosome numbers impose on both cytogenetic and genomic studies.

All birds studied so far show female heterogamety and, consequently, the sex chromosomes are named Z and W. In most cases, the Z chromosome is the 4th in size and the W chromosome is comparatively small and heterochromatic. The extent of morphological

differentiation of the sex chromosomes varies throughout the avian phylogeny: less differentiated sex chromosomes are found in the basal Palaeognathae, especially ratites, while highly heteromorphic sex pairs are present in the rest of contemporary birds included in Neognathae (Ansari et al., 1988; Pigozzi, 1999). The cytogenetics of mitotic and meiotic chromosomes from ancestral avian groups, such as ratites and tinamous from South America, was essential to unravel the main steps of avian sex chromosome evolution (Fridolfsson et al., 1998; Pigozzi, 1999; Tsuda et al., 2007).

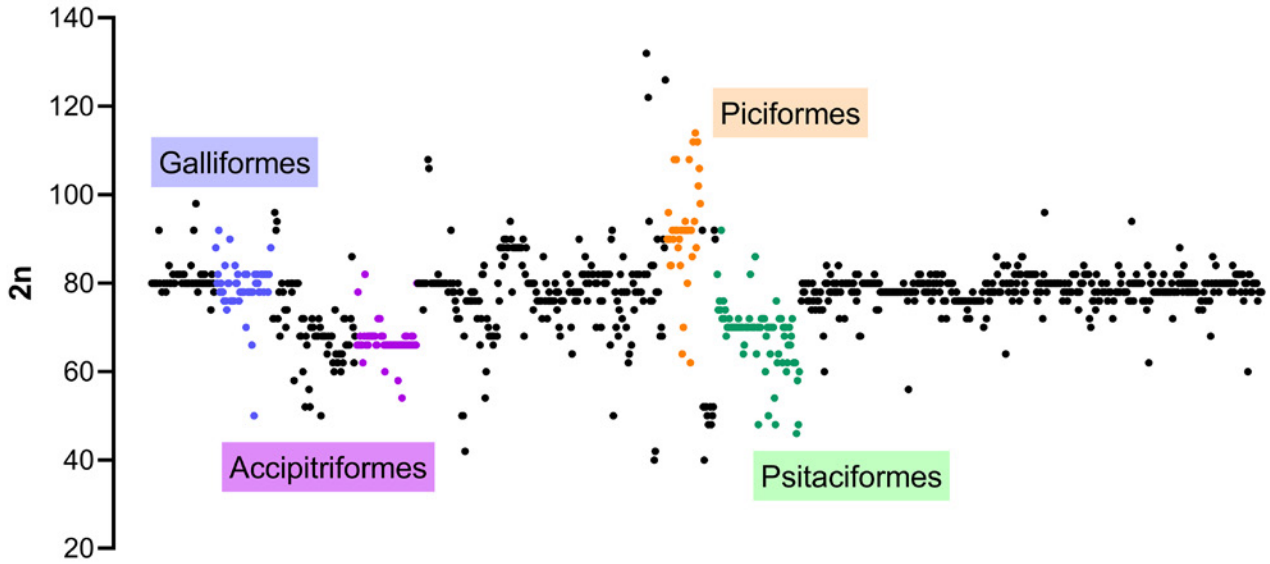

Figure 1. Diploid chromosome numbers in birds. Each dot represents the diploid number (2n) of a species. The groups displayed in colors illustrate orders in which most species have either the typical avian karyotype (Galliformes), or diploid numbers are often below or above the average. Data to build the graph were downloaded from the Bird Chromosome Database (BCD, 2021).

2. Visualization of prophase I events by immunocytology

Prophase I is divided into leptotene, zygotene, pachytene, diplotene and diakinesis. As chromosomes individualize and condense during early prophase (lepto-zygotene), the sister chromatids become organized along structures called axial elements (AEs). Axial elements from homologous chromosomes are "zippered" together by the insertion of the central region. At zygotene, the paired AEs are incorporated into the SC structure as part of the lateral elements (LEs). By the beginning of pachytene, the chromosomes achieve a state known as synapsis, when the four chromatids are aligned and held together by the SC. At this point, the SC consists of the paired LEs, and a central region comprised of transverse filaments distributed asymmetrically between the LEs and the central element, which runs midway through the central region. The meiotic axes components comprise axis-associated proteins, cohesin complexes and cohesin regulators. Homologous proteins have been identified in different species and are particularly well characterized in *Saccharomyces cerevisiae*, *Mus musculus*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, and *Drosophila melanogaster*. While the cohesin components have relatively high degree of homology, the axis-associated proteins are poorly conserved at amino-acid sequence level and were identified as homologs based on *in vivo* and *in vitro* data (Grey and de Massy, 2021).

One of the main mechanisms by which homologous chromosomes are maintained together involves crossing over, which is the result of recombination events that are initiated by DSBs (Gerton and Hawley, 2005). DSBs are introduced by the Spo11 protein, a relative of archaeal topoisomerase VI (Keeney, 2001). These breaks occur during leptotene, in coincidence with axial element formation and homolog pairing. In most organisms, meiotic DSBs are processed to produce single-stranded, recombinase-bound ends that search

for homologous chromosomes and give rise to double Holiday junctions (dHJs) (Sun *et al.*, 1989; Schwacha and Kleckner, 1995). The resulting recombinational interactions are biased to occur between homologous chromosomes, in contrast to somatic recombination, which occurs almost exclusively between sister chromatids (Hunter, 2015). A large number of DSBs are introduced throughout the genome and most of them are repaired following a pathway that gives non-recombinant products (Lam and Keeney, 2014). A subset of DSBs however, are repaired by the formation of one-ended strand-exchange intermediates, called single-end invasions (SEIs), which are considered the earliest detectable crossover-specific joint molecules (Hunter and Kleckner, 2001; Zhang et al., 2014b). The formation of SEIs is coincident with chromosome synapsis, reflecting the interdependence between the initiation of synapsis and the initial differentiation of crossover and non-crossover pathways (Figure 2A). Different organisms rely on different methods for this process of homolog matching and genetic exchange. In yeast and mice, recombination is necessary for recognition and pairing of homologous chromosomes, while other organisms such as *Caenorhabditis elegans, Drosophila melanogaster*, are not entirely dependent on recombination for homologous chromosome synapsis and, instead, recognition and pairing involve *cis* elements, such as heterochromatin, centromeres and pairing centers (McKim *et al.*, 1998; Mahadevaiah *et al.*, 2001; Gerton and Hawley, 2005; Baudat *et al.*, 2013).

Homologous axes and the SC were initially observed by electron microscopy in sections or microspreadings of meiotic nuclei in a large variety of organisms, including yeast, insects, plants, and vertebrates (Solari, 1981; von Wettstein *et al.*, 1984; Stack and Anderson, 2009). Following the identification of SC protein components (see Fraune et al., 2012 for a review of the mammalian SC), it was possible to develop specific antibodies that delineate the meiotic axes during prophase I (Figure 2B). Immunostaining of meiotic axes and other proteins can be used in combination with FISH probes for centromeres, telomeres, or individual chromosome sites, to evaluate the progression of prophase I, the behavior of specific chromosomes, or the time course of events leading to CO designation and resolution (Ashley and Plug, 1998; Sciurano and Solari, 2014; Dia *et al.*, 2017). For example, the protein MEI4, accessory to the endonuclease Spo11, shows the precise moment of DSB formation at leptotene; the complex Rad51/Dmc1 is one reporter of DSB processing and single-strand DNA invading ends; detection of MSH4 and MSH5, implies stable dHJs determined to be COs and, the MutS homolog, MLH1, is a component of late (recombination) nodules so its detection labels the sites of the CO events at pachytene (Ashley *et al.*, 1995; Baker *et al.*, 1996; Lynn *et al.*, 2007; Kumar *et al.*, 2015). The mentioned and many other proteins with key roles in synapsis and recombination are evolutionarily conserved. An advantage of this feature in cytology is that antibodies developed against a protein in a model species such as mice recognize the orthologue in other vertebrates or even in insects, opening the investigation of prophase I events in non-model organisms (Pigozzi, 2001; Viera *et al.*, 2004; Lisachov *et al.*, 2019). More specifically in birds, the immunolocalization of the protein MLH1 has been employed to count CO events on pachytene macro and microbivalents in SC spreads (Figure 3). As previously mentioned, the majority of meiotic COs follow pathways that are conserved in budding yeast, mammals and other organisms. The resolution of the produced dHJs requires the endonuclease activity of the MLH1-MLH3 DNA mismatch repair factor to be resolved exclusively into a CO product. In this conserved pathway, MLH1 protein tag a subset of CO events that show chiasmatic interference, while a second type of COs (non-interfering) follows a molecular pathway lacking MLH1 (reviewed in Pannafino and Alani, 2021). Therefore, the immunostaining for MLH1 labels most CO events, but a fraction escapes the detection with this methodology. Depending on the organism, non-interfering COs represent 5–30% of all CO events (de Boer *et al.*, 2006; Falque *et al.*, 2009). The presence of two classes of COs in birds is not confirmed, but both, recombination nodules and MLH1 foci, show CO interference (Pigozzi and Solari, 1997; Pigozzi, 2001). Comparative counts of CO markers at pachytene and diakinesis -MLH1 foci/ recombination nodules *vs.* chiasmata- in chickens, quails and pigeons show none or only slight differences within the same species supporting the view that MLH1 foci account for most CO in birds (Pigozzi and Solari 1999b; Pigozzi, 2001; Rodionov and Chechik, 2002; del Priore and Pigozzi, 2015).

SC spreads from birds are also useful to determine diploid numbers and to compare mitotic and meiotic (SC) sets. SC karyotyping revealed a germ-line restricted chromosome, first in the zebra finch (*Taeniopygia guttata*), and later in other songbirds (Torgasheva *et al.*, 2019), in a clear example that cytogenetic analyses of mitotic and meiotic chromosomes are not just a companion to genomic studies, but become necessary to provide a comprehensive understanding of any species genome.

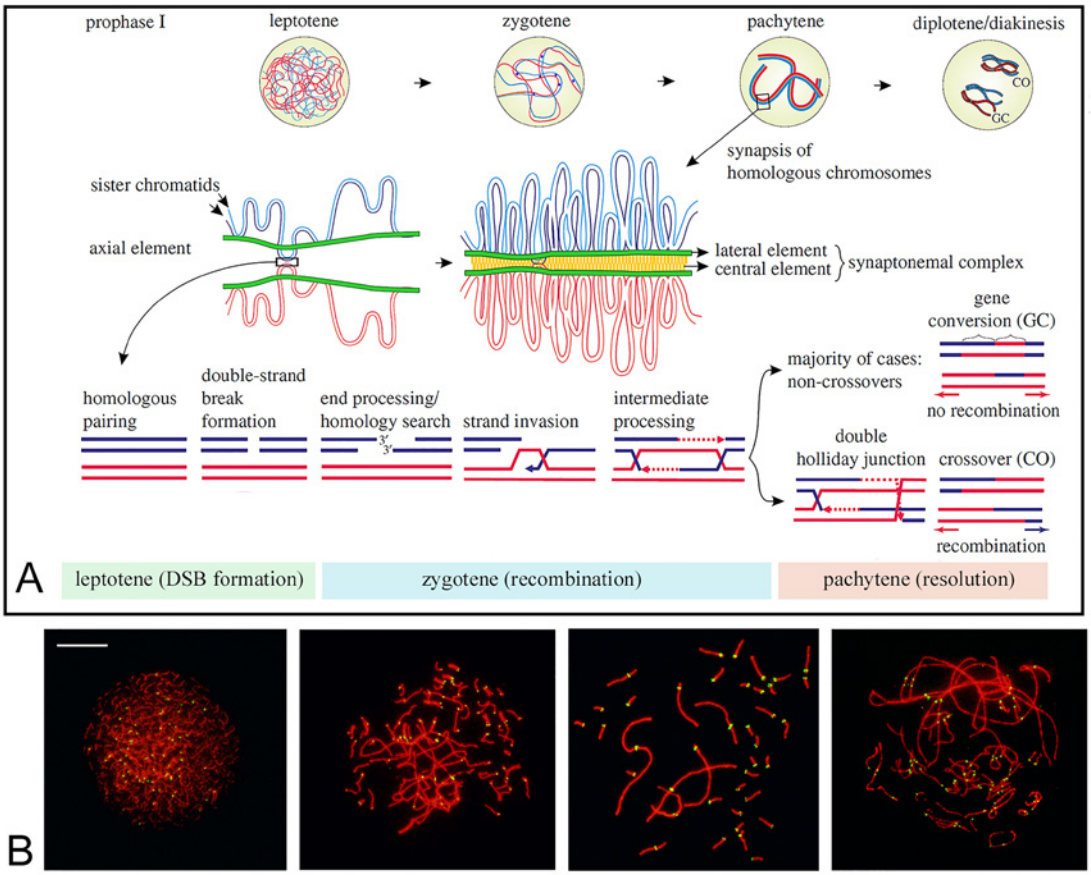

Figure 2. Chromosome axes and main events during meiotic prophase I. A. Chromosome organization during meiotic prophase I is exemplified with two pairs of homologous chromosomes, each split into two sister chromatids (red and blue lines). Following synapsis, the axial elements (in green) become the lateral elements of the synaptonemal complex. The bottom part of the figure shows the events of meiotic recombination at the DNA level. Meiotic recombination starts with the formation of double-strand breaks (DSBs) at leptotene and is completed before the end of pachytene. Reproduced with modifications from Mirzaghaderi and Hörandl (2016); CC by 4.0. B. Immunofluorescence staining of synaptonemal complex and centromere components on chicken oocyte spreads. From left to right the stages are leptotene, zygotene, pachytene and diplotene. An antibody against the cohesin component SMC3 was used to visualize chromosome axes (red) and CREST serum to label centromere proteins (green). Reproduced from del Priore and Pigozzi (2012); Copyright© 2012 Karger Publishers, Basel, Switzerland.

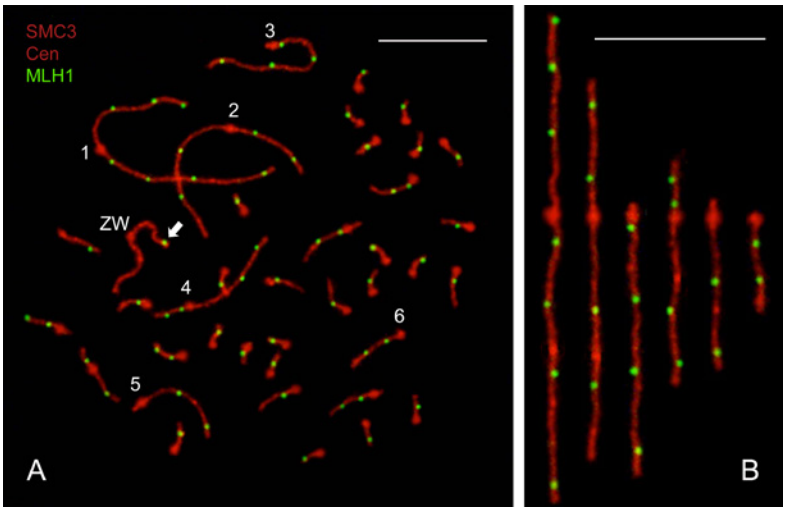

Figure 3. Immunolocalization of recombination events at pachytene. A. immunostained chicken oocyte showing the complete set of synaptonemal complexes labeled with anti-SMC3 and the crossovers detected with anti-MLH1. The ZW pair has a single MLH1 focus located near the homologous end of the bivalent (arrow). The six largest autosomal bivalents have a number next to the centromere signal (red protruding marks). B. The synaptonemal complexes of the six largest bivalents were digitally straightened to enable the comparison in size and position of the centromeres. Bars = 10 µm.

3. The meiotic ZW pair: similarities and differences with the XY pair of mammals

Like in mammals, female meiosis of birds starts during embryonic development. In the chicken, oocytes progress through early first prophase before hatching and most of them reach the pachytene stage around hatching day (Hughes, 1963; Smith *et al.*, 2008; Zheng *et al.*, 2009). A long and incompletely characterized diffuse stage is observed five days after hatching (Wylie. 1972); this stage is followed by the reemergence of individualized chromosomes in the form of lampbrush bivalents that are recognizable in the growing intrafollicular oocytes after 2–3 weeks of post-hatching development, remaining visible in the adult ovary of hens (Gaginskaya and Chin, 1980; Hutchison, 1987).

The synaptic and chiasmatic nature of the ZW pair was first demonstrated in the chicken after staining of SCs and recombination nodules for electron microscopy (Solari, 1977; Rahn and Solari, 1986a). Subsequent studies in other birds with highly heteromorphic sex pairs led to characterization of the meiotic behavior of the sex bivalent, consisting of: a) the formation of a fully synapsed bivalent despite lack of homology in most of the Z and W length; b) the occurrence of a single recombination event at subtelomeric position; c) the existence of a synaptic adjustment of the length of the Z and W axes, a process that now is known to occur in heteromorphic sex chromosomes of other organisms; and, d) the lack of heteropicnosis or condensation of the sex bivalent chromatin (Solari, 1992; Solari and Pigozzi, 1993; Pigozzi and Solari, 1999a). From the observations of the Z and W meiotic axes and the associated chromatin, it was inferred that the sex pair of birds and mammals shared some similarities -*e.g.*, the existence of a pseudoautosomal region, PAR- but also had fundamental differences accordingly to their evolution from different ancestral autosomes in each lineage (Solari, 1993; Bellott *et al.*, 2017).

The empirical and theoretical research about sex chromosome differentiation and the evolutionary forces that shape this process have been reviewed in depth by experts in the field (Rice, 1984; Charlesworth, 1991; Charlesworth *et al.*, 2005; Bachtrog *et al.*, 2014). Here, a brief background is provided for better understanding the role of the meiotic ZW pair analysis in the research on avian sex chromosome differentiation. Heteromorphic sex chromosome pairs are thought to have originated as autosomal homologs following the random acquisition of a mutation that transformed a gene into a sex-determining locus (Rice, 1984; Charlesworth, 1991; Charlesworth *et al.*, 2005; Bergero and Charlesworth, 2009). Recombination suppression may initially encompass only a small chromosomal segment surrounding that locus, but then progressively spreads along Y or W chromosomes. A consequence of this recombination suppression on the Y/W chromosomes is the loss of almost all functional genes present on the ancestral chromosome except for a few loci, often with sex-specific functions, and the acquisition of repetitive sequences and heterochromatin in the non-recombining region of the Y/W (Daish and Grützner, 2019). The region of the sex chromosomes that retains homology is referred to as PAR because this chromosomal segment recombines in both sexes and is functionally not hemizygous in the heterogametic sex (Burgoyne, 1982; Ellis and Goodfellow, 1989). One way to demonstrate homology is to determine the extension of the recombining segment in the sex bivalent of the heterogametic sex, for example using cytogenetic markers of crossing over.

In contrast with mammals, where homomorphic XY pairs are not found, nearly homomorphic ZW pairs are present in all ratites, a group of palaeognathus birds that includes ostriches in Africa, rheas in South America, emus in Australia, cassowaries in Australia and New Guinea, and kiwis in New Zealand. Mapping of recombination nodules/MLH1 foci in SC spreads in both species of rheas showed the existence of homologous recombination over most of the W chromosome, and therefore the presence of a large PAR (Pigozzi and Solari, 1997; Pigozzi and Solari, 1999c; del Priore and Pigozzi, 2017). The homology in the ZW of rheas comprises over 70% of the Z chromosome length, as assessed by recombination nodule and MLH1 focus mapping. Instead, these recombination markers are restricted to a small terminal segment -less than 5% of the Z chromosome- in birds with highly differentiated sex chromosomes (Solari and Pigozzi, 1993; Pigozzi and Solari, 1998b; Pigozzi and Solari, 1999b). Along with ratites, Palaeognathae also includes the monophyletic group of tinamous, that are ground-dwelling birds found in Central and South America. Cytogenetics of female meiotic prophase and sequence comparison of the Z and W chromosome within this avian group revealed important insights into the sex chromosome evolution. Recombination nodule and MLH1 mapping in tinamous uncovered examples of intermediate stages of sex chromosome differentiation (Pigozzi and Solari, 1999c; Pigozzi and Solari, 2005; Pigozzi, 2011). In three of the four tinamid species where meiotic recombination was examined, the PAR is larger compared to neognaths, but shorter compared to ratites, while recombination is restricted to a small segment, comparable to the chicken, in the tataupa tinamou (Figure 4). These results implied different paths of genetic degeneration and the probable existence of different evolutionary strata in the ZW pair of birds. Analyses of sequence read depths between the Z/W relative to autosomes demarcated the PAR and the non-recombining differentiated region in 17 species spanning the entire avian phylogeny (Zhou *et al.*, 2014). The non-recombining regions between

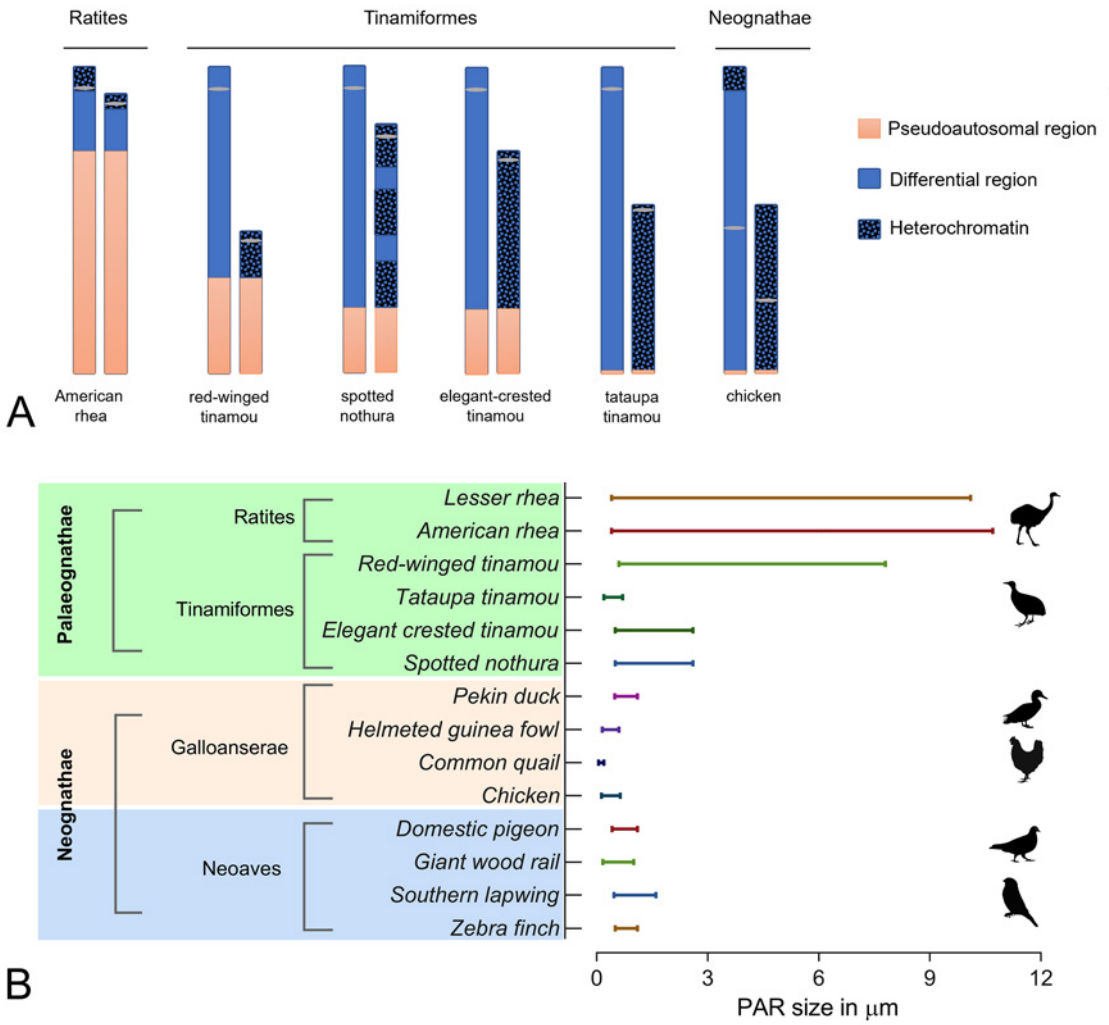

Figure 4. Steps of the sex chromosome differentiation among living birds. A. Spectrum of sex chromosome morphologies in primitive and modern birds. The relative size of the pseudoautosomal region (PAR) in each ZW pair represents the segment with recombination nodules or MLH1 foci in the meiotic bivalent at pachytene. The differential regions are represented in blue, with a black pattern when heterochromatin is present in the C-banded mitotic chromosomes. The ZW pair of chicken is upside down to locate the homologous end towards the same side as in the other species. B. Comparative graph of the PAR sizes in Palaeognathae and Neognathae. Each line represents the size of the PAR in µm, from the homologous telomere of the ZW pair to the more distal recombination nodule or MLH1 focus in each species.

Z and W of many species exhibit a complex pattern of "evolutionary strata" resulting from the suppression of recombination in a stepwise and independent manner among some lineages. Another finding of this study is that the W chromosome sequence in some Neognathae is not completely degenerated and that they have relatively long PARs compared to the chicken where only 28 of the 685 ancestral genes remain on the W chromosome (Bellott *et al.*, 2017). Remarkably, recombination analysis in pachytene also predicts different PAR sizes among Neognathae, even within closely related species (Figure 5). For example, the position of the single recombination nodule in the ZW pair of the domestic quail indicates a smaller recombining segment compared to that of the chicken, and the recombining segment in Southern lapwing (*Vanelus chilensis*, Charadriiformes) is larger compared to that of other neognaths, a feature that was also reported for two other species of the same order (Lisachov *et al.*, 2017).

The sex chromosomes of birds and mammals originated independently from different ancestral autosomes after the two lineages diverged more than 300 million years ago (Daish and Grutzner, 2009; Zhou *et al.*, 2014). The ZW and XY pairs are not orthologous: genes that are sex linked in birds are autosomal in mammals, and vice versa (Nanda *et al.*, 1999; Bellott *et al.*, 2010). During this parallel evolution and resultant to the morphological differentiation process, birds and mammals developed different tolerance to the presence of unpaired, non-homologous segments of the sex chromosomes during the first meiosis. The differential regions of the X and Y chromosomes of mice and other therian mammals remain largely unsynapsed at prophase I, and their chromatin is

condensed forming the XY-body observed in pachytene spermatocytes; instead, the ZW pair of birds undergo complete synapsis and lack any sign of condensation at pachytene (Solari, 1974; Pigozzi, 2016). These differences may respond to different ways to deal with unsynapsed heterologous chromosome segments during gametogenesis. In eukaryotes, an ancestral and evolutionarily conserved response is induced when any pairing problem between homologous chromosomes results in asynapsis. This response involves the silencing and epigenetic modification of the unpaired segments, a phenomenon called meiotic silencing of unpaired chromatin (MSUC) (reviewed in Turner *et al.*, 2005). Its manifestation on the sex chromosomes is the meiotic sex chromosome inactivation or MSCI that occur during prophase I in mammalian spermatocytes due to the unsynapsed heterologous segments of the X and Y. While MSUC is an ancient response to unpaired DNA and part of a checkpoint, MSCI is restricted to the sex chromosomes of therian mammals (Turner *et al.*, 2005; Turner, 2007). A prominent epigenetic marker of MSCI is the histone variant γ-H2AX which decorates the chromatin domains of the asynaptic regions of the X and Y chromosomes at pachytene in marsupials and eutherians (Fernandez-Capetillo *et al.*, 2003; Turner *et al.*, 2004; Franco *et al.*, 2007). Failure of MSCI results in the activation of a pairing checkpoint that ultimately causes spermatocyte elimination by apoptosis, and therefore this phenomenon has direct relevance to the cause of genetic disease and fertility (Turner *et al.*, 2006; Sciurano *et al.*, 2012). In the chicken, the gene expression during the meiotic prophase could not find evidence of an inactivation of sex-linked loci (Guioli *et al.*, 2012). Unlike the mammalian XY, the H2AX phosphorylation in the ZW pair is compatible with the timing of DSB occurrence and processing and it is lost by the pachytene stage. Further, the presence of repressive marks such as H3K9me3 are limited to the W chromosome and the terminal heterochromatin of the Z chromosome (del Priore, 2011; Guioli *et al.*, 2012). Altogether these observations support the notion that MSCI is absent in the avian ZW pair.

The suggestion that heterologous synapsis in the avian ZW pair helps to avoid an unpaired DNA checkpoint in oocytes awaits further investigations. Looking at the synaptic process in heterozygotes for chromosome rearrangements and direct analyses of the checkpoint proteins present during meiotic prophase in birds can provide a means to test this hypothesis.

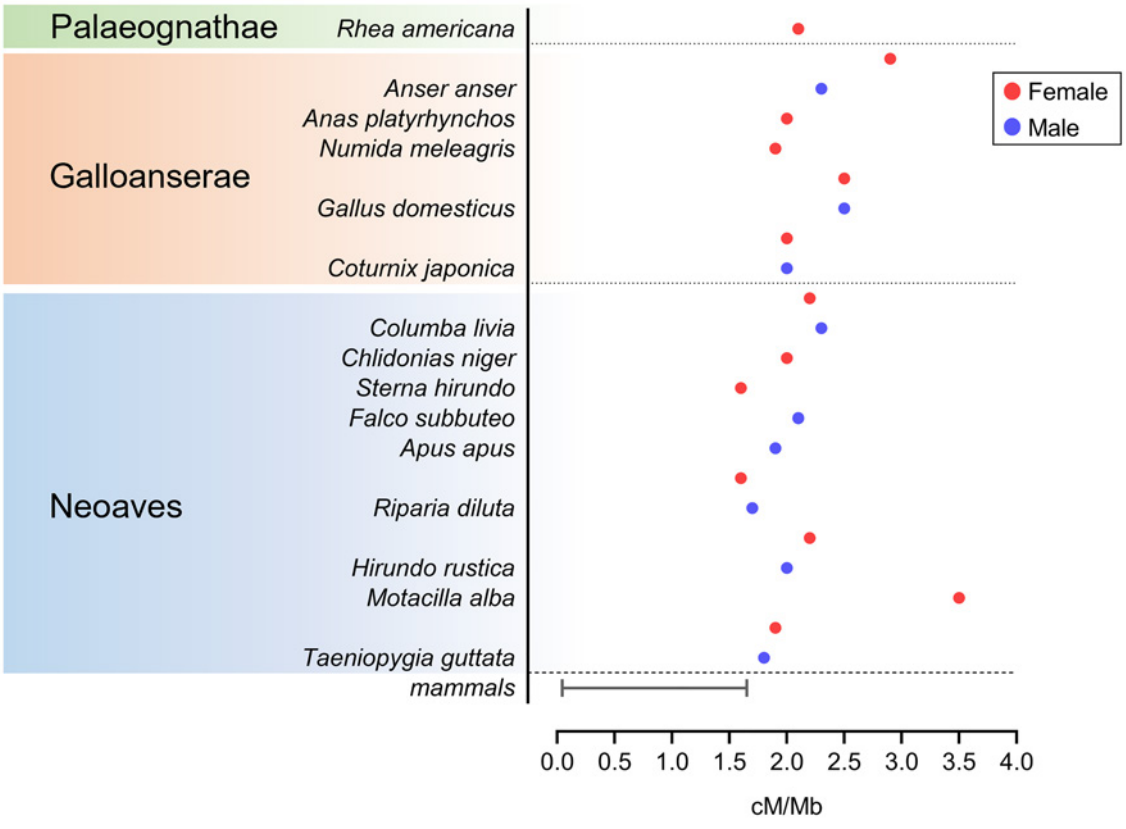

Figure 5. Genome wide recombination rates (RRs) in birds obtained by direct counts of crossovers at pachytene. The number of recombination nodules or MLH1 of foci were multiplied by 50 to obtain the genetic map length in cM and then divided by the genome size (in Mb) of each species to obtain the RRs. The range of the RRs in mammals was obtained from Dumont and Payseur (2008) and from Segura *et al.* (2013).

4. Cytogenetic recombination maps in birds

Recombination rates (RRs), that is, the number of recombination events per Mb per generation, are known to vary between species, between individuals, and even between sexes within the same species. The RRs can be described by the genome wide recombination (how many CO events occur per meiosis) and also by the variations of the CO locations within a genome (recombination landscape). There is a growing interest in comparative studies of the RRs across taxa with the aim to understand how they evolve and how they impact on other evolutionary processes within sexually reproducing organisms (Dumont and Payseur, 2008; Capilla *et al.*, 2016; Stapley *et al.*, 2017). RRs in birds have been studied by using polymorphic DNA markers on the same chromosome in crosses or pedigrees, as well as in genome-wide linkage disequilibrium investigations. (Aslam *et al.*, 2010; Ball *et al.*, 2010; Singhal *et al.*, 2015; Kawakami *et al.*, 2017). These approaches can provide kb-level resolution estimates of recombination, but using this information to compare average genome-wide RRs between taxa is difficult since independent analyses differ in marker density or genome coverage. A method to obtain the global RRs for direct comparisons is to score the number and distribution of cytological markers of crossing over, such as recombination nodules or MLH1 foci at pachytene (see section 2). In birds, recombination nodules were visualized using phosphotungstic acid staining of spread oocytes and spermatocytes at pachytene (Pigozzi and Solari, 1998a; Pigozzi and Solari, 1999a). Even though in certain birds like the domestic pigeon recombination nodules are well-preserved, in other species like the chicken or the zebra finch they are small and their staining differ between nuclei (Rahn and Solari, 1986b; Pigozzi and Solari, 1998b). For this reason, the cytogenetic studies of recombination are mainly represented by immunostaining of MLH1 foci. So far, recombination nodules or MLH1 foci have been scored in oocytes and/or spermatocytes of 14 species from eight orders that belong to the three main avian radiations: Palaeognathae, Galloanserae and Neoaves (Table 1; Figure 5). Even though the total number of species is scarce, some features can be pointed out. First, the average genome-wide RRs in birds are higher compared to mammals and vary within narrower limits: 1.6 cM/Mb and 3.5 cM/Mb for the lowest and highest RRs recorded in birds compared to 0.18 cM/Mb (in the elephant shrew) and 1.78 cM/Mb, in the Chinese muntjac, among mammals (Dumont and Payseur, 2008; Yang *et al.*, 2011). Another feature is that RRs show larger differences between species of the same order than between species of different orders (for example ducks and geese vs. guinea fowl and zebra finch), suggesting that RRs do not follow a phylogenetic trend. In mammals, different types of recombination analyses, including MLH1-focus counts, suggest the existence of a phylogenetic effect in RRs, with more closely related species having more similar average rates of recombination (Dumont and Payseur, 2008; Dumont and Payseur, 2011; Segura *et al.*, 2013). Overall, the evolutionary forces behind the observed distributions of genomic RRs in birds and mammals are not clear. Careful partition of the source variation at the individual, population or species level is needed for better understanding of the genetic and environmental components of RR variations. In order to obtain this information, empirical observations should be obtained in hypothesis-based studies, and in parallel, theoretical models on recombination should be developed to address empirical data (Dapper and Payseur, 2017).

The cytological observation of the crossing over also offers the opportunity to analyze the positions of COs along chromosome arms. In most species, COs are found anywhere along macrochromosome arms occurring at higher frequencies near telomeres. This CO distribution is found in the American rhea, the chicken and the domestic pigeon, among other birds (del Priore and Pigozzi, 2017; Malinovskaya *et al.*, 2019). Another, less common pattern of CO distribution, is a strong polarization of COs towards the chromosome ends, with scarce COs at mid chromosome regions. An extreme example of CO localization is found in the zebra finch (Estrildidae, Passeriformes) where as much as 80% of the total amount of recombination is concentrated on the 20% distal parts of the largest bivalents (Calderón and Pigozzi, 2006; Stapley *et al.*, 2010). Localized COs were also observed in the guinea fowl, a species that is related to domestic chicken, indicating that variable CO landscapes are not related to the phylogenetic position (del Priore and Pigozzi, 2020). The fact that COs are localized in the macro-SCs of the guinea fowl but show a more uniform distribution in the chicken also indicates that broad-scale recombination does not have a strong relationship with large-scale genomic variation, since both species share extensive identity in karyotype and syntenic blocks (Shibusawa *et al.*, 2004; Vignal *et al.*, 2019). At fine-scale, kb level, high recombination rates (hotspots) are related to CpG islands that are associated to gene promoter regions in birds. These regions have an open chromatin state that could favor the access of the recombination machinery (Singhal *et al.*, 2015; Kawakami *et al.*, 2017). However, unlike the recombination maps observed at cytological level, the distribution of genes does not follow a localized pattern, indicating that hotspots at fine scale do not correlate with broad-scale maps of crossing over. Epigenetic modifications acting to regulate the strength of hotspots might explain the differences in the fine *vs*. broadscale recombination landscapes, as well as differences in recombination observed between closely related species (Kawakami *et al.*, 2017).

Another aspect that can be approached by cytological examination of CO events is the presence and extent of heterochiasmy, that is the situation in which both sexes recombine, but at different rates. Variant recombination amount and diverse CO patterns between sexes are widely extended among vertebrates and many other eukaryotes (Lenormand, 2003; Sardell and Kirkpatrick, 2020). In birds, male *vs.* female recombination rates have relatively small discrepancy between sexes, but in a few cases, differences are more noticeable (Table 1; Figure 5). When intersex recombination differences were detected, the number of COs could be higher in either sex; in the case of different CO distributions in males and females, they could be located more frequently near the centromeres or towards the telomeres, depending on the species (Malinovskaya *et al.*, 2020 and references there in). A common feature underlying intersex recombination differences is the presence of longer SCs in the sex with higher recombination frequency (Lisachov *et al.*, 2017; Torgasheva and Borodin, 2017), in agreement with the interplay between meiotic axial length, arrangement of DNA loops and the frequencies of recombination intermediates that are solved as COs (Kleckner *et al.*, 2003; Ruiz-Herrera *et al.*, 2017).

Overall, the CO patterning observed in birds indicates that different mechanisms operate at wide range scale from the DNA level to the chromosomal level.

The karyotype of birds has low rates of interchromosomal changes, when compared to mammals and non-avian reptiles. Even though the intrachromosomal rearrangements are common, there are very few examples of interchromosomal rearrangements at the level of macrochromosomes and high degree of synteny, even between distantly related avian lineages (O'Connor *et al.*, 2018). This creates an apparent paradox between karyotype and genome conservation and the morphological, physiological and adaptive diversity of birds. A possibility that makes compatible these two facts is that the organization of the bird genome in numerous chromosomes favors genetic diversity by increasing recombination rates and increasing the capacity for random segregation due to the presence of microchromosomes (Ellegren, 2013). Cytological maps of broad-scale recombination supports the idea that karyotype homogeneity does not restrict the advent of recombination variants in macrochromosomes. In other words, variant recombination landscapes found in macrochromosomes (that is, restricted *vs.* non-restricted recombination) also create genetic variation, which contributes to the great phenotypic diversity observed among birds.

Table 1. Recombination rates estimated from recombination nodules (RNs) or MLH1-focus analysis in birds.

Common name	Latin name	Order	Sex	COs per autosomal set		Genetic map (cM)	C value (pg)	Genome size (Mb)	RR (cM/Mb)	Reference
				X	*SD*					
Greater rhea	*Rhea americana*	Rheiformes	F	58.8	4.4	2940	1.46	1428	2.1	del Priore and Pigozzi, 2017
Greylag goose	*Anser anser*	Anseriformes	F	72.6	7.8	3632	1.3	1408	2.9	Torgasheva and Borodin, 2017
			M	57.9	7.6	2897			2.3	Torgasheva and Borodin, 2017
Domestic duck	*Anas platyrhynchos*	Anseriformes	F	55.9	3.8	2795	1.54	1506	2.0	del Priore and Pigozzi, 2016
Guinea fowl	*Numida meleagris*	Galliformes	F	44.4	1.6	2220	1.22	1193	1.9	del Priore and Pigozzi, 2020
Chicken	*Gallus domesticus*	Galliformes	F	62.1	5.4	3105	1.25	1223	2.5	del Priore and Pigozzi, 2020
			M	61.5	5.6	3075			2.5	Malinovskaya *et al.*, 2019
Japanese quail	*Coturnix japonica*	Galliformes	F	55.3	2.1	2765	1.41	1379	2.0	del Priore and Pigozzi, 2015
			M	56.3	1.8	2815			2.0	del Priore and Pigozzi, 2015
Domestic pigeon	*Columba livia*	Columbiformes	F	62.7	4.9	3135	1.44	1408	2.2	Pigozzi and Solari, 1999a
			M	64.7	4.8	3235			2.3	Pigozzi and Solari, 1999b
Black tern	*Chlidonias niger*	Charadriiformes	F	43.1	5	2155	1.4	1369	2.0	Lisachov *et al.*, 2017
Common tern	*Sterna hirundo*	Charadriiformes	F	52	4.2	2600	1.4	1369	1.6	Lisachov *et al.*, 2017
Eurasian hobby	*Falco subbuteo*	Falconiformes	M	51.1	6.6	2555	NA	1242[a]	2.1	Malinovskaya *et al.*, 2018
Common swift	*Apus apus*	Apodiformes	M	51.4	4.3	2570	NA	1350[b]	1.9	Malinovskaya *et al.*, 2018
Barn swallow	*Hirundo rustica*	Passeriformes	F	55.6	6.3	2780	1.38	1252	2.2	Malinovskaya *et al.*, 2020
			M	49	4.5	2450			2.0	Malinovskaya *et al.*, 2020
Pale martin	*Riparia diluta*	Passeriformes	F	44.6	3.6	2230	1.45[c]	1418	1.6	Malinovskaya *et al.*, 2020
			M	48.9	2.4	2445			1.7	Malinovskaya *et al.*, 2020
White wagtail	*Motacilla alba*	Passeriformes	F	76.1	8.6	3805	NA	1072[d]	3.5	Semenov *et al.*, 2018
Zebra finch	*Taeniopygia guttata*	Passeriformes	F	45.7	0.4	2285	1.25	1223	1.9	Calderón and Pigozzi, 2006
			M	45.2	0.2	2260			1.8	Calderón and Pigozzi, 2006

COs crossovers; *X*, average; *SD*, standard deviation; RR, recombination rate.

[a, b, c] Genome sizes were derived from C values reported in Gregory (2021). Genome sizes are unknown for *F. subbuteo*, *A. apus* and *R. diluta*. The available genome size of the closest species was used; they are *F. eleonorae* (1.27 pg), *A. affinis* (1.38) and *R. riparia* (1.45), respectively.

[d] From *M. alba* genome assembly (https://www.ncbi.nlm.nih.gov/assembly/GCF_015832195.1/#/st).

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CAUSES AND CONSEQUENCES OF DNA CONTENT VARIATION IN *ZEA*

CAUSAS Y CONSECUENCIAS DE LA VARIACIÓN DEL CONTENIDO DE ADN EN *ZEA*

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ABSTRACT

Cytogenetic evidence indicates that *Zea*, which comprises maize (*Z. mays* ssp. *mays*) and its wild relatives, is an allopolyploid genus. Our research group has carried out numerous cytogenetic studies on *Zea* species, mainly focused on native Argentinian and Bolivian maize landraces. We found a wide inter- and intraspecific genome size variation in the genus, with mean 2C-values ranging between 4.20 and 11.36 pg. For the maize landraces studied here, it varied between 4.20 and 6.75 pg. The objectives of this work are to analyze the causes of genome size variation and to discuss their adaptive value in *Zea*. This variation is mainly attributed to differences in the heterochromatin located in the knobs and to the amount of interspersed DNA from retrotransposons. Polymorphisms in presence or absence of B-chromosomes (Bs) and the population frequency of Bs are also a source of genome size variation, with doses ranging between one and eight in the landraces analyzed here. Correlation analysis revealed that the percentage of heterochromatin is positively correlated with genome size. In addition, populations cultivated at higher altitudes, which are known to be precocious, have smaller genome sizes than do those growing at lower altitudes. This information, together with the positive correlation observed between the length of the vegetative cycle and the percentage of heterochromatin, led us to propose that it has an adaptive role. On the other hand, the negative relationship found between Bs and heterochromatic knobs allowed us to propose the existence of an intragenomic conflict between these elements. We hypothesize that an optimal nucleotype may have resulted from such intranuclear conflict, where genome adjustments led to a suitable length of the vegetative cycle for maize landraces growing across altitudinal clines.

Key words: B chromosomes, heterochromatin, intragenomic conflict, knobs, maize landraces

RESUMEN

La evidencia citogenética indica que el género *Zea*, el maíz (*Z. mays* ssp. *mays*) y sus parientes silvestres, posee un origen alopoliploide. Nuestro grupo de investigación ha realizado numerosos estudios en especies de *Zea*, principalmente en maíces nativos de Argentina y Bolivia. En este género, hallamos una amplia variación inter e intraespecífica en el tamaño del genoma, con valores 2C medios que oscilan entre 4,20 y 11,36 pg. El valor 2C medio de los maíces nativos estudiados varió entre 4,20 y 6,75 pg. Los objetivos de este trabajo son analizar las causas de la variación del tamaño del genoma en *Zea* y discutir su valor adaptativo. Esta variación se atribuye principalmente a las diferencias en la heterocromatina de los *knobs* y en la cantidad de ADN intercalado de los retrotransposones. Otras fuentes de variación son los polimorfismos para presencia/ausencia de cromosomas B (Bs) y para la frecuencia poblacional de Bs en las razas analizadas, con dosis que oscilan entre uno y ocho Bs. El porcentaje de heterocromatina se correlaciona positivamente con el tamaño del genoma. Las poblaciones cultivadas en altitudes altas, que son precoces, tienen tamaños de genoma más pequeños que las que crecen en bajas altitudes. Esta información, junto con la correlación positiva observada entre la duración del ciclo vegetativo y el porcentaje de heterocromatina, nos llevó a proponer el rol adaptativo de la heterocromatina. Por otro lado, la relación negativa encontrada entre Bs y *knobs* heterocromáticos nos permitió proponer la existencia de un conflicto intragenómico entre estos elementos. Hipotetizamos que de este conflicto intranuclear habría resultado el nucleotipo óptimo, donde ajustes genómicos condujeron a una duración adecuada del ciclo vegetativo en las razas de maíz que crecen a lo largo de clines altitudinales.

Palabras clave: conflicto intragenómico, cromosomas B, heterocromatina, *knobs*, maíz nativo

INTRODUCTION

The genus *Zea* (Poaceae - Maydeae) includes the perennial species *Z. perennis* (Hitch.) (Reeves & Mangelsdorf) and *Z. diploperennis* Iltis (Doebley & Guzman), and the annual species *Z. luxurians* (Durieu & Ascherson) (Bird), *Z. nicaragüensis* (Iltis & Benz) and *Z. mays* L. The latter has four subspecies: *Z. mays* ssp. *mays* (the maize), *Z. mays* ssp. *parviglumis*, *Z. mays* ssp. *huehuetenanguensis* and *Z. mays* ssp. *mexicana* (Doebley, 1990; Iltis and Benz, 2000). All taxa have 2n=20, except for *Z. perennis* with 2n=40.

Cytogenetic evidence indicates that maize and its wild relatives (the teosintes) are cryptic polyploids. In the pioneering studies of Anderson (1945) maize was considered as allotetraploid 2n=4x=20, derived from extinct ancestors with 2n=10. Meiotic studies of intra- and interspecific hybrids confirmed their allopolyploid nature, indicating that maize and its wild relatives are tetraploids, except for the octoploid *Z. perennis*, with x=5 being the basic chromosome number (revisited in Poggio *et al.*, 2005; Poggio and González, 2018). Further molecular studies provided compelling evidence for the allopolyploid nature of maize (Moore *et al.*, 1995; Gaut and Doebley, 1997; White and Doebley, 1998; Swigonova *et al.*, 2004).

Our research group has carried out numerous cytogenetic studies on *Zea* species, mainly focused on native Argentinian and Bolivian maize landraces (Tito *et al.*, 1991; Quintela Fernández *et al.*, 1995; Poggio *et al.*, 1998, 1999a, 1999b, 2000a, 2000b, 2005; Rosato *et al.*, 1998; González *et al.*, 2004, 2006, 2013; González and Poggio, 2011, 2015, 2021; Realini *et al.*, 2016, 2018, 2021; Fourastié *et al.*, 2017; Poggio and González, 2018).

Maize is one of the most important cereal crops worldwide, growing in a broad range of agro-ecological regions. In South America, many landraces are adapted to a great variety of climatic conditions at different growing altitudes (Roberts *et al.*, 1957; Wellhausen *et al.*, 1957; Timothy *et al.*, 1961; Rodríguez *et al.*, 1968; Salhuana and Machado, 1999; Sánchez *et al.*, 2007; Cámara Hernández *et al.*, 2011; Orozco-Ramírez *et al.*, 2016). Cámara Hernández *et al.* (2011) described more than 50 morphological native landraces from Northern Argentina, which are cultivated using ancestral farming practices. Of these, 36 landraces grow from lowlands to highlands in Northwestern Argentina (NWA), while 15 are cultivated at lower altitudes in Northeast Argentina (NEA). They are potential sources of genetic variation, constituting useful reservoirs of tolerance and resistance alleles for biotic and abiotic stresses.

The objectives of this work are to analyze genome size variation in the genus *Zea* based on studies carried out by our research group, to discuss the causes of such variation and to explore the relationship between genome size and cytological, phenological and environmental characteristics.

INTER- AND INTRASPECIFIC VARIATION IN THE GENOME SIZE OF *ZEA*

Genome size plays an important role in the cytogenetic variation of a taxon or group of taxa. This character is measured by microdensitometry with Feulgen's stain or, most often, using flow cytometry (Tito *et al.*, 1991; Dolezel *et al.*, 2007). DNA content is usually expressed in picograms (pg) as the 2C-value, which refers to the amount of genomic DNA in unreplicated somatic cells (Poggio *et al.*, 2010).

In genus *Zea*, the interspecific variation in the mean DNA content has been reported to range between 4.2 and 11.36 pg (Tables 1 and 2) (Laurie and Bennett, 1985; Tito *et al.*, 1991; Fourastié *et al.*, 2017). In maize, intraspecific variability in the DNA content has been recorded in native landraces from the following sites: a) NEA, cultivated in lowlands from 98 to 591 m.a.s.l.; b) NWA, growing along a broad altitudinal cline from 80 to 3900 m.a.s.l.; and c) Bolivia, cultivated from 200 to 3250 m.a.s.l. (Table 2) (Quintela Fernández *et al.*, 1995; Rosato *et al.*, 1998; Realini *et al.*, 2016; Fourastié *et al.*, 2017; González and Poggio, 2021). Table 2 shows the range of mean 2C-values (4.20-6.75 pg) for these landraces. A similar genome size variation has been obtained for other American maize populations growing along altitudinal clines (Laurie and Bennett, 1985; Rayburn and Auger, 1990; Díez *et al.*, 2013; Bilinsky *et al.*, 2018).

Table 1. DNA content in *Zea* species. Data from Tito *et al.* (1991).

Species (somatic chromosome number)	Mean 2C-value (pg) (±SD)
Zea luxurians (2n=20)	8.83 (± 0.08)
Zea diploperennis (2n=20)	6.36 (± 0.06)
Zea perennis (2n=40)	11.36 (± 0.11)
Zea mays ssp. mays (2n=20) C-tester line	5.86 (± 0.05)
Zea mays ssp. mexicana (2n=20)	6.79 (± 0.05)

Table 2. Genome size and B-chromosome variations in maize landraces.

Source of maize landraces	Altitude m.a.s.l. (range)	2C-Value* (range)	Dose of Bs (range)	Frequency of Bs (range)
NWA	80-3900	4.20-6.75 pg	1 - 8	0-100%
Bolivia	200-3250	5.26-6.23 pg	1 - 5	16.6-81.8%
NEA	98-591	4.62-6.29 pg	-------	-------

Ref.: NWA: Northwestern Argentina, NEA: Northeastern Argentina, m.a.s.l.: meters above sea level. *The 2C-value was measured in plants without B chromosomes (A-DNA). Data from: Rosato *et al.*, 1998; Realini *et al.*, 2016; Fourastié *et al.*, 2017; González *et al.*, 2021.

CAUSES OF GENOME SIZE VARIATION

In *Zea*, genome size variation has been mainly attributed to differences in the heterochromatin located in knobs and to interspersed DNA amount (e.g. retrotransposon families), making up over 70% of the nuclear genome (SanMiguel *et al.*, 1998; Meyers *et al.*, 2001; Realini *et al.*, 2016; Fourastié *et al.*, 2017). The presence of accessory chromosomes (B chromosomes; Bs) also contribute to DNA content variation, as discussed later (Rosato *et al.*, 1998; Fourastié *et al.*, 2017; González and Poggio, 2021).

Chromosomes of *Zea* species have blocks of heterochromatin called knobs (Kato, 1976; McClintock, 1978). This heterochromatin is highly condensed and composed of highly repeated DNA sequences (Peacock *et al.*, 1981). Knobs can be visualized in dividing cells as well as in interphase nuclei as chromocenters (Wan and Widholm, 1992). During pachytene of *Zea* species, knobs can be found at 34 different chromosomal positions in the karyotype (Kato, 1976). They are detected using C and DAPI chromosome banding (González *et al.*, 2013). At the molecular level, knobs are composed of two sequence families of 180 base pairs (180-bp) and 350 base pairs (TR-1), and may contain several retrotransposons (Dennis and Peacock, 1984; Ananiev *et al.*, 1998). The sequence families, which are represented by thousands to millions of tandemly arranged copies, conform the different knob types (knobs exclusively containing 180-bp repeats, knobs exclusively containing TR-1 repeats and knobs with different proportion of both sequences) (Ananiev *et al.*, 1998).

As mentioned above, DAPI banding identifies knobs as DAPI-positive bands that are A-T rich (Figure 1 A, B, C, F). However, this technique does not provide information on knob sequences. The fluorescent *in situ* hybridization (FISH) allows the detection and localization of specific sequences on interphase nuclei and metaphase chromosomes (Poggio *et al.*, 1999b; 2005; González *et al.*, 2006; González and Poggio, 2011; 2015). The hybridization of the 180-bp and TR-1 knob sequences on mitotic metaphases of *Zea* is used to determine the sequence composition of each DAPI-positive band (*i.e.* each knob) (Figure 1 D, E) (Albert *et al.*, 2010; González *et al.*, 2013).

Different maize landraces and teosintes show a wide variation in the size, number, chromosome position and sequence composition of heterochromatic knobs (Kato, 1976; McClintock *et al.*, 1981; González *et al.*, 2013), thus serving as valuable cytological markers. For example, they have been used for the cytogenetic characterization of landraces from Northern Argentina (Realini *et al.*, 2016; Fourastié, 2017).

In *Zea*, DNA content is positively correlated with the number and size of knobs, as well as with the percentage of heterochromatin in the karyotype (Laurie and Bennett, 1985; Tito *et al.*, 1991; Poggio *et al.*, 1998; Realini *et al.*, 2016; Fourastié *et al.*, 2017; González and Poggio, 2021). The percentage of heterochromatin in a *Zea* karyotype is calculated by summing all chromosomal portions occupied by the DAPI-positive bands detected in the chromosomal complement. *Z. luxurians* has the highest DNA content of the 2n=20 species within the genus (Table 1), possibly due to the larger number and size of knobs, which are at terminal position on almost all chromosomes (Figure 1 C, D) (González and Poggio, 2011; González *et al.*, 2013). On the contrary, DAPI-banding and FISH experiments did not detect conspicuous knobs in the octoploid *Z. perennis*, showing the lowest DNA content per basic genome (Cx) among *Zea* species (Kato and López, 1989; Tito *et al.*, 1991; González *et al.*, 2013) (Table 1; Figure 1 F). The small quantity of knob sequences in *Z. perennis* was postulated to be a consequence of the genome downsizing occurring during the process of secondary polyploidization in this species (Poggio *et al.*, 2005; González and Poggio, 2015).

In maize landraces, the percentage of heterochromatin

is positively correlated with the genome size (González and Poggio, 2011; Realini *et al.*, 2016; Fourastié *et al.*, 2017), suggesting that the variation in DNA content is mainly due to differences in the size, and to a lesser extent, in the number of knobs. However, some authors provided evidence for the presence of other sources than heterochromatin knobs contributing to genome size (revisited in Realini *et al.*, 2016; 2018). Transposable elements (TEs) play a role in the dynamics of the nuclear genome, either through polymorphic insertions and deletions or by mediating ectopic recombination events leading to structural variation in the genome (SanMiguel and Bennetzen, 1998; Meyers *et al.*, 2001). Recently, Coutinho Silva *et al.* (2020) demonstrated that in maize a higher 2C-value is associated with a more abundant distribution of LTR-retrotransposons in the karyotype, mainly from the *Grande* family. Further studies will enhance the knowledge on the differential composition of retrotransposon families in native maize landraces from Argentina, and its influence on genome size variation.

NEGATIVE CORRELATION BETWEEN DNA CONTENT AND ALTITUDE OF CULTIVATION IN MAIZE LANDRACES

In NWA, Rosato *et al.* (1998) reported a significant negative correlation between DNA content and altitude of cultivation in landraces growing along an altitudinal cline. This was further supported by Fourastié *et al.* (2017) for other NWA populations. Recently, González and Poggio (2021) observed that the Bolivian landraces cultivated at higher altitudes have lower DNA content than those growing at lower altitudes (Figure 2). Negative correlations between genome size and altitude of cultivation were also detected in different altitudinal clines from the American continent (Rayburn and Auger, 1990; Díez *et al.*, 2013; Bilinsky *et al.*, 2018).

HETEROCHROMATIN HAS ADAPTIVE SIGNIFICANCE ALONG AN ALTITUDINAL CLINE

Realini *et al.* (2016) observed a positive correlation between the length of the vegetative cycle and the percentage of heterochromatin in maize landraces from lowlands in NEA. Knob heterochromatin is the latest component to finish DNA replication because increased DNA packaging extends DNA synthesis, leading to a longer cell cycle, which may affect the rate of cell division and plant development (Pryor *et al.*, 1980; Buckler *et al.*, 1999; Greilhuber and Leitch, 2013). On this basis, length of the vegetative cycle was proposed to be optimized through artificial selection for an appropriate

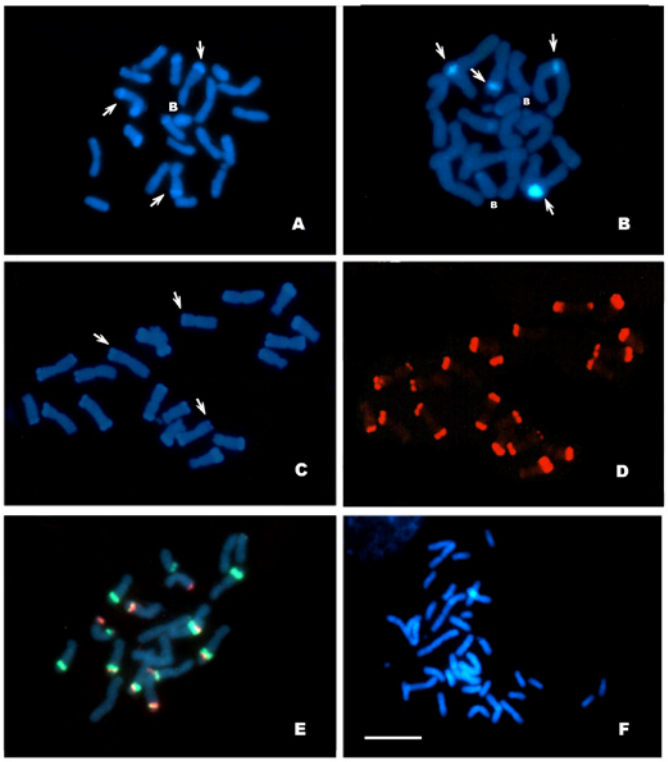

Figure 1. Mitotic metaphases of *Zea*. **A:** DAPI banding on NWA maize Blanco y Ocho Rayas landrace, with 1 B chromosome. **B:** DAPI banding on NWA maize Amarillo Chico landrace, with 2 B chromosomes. **C:** DAPI staining on *Z. luxurians*. **D:** Fluorescent *in situ* hybridization (FISH) on mitotic metaphase of *Z. luxurians* hybridized with 180bp knob sequence detected with Cy3 (red). **E:** FISH on mitotic metaphase of NEA maize Pipoca Colorado landrace simultaneously hybridized with 180bp knob sequence detected with antidigoxigenin-FITC (green) and with TR-1 knob sequence detected with Cy3 (red). **F:** DAPI banding of *Z. perennis*. Ref.: white arrows show some DAPI-positive bands (knobs). Bar=10 µm.

percentage of heterochromatin (Realini *et al.*, 2016; 2021; Bilinsky *et al.*, 2018).

As mentioned above, the percentage of heterochromatin is positively correlated with genome size. In addition, DNA content is higher in inbred lines with long vegetative cycles than in precocious ones, and their F1 hybrids have an intermediate genome size (González and Poggio, in prep.). Jian *et al.* (2017) also observed a high correlation between genome size and flowering time in inbred lines growing under tropical conditions. These results may explain the negative correlation detected between genome size and percentage of heterochromatin with cultivation altitude (Figure 2) (Tito *et al.*, 1991; Poggio *et al.*, 1998; Fourastié *et al.*, 2017; González and Poggio, 2021). Thus, the fact that maize landraces at high altitudes are precocious and show a reduction in heterochromatin percentage most likely represents an adaptation to a shorter growing season typical of highlands, with natural selection acting on the flowering time across altitudinal clines (Bilinsky *et al.*, 2018). This reinforces the hypothesis that the percentage of heterochromatin has adaptive value along altitudinal clines.

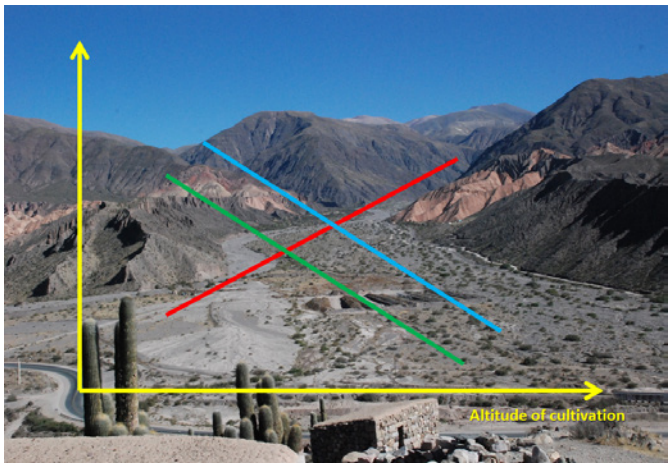

Figure 2. Relationships of DNA content (blue), percentage of heterochromatin (green) and dose and frequency of B chromosomes (red) with cultivation altitude in maize landraces. This graph only represents the trends for the relationships. Photograph taken by Julián Cámara Hernández.

B-CHROMOSOME POLYMORPHISMS AS SOURCES OF GENOME SIZE VARIATION

In *Zea*, B chromosomes (Bs) are also regarded as a source of genome size variation (McClinctock *et al.*, 1981; Kato and López, 1990; Poggio *et al.*, 1998; Rosato *et al.*, 1998; Cheng and Lin, 2003; Lamb *et al.*, 2007; Rosado *et al.*, 2009). Supernumerary Bs are dispensable chromosomes lacking homology with any member of the normal complement, the A-chromosome (A) set. These accessory chromosomes represent a specific type of selfish genetic element with mechanisms of drive which allow them to increase their transmission rates through different processes of non-mendelian inheritance (Jones and Houben, 2003; Houben *et al.*, 2014; Blavet *et al.*, 2021). Although Bs follow their own species-specific evolutionary pathways, it is widely accepted that they are derived from their respective A complement (revisited in Houben *et al.*, 2014).

Polymorphisms for presence/absence and different doses of Bs have been reported in annual *Zea* species (Longley, 1937; Ting, 1976; Kato, personal communication). Polymorphisms are well documented in maize, particularly for landraces from NWA and Bolivia (McClinctock *et al.*, 1981; Quintela Fernández *et al.*, 1995; Rosato *et al.*, 1998; Fourastié *et al.*, 2017; González and Poggio, 2021). In these landraces, a wide variation of population frequency of Bs was observed. The doses range between one and eight Bs per plant, with two Bs per plant being the most frequently reported dose (Table 2). Fourastié *et al.* (2017) proposed that the frequency of Bs depends not only on the cultivation altitude, but also on the genotypical and nucleotypical backgrounds of the landraces.

In NWA and Bolivian landraces, which grow along a wide altitudinal cline, the mean number and frequency of Bs are significantly and negatively correlated with the 2C-value of the A-chromosome complement (A-DNA) and positively correlated with altitude of cultivation (Figure 2) (Quintela Fernández *et al.*, 1995; Poggio *et al.*, 1998; Rosato *et al.*, 1998; Fourastié *et al.*, 2017; González and Poggio, 2021). These authors also found a significant negative correlation between the mean number of Bs and the percentage of knob heterochromatin. Moreover, they hypothesized that Bs are maintained at higher frequencies in populations with low percentage of heterochromatin to preserve an optimal nucleotype (*sensu* Bennett, 1972). Such term defines the conditions of the nucleus affecting cell and developmental parameters such as cell volume, nuclear volume, chromosome size, mitotic cycle time, duration of meiosis and minimum generation time (Bennett, 1987; Poggio *et al.*, 1998). The negative association observed between the frequency of Bs and the percentage of heterochromatin suggests that there is a maximum limit on the mass of nuclear DNA that allows the optimum nucleotype (Rosato *et al.*, 1998; Fourastié *et al.*, 2017). Based on the analysis of many landraces from NWA and Bolivia, González and Poggio (2021) proposed that the optimal nucleotype is the result of an intragenomic conflict between Bs and heterochromatin knobs, where genome adjustment may lead to an appropriate length of the vegetative cycle for maize landraces growing across altitudinal clines.

A better understanding of the causes accounting for genome size variation and their adaptive significance in maize landraces is essential for the development of successful breeding and conservation programs.

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DNA CONTENT AND CYTOGENETIC CHARACTERISTICS OF *Gymnocalycium quehlianum* (CACTACEAE) ALONG AN ALTITUDINAL GRADIENT

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ABSTRACT

Important changes in vegetation types occur along elevational gradients. The genus *Gymnocalycium* is endemic to southern South America, and its species are distributed along elevational gradients. In particular, *Gymnocalycium quehlianum* is a globular cactus endemic to the Sierras de Córdoba. Studying cytogenetic aspects and DNA content in populations throughout their distribution is key to understanding the species. DNA content and cytogenetic characteristics were analyzed in four populations of *G. quehlianum* (615, 744, 948 and 1257 masl). The genome size in the four populations varied between 3.55 and 4.30 pg. The populations were diploid (2n = 22). All populations showed the karyotype formula of 10 metacentrics (*m*) + 1 submetacentric (*sm*). The species presented symmetrical karyotypes and constitutive heterochromatin CMA+/DAPI- associated with nucleolar organizing regions, always found in the first pair of m chromosomes. The 18-5.8-26S rDNA locus is found in the terminal regions of the first pair of chromosomes m, and the 5S locus is adjacent to the 18-5.8-26S locus. A tendency for DNA content to decrease with increasing altitude was observed.

Key words: *Gymnocalycium quehlianum*, chromosome number, cytogenetic, DNA content, altitudinal gradient

RESUMEN

A lo largo de los gradientes altitudinales se producen cambios importantes en los tipos de vegetación. El género *Gymnocalycium* es endémico del sur de América del Sur y sus especies se distribuyen en gradientes altitudinales. En particular, *Gymnocalycium quehlianum* es un cactus globular endémico de las Sierras de Córdoba. Estudiar aspectos citogenéticos y de contenido de ADN en las poblaciones a lo largo de su distribución es clave para comprender a la especie. En cuatro poblaciones de *G. quehlianum* (615, 744, 948 y 1257 msnm) se analizaron el contenido de ADN y las características citogenéticas. El tamaño del genoma en las cuatro poblaciones varió entre 3,55 y 4,30 pg. Las poblaciones resultaron diploides (2n=22). Todas las poblaciones presentaron la fórmula del cariotipo de 10 metacéntricos (*m*) + 1 submetacéntrico (*sm*). La especie presentó cariotipos simétricos y heterocromatina constitutiva $CMA^+/DAPI^-$ asociados con regiones organizadoras nucleolares, que siempre se encontraban en el primer par de cromosomas *m*. El locus de ADNr 18-5.8-26S se encuentra en las regiones terminales del primer par de cromosomas *m* y el locus de 5S está adyacente al locus 18-5.8-26S. Se observó una tendencia del contenido de ADN a disminuir con el aumento de la altitud.

Palabras clave: *Gymnocalycium quehlianum*, número cromosómico, citogenética, contenido de ADN, gradiente altitudinal

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INTRODUCTION

Gymnocalycium (subfamily Cactoideae) is a genus endemic to southern South America and comprises about 50 species, most of them with a narrow geographical distribution (Charles, 2009). In Argentina, it is the Cactaceae genus with the highest number of species (41), representing 18% of the total richness of the subfamily in the country (Kiesling *et al.*, 2008). *Gymnocalycium* is divided into subgenera based on the characteristics of seeds, floral anatomy and fruits (Schütz, 1986; Demaio *et al.*, 2011). *G. quehlianum* (subgenus Trichomosemineum) is endemic to Córdoba province (Argentina) and is distributed along the Sierra Chica up to the Sierra Norte (Figure 1). It is abundant in mountain environments between 500 and 1200 m a.s.l. (Charles, 2009; Gurvich *et al.*, 2014). It has depressed grayish green conical stems, ribs consisting of a hump with small radial spines, and white flowers with a reddish throat (Charles, 2009, Kiesling and Ferrari, 2009)

The altitudinal gradient is the main factor that influences vegetation patterns in mountain environments. As altitude increases, temperature decreases and solar radiation becomes more intense. However, the effects of precipitation are variable, depending on the different mountain ranges (Körner, 2007). These environmental changes condition the presence of different species along the gradient (Cabido *et al.*, 2010), although many species have wide distribution ranges (Knight and Ackerly, 2002, Bauk *et al.*, 2015). Climate change will lead to changes in distribution range of many species and, therefore, to the loss of genetic diversity within each species. When addressing the impacts of climate change on biological diversity, each species is considered as one unit in most studies, neglecting intraspecific genetic variations (Thuiller *et al.*, 2008). Maintaining genetic diversity within a species is crucial for adaptation in the short- and long-term (Jump *et al.*, 2009).

Genome size and ploidy level, two important variables that determine genetic diversity, have been related to ecological characteristics (Knight and Ackerly, 2002; Ramsey and Ramsey, 2014). Genome size (C-value) is a feature that may change between populations, varying between 0.05 and 127.4 pg in Angiosperms; however, this variation is not necessarily related to ploidy level (Bennett and Leitch, 2005). Genome size has been related to different features, such as minimum cell generation time, life history, plant phenology, and some important parameters for plant breeders, such as frost resistance, biomass production, ecological adaptations, cellular cycle time, and DNA synthesis, all of which could affect plant growth rate (Ohri, 1998; Burton and Husband, 2001). Some genetic traits, like DNA content and ploidy level, may vary along environmental gradients (Knight and Ackerly, 2002). Numerous studies have related

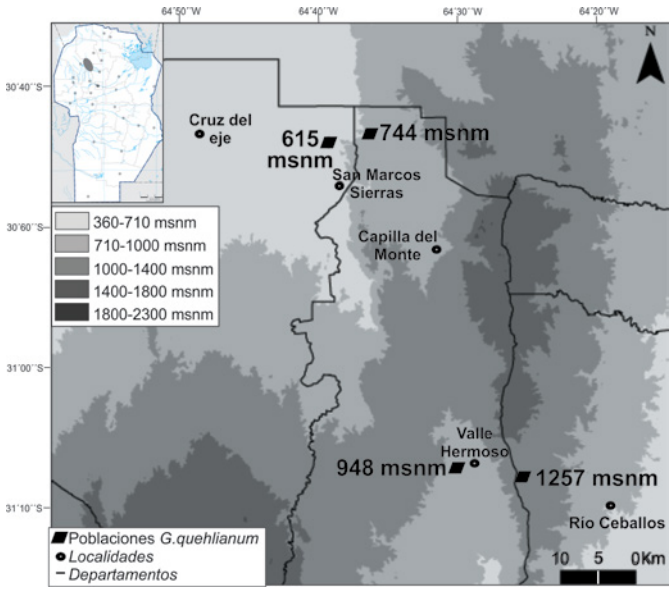

Figure 1. Location of the four collection sites (altitudinal provenances: 615, 744, 948, 1257 m a.s.l.) of *G. quehlianum* along an altitudinal gradient in Córdoba Mountains (Córdoba province, Argentina). Map obtained with DIVA-GIS 2 (Hijmans *et al.* 2002).

C-value changes to morphological characters, habitat and distribution (Bennett, 1976; Knight and Ackerly, 2002; Bennett and Leitch, 2005; Slovák *et al.*, 2009). However, the functional significance of this variation and the mechanisms of these changes are diverse. When comparing species, Knight and Ackerly (2002) found that those having a higher 2C content were more frequent at intermediate latitudes and altitudes. Cx values, representing the DNA content of one non-replicated monoploid genome with the chromosome number x (Greilhuber et al. 2005) There are few cacti examined to date, presenting a range of Cx- values from 1.53 to 8.94 pg (Palomino *et al.*, 1999; Zonneveld *et al.*, 2005; Del Angel *et al.*, 2006; Negron-Ortiz, 2007; Las Peñas *et al.*, 2014, 2017; Bauk *et al.*, 2016).

Eupolyploidy refers to the possession of three or more complete sets of chromosomes representing the haploid genome (Ramsey and Schemske, 1998; Soltis *et al.*, 2003). Ploidy level has played significant roles in diversification and speciation processes in flowering plants (Stebbins, 1971; Grant, 1981; Leitch and Bennett, 1997; Levin, 2002; Coghlan *et al.*, 2005; Leitch and Leitch, 2008; Soltis *et al.*, 2009; Soto-Trejo *et al.*, 2013). Chromosome counts have been performed only in a few of the 1,400 species of Cactaceae (of which 26.2% are diploid, 13.4% are both diploid and polyploid, and 60.4% are polyploid), confirming that the frequency of genome duplication in the group is far more common than diploidy (Pinkava, 2002; Las Peñas *et al.*, 2019).

The cytogenetic data available for *G. quehlianum* indicate that it is diploid (2n=22) and its DNA content

Table 1. Content DNA and cytogenetic characteristic in four populations of *G. quehlianum*. Voucher data (all from Argentina, Córdoba province), 2C (diploid DNA amount), Cx (basic DNA amount), 2n (somatic chromosome number), FK (karyotype formulae), TLH (mean total haploid chromosome length), C (mean chromosome length), A_1 (intrachromosomal asymmetry index), A_2 (interchromosomal asymmetry index), FISH (fluorescent *in situ* hybridization, 45S: number of rDNA 18–5.8–26S loci, 5S: number of rDNA 5S loci).

Population	Voucher specimens	Content DNA (pg)									FISH	
G. quehlianum		2C	4C	8C	Cx	2n	KF	TLH (µm)	C (µm)	A_1	A_2	45S	5S
San Marcos Sierras (615 msnm)	Las Peñas *et al*. 835	4.3	9.12	17.52	2.15	22	10 m*+1sm	29.02	2.64	0.19	0.13	2	4
Capilla del monte (744 msnm)	Las Peñas *et al.* 836	3.84	7.78	15.63	1.92	22	10 m*+1sm	45.26	4.11	0.19	0.18	-	-
Valle Hermoso (948 msnm)	Las Peñas *et al.* 837	3.89	7.77	15.43	1.94	22	10 m*+1sm	34.65	3.15	0.2	0.16	2	4
Camino del Cuadrado (1257 msnm)	Las Peñas *et al.* 838	3.55	7.23	14.68	1.78	22	10 m*+1sm	34.65	3.15	0.19	0.14	2	4

is 2C=6.46 pg (Das and Das, 1998). Ploidy level is an important characteristic that may be affecting speciation and patterns of species diversity. Chromosome number of a species may vary with altitude (Grant, 1981; Levin, 2002; Morales Nieto *et al.*, 2007). The main aim of this work was to explore possible relationships between altitudinal range, cytogenetic characteristics, and DNA content in *G. quehlianum.*

MATERIALS AND METHODS

Species and study area

Gymnocalycium quehlianum occurs in rocky outcrops from 600 to 1200 m a.s.l. (Demaio *et al.*, 2011; Gurvich *et al.*, 2014). We studied four populations from Córdoba (Argentina) located along an altitudinal gradient between the localities of San Marcos Sierra (31° 28´ S, 64° 34 W) and Camino del Cuadrado (31° 41´ S, 64° 50´ W), at 615, 744, 948, and 1257, m a.s.l. (Figure 1). Vegetation varies from subtropical dry forest to temperate grasslands at the extreme sites (Giorgis *et al.*, 2011).

Collection data of the studied populations are presented in Table 1. Voucher specimens were deposited in the herbarium of the Museo Botánico de Córdoba (CORD). Living plants were placed in earthenware pots in an equal part mixture of sand and potting soil in the Experimental Garden of Museo Botánico (Córdoba, Argentina) to obtain adventitious roots.

Nuclear DNA content analyses

The amount of DNA was measured by flow cytometry in three individuals per population and three runs per individual. DNA content was measured by obtaining nuclear suspensions, according to Doležěl *et al.*, (2007), with minor modifications. Small pieces of fresh leaves from each sample individual and from *Zea mays* L. CE-777 (2C = 5.43 pg), which was used as internal standard, were co-chopped with a sharp razor blade in a glass petri dish containing 0.5 ml of Otto I solution (0.1 M citric acid 0.5% Tween 20) and 0.2 ml of 5 % PVP (PVP 40, Sigma-Aldrich). Nuclear suspensions were then filtered through a 45µm mesh nylon membrane and maintained at room temperature for 10–60 min. After that, 0.5 mL of Otto II buffer (0.4 M Na_2HPO_4—12 H_2O), propidium iodide (50 µg mL^{-1}), and RNAse (50 µg mL^{-1}) were added to stain DNA and avoid the labeling of double stranded RNA. Samples were kept at room temperature and analyzed after 10 min in a Bd Accuri™ C6 Flow Cytometer equipped with a 488 nm and a 633 nm Laser. Three DNA estimations were made for each plant (5,000 or 10,000 nuclei per analysis) on three days. Nuclear DNA content was calculated as (Sample peak mean/Standard peak mean) *2C DNA content of the standard (in pg). Cx values, were calculated as the 2C nuclear DNA amount divided by ploidy level (Greihuber *et al.*, 2005).

1.1. Cytogenetic analyses

Metaphase chromosomes were prepared from adventitious root tips pretreated with 2 mM 8-hydroxyquinoline for 24 h at 4°C and fixed in 3:1 ethanol:acetic acid. For slide preparation, root tips were washed twice in distilled water (10 min each), digested with a pectinex solution for 45 min at 37°C, and squashed in a drop of 45% acetic acid. The coverslip was removed in liquid nitrogen and then the slides were stored at -20°C.

Karyotype analysis. Slide preparations were stained with Giemsa and permanent mounts were made with Entellan© (Merck, Germany). Ten metaphases of different individuals per population were photographed with a phase contrast optic Olympus BX61 with software Cytovision® (Leica Biosystems) and camera JAI® model CV-M4+ CL monochromatic. The following measurements were taken: length of the short arm (s) and long arm (l), and total chromosome length (c) for each pair. The arm ratio (r = l/s) was calculated and used to classify chromosomes and determine homologs, according

to Levan *et al.*, (1964). Karyograms were constructed by organizing the chromosomes into groups according to their arm ratio and ordering them by decreasing length within each category. The resulting idiograms were based on the mean values obtained from the measurements of all individuals of each population. Karyotype asymmetry was estimated using the intrachromosomal (A1 = 1 - [P(b/B)/n]) and interchromosomal (A2 = s/x) indices of Romero Zarco (1986), where b and B are the mean length of short and long arms of each pair of homologues, respectively, n is the number of homologues, s is the standard deviation, and x the mean chromosome length.

Chromosome banding. Slides for fluorescent banding were stained with a drop of 0.5 mg/ml Chromomycin A_3 (CMA) in McIlvaine's buffer, pH 7.0, and distilled water (1:1) containing 2.5 mM MgCl for 90 min, subsequently stained with 2 µg/ml 4′-6-diamidino-2-phenylindole (DAPI) (both Sigma-Aldrich, Austria) for 30 min, and finally mounted in McIlvaine's buffer–glycerol v/v 1:1 (Schweizer, 1976; Schweizer and Ambros, 1994). The relative lengths of short and long chromosome arms (data not shown) and bands were calculated (considering haploid karyotype length = 100%) in five metaphases per population, each from a different individual. The amount of heterochromatin was expressed as percentage of the total length of the haploid karyotype.

Fluorescent *in situ* hybridization (FISH). The protocol of Schwarzacher and Heslop-Harrison (2000) was used with the p*Ta*71 probe to identify the 18S-5.8S-26S rDNA loci (Gerlach and Bedbrook, 1979) labeled with biotin-14-dUTP by nick translation (Bionick, Invitrogen) and subsequently detected with avidin-FITC (Sigma). For analysis of the 5S rDNA loci, a specific probe from *Pereskia aculeata* was used (Las Peñas *et al.*, 2011). These fragments were labeled with Digoxigenin-11-dUTP (DIG Nick translation mix, Roche) and detected with Anti-DIG-Rhodamine (Roche, USA). The slides were mounted with Vectashield antifade (Vector Laboratories) containing DAPI.

1.2. Statistical analyses

The analysis of variance or its nonparametric equivalent Kruskall Wallis was used for the analysis of DNA content and cytogenetic variables, followed by a comparison of means (Tukey, p <0.05). The analyses were performed using the INFOSTAT statistical package (Di Rienzo *et al.*, 2012).

RESULTS

Genome size of *G. quehlianum* varied between 3.55 pg and 4.30 pg in the four populations, with no significant differences (p = 0.11) among them (Table 1). However, a trend of DNA content decrease with increasing altitude was observed. In addition, endopolyploidy with peaks for 4C and 8C (Table 1) was observed in all the analyzed populations.

Regarding the cytogenetic characteristics, all the populations presented 2n = 22 (Table 1; Figure 2). No differences were found in the 10 *m* and 1 *sm* karyotype formula; the first *m* pair had a terminal satellite on the short arms, which was detected in 75% of the examined cells (Figure 2).

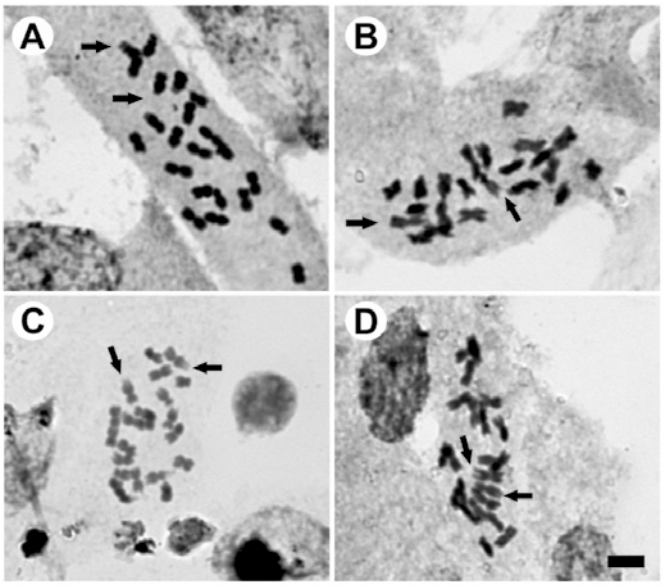

Figure 2. Somatic metaphases of *G. quehlianum* with Giemsa staining. **A** San Marcos Sierra (615 m a.s.l), **B** Capilla del monte Km 101 (744 m a.s.l), **C** Valle Hermoso (948 m a.s.l), **D** Camino del Cuadrado (1257 m a.s.l). Arrows indicate satellites. Bar = 5 µm.

The average chromosomal length was 3.26 µm. The highest chromosome length value (5.50 µm) was found in pair 1 from Capilla del Monte (744 m a.s.l.), and the lowest value (2.27 µm) was found in pair 10 fromValle Hermoso(948 m a.s.l). Statistically significant differences were found in C values (p <0.0001), with the shortest chromosomes (2.64 µm) being detected in the population from San Marcos Sierra (615 m a.s.l.), and the longest ones (4.11 µm), at Capilla del Monte (744 m a.s.l). The length of the haploid genome (TLH) varied between 29.02 µm (615 m a.s.l) and 45.26 µm (744 m a.s.l). No significant differences in intra- and inter-chromosomal asymmetry indices (A_1 and A_2), respectively, were observed (Table 1).

The populations of *G. quehlianum* at 615, 948, and 1257 m a.s.l were analyzed with the banding technique and FISH. All of them presented constitutive heterochromatin bands $CMA^+/DAPI^-$ associated with nucleolar

organizing regions (NORs) in the first pair of *m* chromosomes (Figure 3). On the other hand, FISH showed that the probe for 18-5.8-26S ribosomal genes hybridized in the terminal regions of the first *m* pair, coinciding with the $CMA^{+}/DAPI^{-}$ bands described above (Figure 3). The 5S locus was located in two chromosome pairs: in the first *m* pair below the 18-5.8-26S gene and in the last *m* pair in a paracentromeric position (Figure 3, 4). Both genes had similar sizes, being homomorphic. No differences between populations were observed when using either the banding or the FISH techniques (Figure 4).

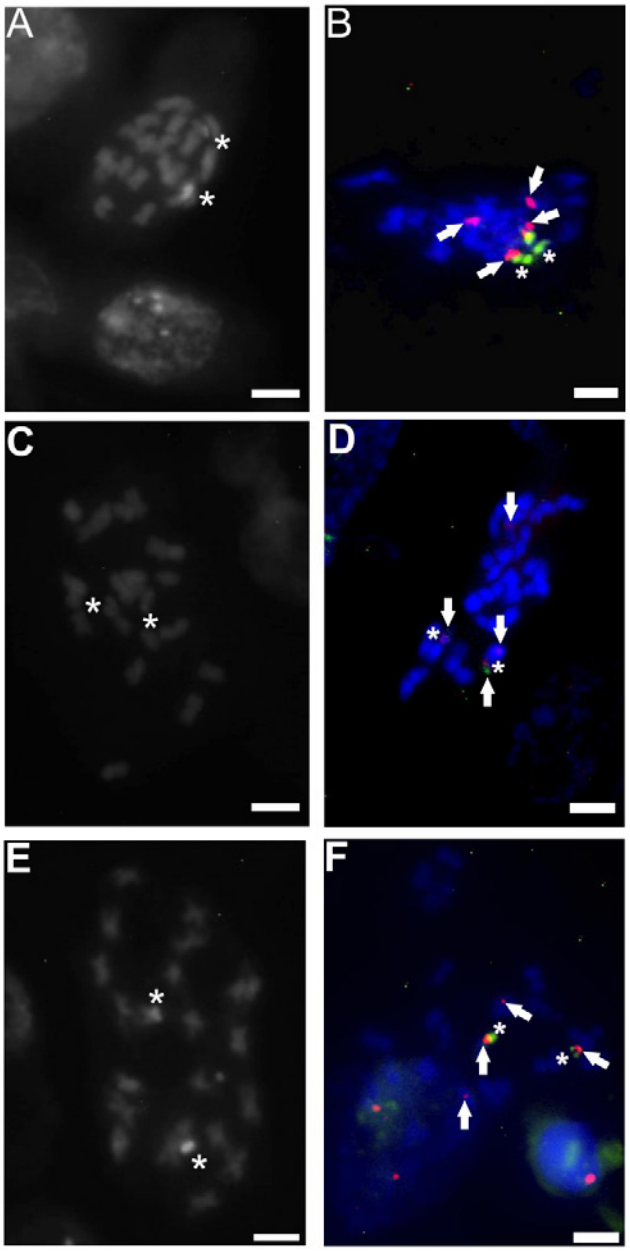

Figure 3. Populations of *G. quehlianum*: **A** and **B**. San Marcos Sierras (615 m a.s.l); **C** and **D** Valle Hermoso (948 m a.s.l); **E** and **F**: Camino del Cuadrado (1257 m a.s.l). **A**, **C** and **E** Fluorochrome chromosome banding CMA/DAPI; **B**, **D** and **F.** FISH using 18–5.8–26S (green) and 5S rDNA (red) probes. Arrows: rDNA 18–5.8–26S and CMA+/DAPI-/NORs, Asterisks: rDNA 5S. Bar= 5 µm.

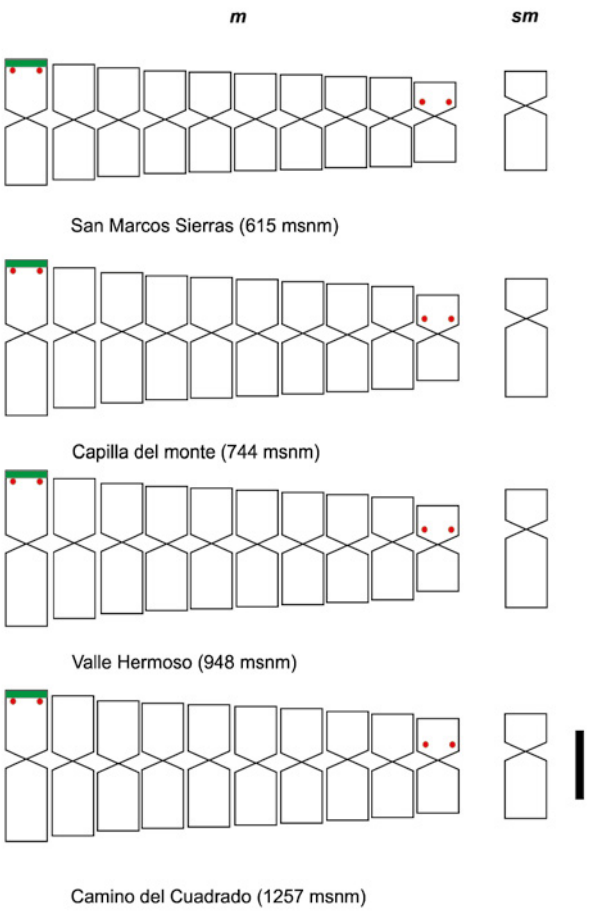

Figure 4. Idiograms with physical location of repetitive segments in *G. quehlianum* rDNA 18S–5.8S–26S /CMA+/DAPI- /NORs (green), and 5S rDNA (red). Bar = 2 µm.

DISCUSSION

The DNA content has been related to ecological features along altitudinal gradients in some Angiosperms (Knight and Ackerly, 2002; Šmarda and Bureš, 2006). In previous data on DNA content in *G. quehlianum*, Das and Das (1998) obtained a value of 6.46 pg for 2C using the Feulgen densitometry method. This value is higher than the one obtained in this study, which yielded an average of 3.86 pg among the four populations. These differences in the results could be due to the use of different methodological techniques to obtain the values of DNA content. The Feulgen densitometry method may not be as precise as flow cytometry (Dolezer and Bartos, 2005).

Species of several genera of New Zealand grasslands (*Agrostis, Festuca, Poa, Puccinellia*) with the highest C values were found in extreme environments, such as the sub-Antarctic region (Murray *et al.*, 2005). Knight and Ackerly (2002) analyzed the correlation between nuclear DNA content and environmental gradients in

different species of California, USA. Those with a higher content were more frequent in intermediate locations of the gradients, with lower contents being found at both extremes. In G. quehlianum, a decrease in DNA content values with increasing altitude was observed (4.30 pg at 615 m a.s.l., and 3.55 pg at 1257 m a.s.l.). Non significant statistical differences were found in the DNA content values among populations.Furthermore, in the analysis of the DNA content of all populations, a mixture of nuclei with three peaks, 2C, 4C and 8C, was observed, which suggests endopolyploidy. This process is considered an important mechanism of adaptation to high temperatures and water scarcity (Nagl, 1978; Negron-Ortiz, 2007; Leitch and Leitch, 2013). This pattern occurs in species with a small genome, which also gives them advantages in arid environments (Palomino *et al*., 1999; Del Angel *et al*., 2006). In Cactaceae, endopolyploidy was found in the subfamily Opuntioideae (Negron-Ortiz, 2007; Segura *et al*., 2007) as well as in some species of Cactoideae (Palomino *et al*., 1999; Del Angel *et al*., 2006; Bauk *et al*., 2016). However, it has not been observed in *Gymnocalycium*. The processes of endoduplication of the genome can vary among individuals of the same species in response to the effects of different environmental conditions (Barow, 2006; Leitch and Leitch, 2013).

The most common basic chromosome number in Cactaceae is x=11 (Pinkava, 2002; Goldblatt and Johnson, 2006; Das and Mohanty, 2006; Las Peñas *et al*., 2009, 2014). In all populations, the chromosome number of *G. quehlianum* was 2n=2x = 22, except for one individual of the population at 948 m a.s.l., indicating that a greater number of individuals should be analyzed in each population in order to explore whether the different chromosome numbers are associated with the altitudinal gradient. The chromosome numbers reported here coincide with meiotic studies that included the determination of pollen stem cells (Das and Das, 1998). Furthermore, no significant differences in the cytogenetic variables were detected among populations. Thus, *G. quehlianum* chromosomes had an average length of 2.98 µm and a symmetric karyotype, with most of the chromosomal pairs being metacentric. These characteristics coincide with the karyotypic homogeneity presented for the family (Cota and Philbrick, 1994; Cota and Wallace, 1995; Das *et al*., 2000; Das and Mohanty, 2006; Las Peñas *et al*., 2008, 2009, 2014).

Results of the CMA/DAPI chromosome banding and FISH techniques show that *G. quehlianum* presented the 18-5.8-26S loci in the terminal regions of the first pair of metacentric chromosomes, coinciding with the CMA^+/$DAPI^-$ band pattern. No differences among populations were observed along the altitudinal gradient. The location of this gene is highly conserved in Cactaceae (Las Peñas *et al*., 2009, 2014, 2017). On the other hand, the 5S probe hybridized in the first *m* pair next to the 18-5.8-26S, and in the smallest *m* pair. In most of Cactoideae, a pair of 18-5.8-26S and 5S loci is present in a haploid genome (Bauk *et al*.,2016; Las Peñas *et al*., 2016, 2017). In the genus *Pfeifera,* the 5S gene is duplicated in a chromosome different from the one carrying the 18-5.8-26S gene (Moreno *et al*., 2015), as it was found in this work. The 5S rDNA unit is located independently of other rDNA sequences, which can be related to the fact that it is transcribed by RNA polymerase III, whereas the polycistronic 45S rDNA uses RNA polymerase I (Garcia and Kovařík, 2013; Brasileiro-Vidal *et al*., 2007).

In most Angiosperms, the 18-5.8-26S and 5S sites are found in different chromosomes (Roa and Guerra 2015). However, in this work, the 5S locus was located adjacent to the 18-5.8-26S gene and in the last *m* pair.

The dispersion of 5S sites here reported for *G. quehlianum* may be attributed to several factors, including structural chromosomal rearrangements, such as translocations (Hayashi *et al*., 2001); dispersion of rDNA repeats; and amplification of new minor loci and deletion or not of original major loci (Pedrosa-Harand *et al*., 2006). The latter two factors were proposed as the mechanisms by which 5S rDNA loci repeatedly changed position during the radiation of species, without changing the co-linearity of other markers. These position changes can be mediated by mobile elements (Kalendar *et al*., 2008; Raskina *et al*., 2008).

This work makes one of the first contributions of information about DNA content and cytogenetic characteristics (karyotype, heterochromatin distribution and position of ribosomal genes) of *G. quehlianum* along an altitudinal gradient. Furthermore, we previously reported greater variation in ecological characters (Martino *et al*., 2021) than in cytogenetic ones among the four populations; this result may be attributed to the great phenotypic plasticity of morphological traits in response to environmental differences of this species. At the same time, our results indicate that changes in DNA content as well as cytogenetic changes are due to cryptic chromosome rearrangements for the species adaptation to the altitudinal gradient.

Climate change is affecting organisms throughout the world. Therefore, understanding the relationships between species characteristics and environment would help predict species responses to climate change (Gurvich *et al*., 2002; Aragón-Gastélum *et al*., 2014). Species with wide distributions along climate gradients would be less affected by climate than species with more limited distribution, such as *G. quehlianum*. In order to conserve this species, it is important to know its genetic characteristics all across its distribution range. Further research of these aspects is necessary to predict species responses to environmental changes.

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AUTHOR CONTRIBUTIONS

Design of the research: Martino P.; Gurvich G.; Las Peñas M. L.; Performance of the research: Martino P.; Gurvich G.; Las Peñas M. L.

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CYTOGENETIC STUDY IN SAND SPIDERS (SICARIIDAE) FROM THE BRAZILIAN CAATINGA: SEX CHROMOSOME SYSTEM DIVERSITY IN CLOSELY RELATED SPECIES

ESTUDIO CITOGENÉTICO EN ARAÑAS DE ARENA (SICARIIDAE) DE LA CAATINGA BRASILEÑA: DIVERSIDAD DEL SISTEMA DE CROMOSOMAS SEXUALES EN ESPECIES ESTRECHAMENTE RELACIONADAS

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ABSTRACT

In this study, we investigated the chromosomes of three species of *Sicarius* spiders from the Brazilian Caatinga, using classical and molecular cytogenetic techniques. Based on the phylogenetic approach, we also discussed about the variation of diploid number, types of sex chromosome system and changes in the localization of ribosomal genes of Scytodoidea. *Sicarius* are Synspermiata spiders that together with the genera *Loxosceles* and *Hexophthalma* constitute the family Sicariidae. In this group, the available cytogenetic data showed a low diploid number range (2n♂=18 to 2n♂=23) and the presence of only multiple sex chromosome systems (X_1X_2Y and X_1X_20). Mitotic metaphase cells exhibited 2n♂=16+X_1X_2Y for *Sicarius cariri* and *S. ornatus*, and 2n♂=18+XY for *S. tropicus*. In these species, silver impregnation revealed nucleolar organizer region (Ag-NOR) on the terminal region of pair 1. In *S. ornatus* and *S. tropicus*, the results obtained with fluorescent *in situ* hybridization (FISH) using 18S rDNA probe were similar to Ag-NOR, however in *S. cariri*, the ribosomal sites were localized in the terminal region of the X_1 sex chromosome. In this work, we presented the first description of a simple sex chromosome system for Sicariidae, helping to understand how the XY sex chromosome system evolved from the X_1X_2Y system. Additionally, FISH data incongruous with Ag-NOR indicate that the cytogenetic studies in Sicariidae allow investigating the relation between the karyotype evolution and the distribution and the activity of rDNA genes.

Key words: karyotype, mitosis, nucleolar organizer region, rDNA, *Sicarius*

RESUMEN

En este estudio, investigamos los cromosomas de tres especies de arañas *Sicarius* de la Caatinga brasileña, utilizando técnicas de citogenética clásica y molecular. Usando un enfoque filogenético, también discutimos la variación del número diploide, los tipos de sistema cromosómico sexual y los cambios en la localización de los genes ribosómicos en Scytodoidea. Los *Sicarius* son arañas Synspermiata que, junto con los géneros *Loxosceles* y *Hexophthalma*, constituyen a la familia Sicariidae. En este grupo, los datos citogenéticos disponibles mostraron un rango de número diploide bajo (2n♂=18 a 2n♂=23) y únicamente la presencia de sistemas de cromosomas sexuales múltiples (X_1X_2Y y X_1X_20). Las células mitóticas en metafase mostraron 2n♂=16+X_1X_2Y para *Sicarius cariri* y *S. ornatus*, y 2n♂=18+XY para *S. tropicus*. En estas especies, la impregnación de plata reveló la región organizadora nucleolar (Ag-NOR) en la región terminal del par 1. En *S. ornatus* y *S. tropicus*, los resultados obtenidos con la hibridación in situ fluorescente (FISH) utilizando la sonda de ADNr 18S fueron similares a los de Ag-NOR, sin embargo, en *S. cariri* los sitios ribosomales se localizaron en la región terminal del cromosoma sexual X_1. En este trabajo, presentamos la primera descripción de un sistema cromosómico sexual simple para Sicariidae, ayudando a entender cómo el sistema cromosómico sexual XY evolucionó a partir del sistema X_1X_2Y. Además, los datos de FISH incongruentes con Ag-NOR indican que los estudios citogenéticos en Sicariidae permiten investigar la relación entre la evolución del cariotipo y la distribución y la actividad de los genes de ADNr.

Palabras clave: cariotipo, mitosis, región organizadora nucleolar, ADNr, *Sicarius*

INTRODUCTION

The spider family Sicariidae is considered of medical importance in the world (Lotz, 2012), including sedentary species, which can be ground-dwelling hunters or web-weavers (Dias *et al.*, 2010). Sicariidae includes 171 species distributed into three genera: *Hexophthalma* composed of eight species, *Sicarius*, with 21 species, and *Loxosceles*, the most diversified genera with 142 representatives (World Spider Catalog, 2021). This latter genus is well known due to the toxicity of its venom, causing skin necrosis, renal failure and haemolysis (Silva *et al.*, 2004; Vetter, 2008). *Hexophthalma* spiders occur only in southern Africa while *Loxosceles* presents widest distribution, with species described in America, Africa, Mediterranean Europe and Asia; however, the largest diversity of species is recorded in the American continent (World Spider Catalog, 2021). *Sicarius* is distributed in South and Central America and is restricted to xeric habitats, mainly deserts and tropical dry forests (Magalhaes *et al.*, 2013).

For many years, in Brazil only one *Sicarius* species was known, *S. tropicus* (Mello-Leitão, 1936). However, recently Magalhaes *et al.* (2013, 2017) described other species from this country, namely *S. boliviensis* Magalhaes, Brescovit & Santos, 2017, *S. cariri* Magalhaes, Brescovit & Santos, 2013, *S. diadorim* Magalhaes, Brescovit & Santos, 2013, *S. jequitinhonha* Magalhaes, Brescovit & Santos, 2017, *S. ornatus* Magalhaes, Brescovit & Santos, 2013, and *S. saci* Magalhaes, Brescovit & Santos, 2017. The monophyly of Sicariidae is well supported by morphological (Platnick *et al.*, 1991; Binford *et al.*, 2008; Labarque y Ramírez, 2012; Magalhaes *et al.*, 2013, 2017) and molecular data (Wheeler *et al.*, 2017 *contra* Binford *et al.*, 2008). Some characteristics considered as synapomorphies for sicariids are modifications in chelicerae setae, tarsal claws, abdominal entapophysis, and the venom protein sphingomyelinase D, which is responsible for the envenomation symptoms (Binford y Wells, 2003; Magalhaes *et al.*, 2017).

Sicariidae belongs to the monophyletic superfamily Scytodoidea composed by (Sicariidae (Drymusidae + Periogopidae) (Ochyroceratidae + Scytodidae))) (Labarque y Ramírez, 2012; Wheeler *et al.*, 2017). In this group, Scytodidae is the most diverse, including a total of five genera and 245 known species (World Spider Catalog, 2021), but only five of them belonging to *Scytodes* were analyzed from the cytogenetic point of view (Araujo *et al.*, 2021). The scytodids present a high variability in diploid number, from 2n♂=13 to 2n♂=31, but a simple and conserved sex chromosome system of the X0 type. The exception is *Scytodes globula* Nicolet, 1849 that revealed an intraspecific variation due to the occurrence of X0 and $X_1X_2$0 systems (Diaz y Saez, 1966; Rodríguez-Gil *et al.*, 2002; Araujo *et al.*, 2008).

Ochyroceratidae possess 168 species described into 10 genera, but only a North American undetermined species of *Ochyrocera* was cytogenetically analysed, exhibiting 2n♂=13 and X0 sex chromosome system (Král *et al.*, 2006). The family Drymusidae includes 17 species with chromosomal data only for *Izithunzi capense* (Simon, 1893), from South Africa, with 2n♂=37+X_1X_2Y (Král *et al.*, 2006). Periegopidae is known only by three species from Queensland and New Zealand (World Spider Catalog, 2021), and there are no cytogenetic data for this family.

The family Sicariidae has karyotype information for 15 representatives, showing low diversity in the diploid number (2n♂=18 to 2n♂=23) and the occurrence of only multiple sex chromosome systems of the X_1X_2Y and $X_1X_2$0 types (Araujo *et al.*, 2021). *Hexophthalma* only has the diploid number 2n=20 described for females of an undetermined species (Král *et al.*, 2019). The genus *Loxosceles* presents 12 species chromosomally characterized, in which the following diploid numbers were identified: 2n♂=18 in *L. reclusa* Gertsch & Mulaik, 1940; 2n♂=19 in *L. spinulosa* Purcell, 1904; 2n♂=20 in *L. rufipes* (Lucas, 1834); 2n♂=20-21 in *L. rufescens* (Dufour, 1820); 2n♂=23 in *L. amazonica* Gertsch, 1967, *L. gaucho* Gertsch, 1967, *L. hirsuta* Mello-Leitão, 1931, *L. intermedia* Mello-Leitão, 1934, *L. laeta* (Nicolet, 1849), *L. puortoi* Martins, Knysak & Bertani, 2002, *L. similis* Moenkhaus, 1898 and *L. variegata* Simon, 1897. All these species showed X_1X_2Y sex chromosomes system, except *L. rufipes* and *L. reclusa* that exhibited $X_1X_2$0 system (Beçak y Beçak, 1960; Diaz y Saez, 1966; Hetzler, 1979; Silva, 1988; Tugmon *et al.*, 1990; Oliveira *et al.*, 1996, 1997; Silva *et al.*, 2002; Král *et al.*, 2006; Kumbiçak, 2014; Araujo *et al.*, 2020).

In the genus *Sicarius*, only two species were investigated, *S. tropicus* (2n♂=19, X_1X_2Y) from Brazil and an undetermined species (2n=21, X_1X_2Y) from Cusco, Peru (Franco y Andía, 2013; Araujo *et al.*, 2021), more likely to be *Sicarius boliviensis*, owing to the sampling locality (Magalhaes *et al.*, 2017). Nevertheless, the cytogenetic data of *S. tropicus* could be considered preliminary because the karyotype information is restricted to a brief description of diploid number and sex chromosome system (Franco y Andía, 2013).

A cytogenetic analysis of three *Sicarius* species from the Brazilian fauna was accomplished in the present study, using standard staining, silver impregnation to reveal the active nucleolar organizer regions (NORs), and fluorescent *in situ* hybridization (FISH) with 18S rDNA probe to map the number and localization of the major ribosomal genes. Among the 21 Scytodoidea spiders karyotyped, only 10 species were examined regarding to the NOR distribution (Král *et al.*, 2006; Araujo *et al.*, 2008, 2020). Additionally, based on the phylogenetic approach, we discussed about the chromosome evolution of Scytodoidea, focusing in the variation of diploid number, types of sex chromosome system and change in the localization of ribosomal genes.

MATERIALS AND METHODS

A sample of 35 specimens was analyzed in this work. The data concerning the number of individuals and the collection localities in Brazil are shown in Table 1. The vouchers were deposited in the arachnid collection of the Instituto Butantan, São Paulo, (IBSP; curator A.D. Brescovit); Coleções Taxonômicas of the Universidade Federal de Minas Gerais, Belo Horizonte (UFMG; curator A.J. Santos), and Coleção de História Natural of the Universidade Federal do Piauí, Floriano (CHNUFPI; curator L.S. Carvalho), in Brazil.

The cytological preparations were obtained following the procedures of Araujo *et al.* (2005). The chromosome slides were stained with 3% Giemsa solution (3% commercial Giemsa solution and 3% phosphate buffer pH 6.8, in distilled water), silver-impregnated (Howell y Black, 1980) to detect the NORs and submitted to FISH with 18S rDNA probes to localize the major ribosomal gene. The morphological classification of chromosomes followed the nomenclature proposed by Levan *et al.* (1964).

The 18S rDNA probes were obtained by PCR using the DNA of *Physocyclus globosus* (Taczanowski, 1874) (Pholcidae) and the primers 18S-F 5' CGAGCGCTTTTATTAGACCA and 18S-R 5' GGTTCACCTACGGAAACCTT (Forman *et al.*, 2013). Probes were labeled with 11-dUTP-digoxigenin by PCR. The FISH technique was performed according Pinkel *et al.* (1986). The chromosomal DNA was denatured in 70% formamide for 5 min at 70° C and the hybridization solution was denatured in a termal cycler for 10 min at 95°C. Probes were detected with anti-digoxigenin antibody conjugated to rhodamine. Chromosome spreads were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) and the slides were mounted with antifading solution. The images were captured using a Zeiss Imager A2 microscope, coupled to a digital camera and the Axio Vision software.

The ancestral condition of diploid number, sex chromosome system and number of rDNA sites was reconstructed in Mesquite (Maddison y Maddison, 2011), using the maximum parsimony approach and the phylogenetic proposal of Wheeler *et al.* (2017). The chromosome data were obtained from the present study and spider cytogenetic database (Araujo *et al.*, 2021).

Table 1. *Sicarius* species cytogenetically analyzed in this work, including the number of specimens and collection localities in Brazil. PI=state of Piauí; SE=state of Sergipe; PB=state of Paraíba.

Species	Number of individuals	Locality
Sicarius cariri	3♂/1♀	Parque Nacional da Serra da Capivara (8°49'48.0"S, 42°33'16.0"W), São Raimundo Nonato, PI
	1♀	(7°9'39.4"S, 41°28'2.5"W), Picos, PI
	2♀	Horto Florestal (4°16'19.0"S, 41°41'18.9"W), Piripiri, PI
	2♂	Povoado Saquinho (2°46'2.5"S, 41°48'19.3"W), Ilha Grande do Piauí, PI
	2♂	Parque Nacional da Serra das Confusões (8°56'16.9"S, 43°51'48.1"W), Cristino Castro, PI
	15♂/1♀	Parque Municipal Pedra de Castelo (5°12'5.9"S, 41°41'14.0"W), Castelo do Piauí, PI
Sicarius ornatus	2♂	Parque Nacional da Serra de Itabaiana (10°44'57.3"S, 37°20'20.1"W), Itabaiana, SE
Sicarius tropicus	3♂	Reserva Particular de Patrimônio Natural Fazenda Almas (7°23'16.9"S, 36°48'31.8"W), São José dos Cordeiros, PB
	3♂	Parque Municipal Pedra de Castelo (5°12'5.9"S, 41°41'14.0"W), Castelo do Piauí, PI

RESULTS

Chromosome characterization

Mitotic metaphase cells of male and female specimens of *S. cariri* showed the diploid number and sex chromosome system 2n=16+X_1X_2Y and 2n=16+$X_1X_1X_2X_2$, respectively (Fig. 1A-B). All the chromosomes presented metacentric morphology, with exception of submetacentric pair 2. Regarding to the size, the autososomal chromosomes could be classified into three categories: large (pairs 1 and 2), medium (pairs 3 and 4) and small (pairs 5 to 8). In spermatogonial cells, the X_1 and Y sex chromosome were easily identified as unpaired elements, which corresponded to the largest and smallest chromosomes of the karyotype. The X_2 chromosome showed an intermediary size between the 2nd and 3rd autosomal pairs (Fig. 1A).

Sicarius ornatus also presented 2n♂=19. In the karyotype, three unpaired chromosomes were identified, which were similar to the sex chromosomes of *S. cariri*. Thus, *S. ornatus* should also display a X_1X_2Y sex chromosome system. However, in this species, all autosomal pairs revealed metacentric morphology, the X_1 and X_2 sex chromosomes were submetacentric and the Y was a tiny acrocentric chromosome (Fig. 1C). The autosomal pair 1 exhibited large size, compared to the medium-sized pairs 2 to 5 and the smallest elements of the karyotype, pairs 6 to 8. The X_1 chromosome presented a similar size to the pair 1, the X_2 chromosome was larger than the pair 2 and the Y was the smallest chromosome of the karyotype (Fig. 1C).

In *S. tropicus*, the mitotic metaphase cells evidenced 2n♂=20, with two unpaired chromosomes, one large and other small-sized. The karyotype comparison with *S. cariri* and *S. ornatus* and the analysis of meiotic cells permitted us interpreted these unpaired elements as X and Y sex chromosomes. In *S. tropicus*, all chromosomes presented metacentric morphology. The pair 1 was

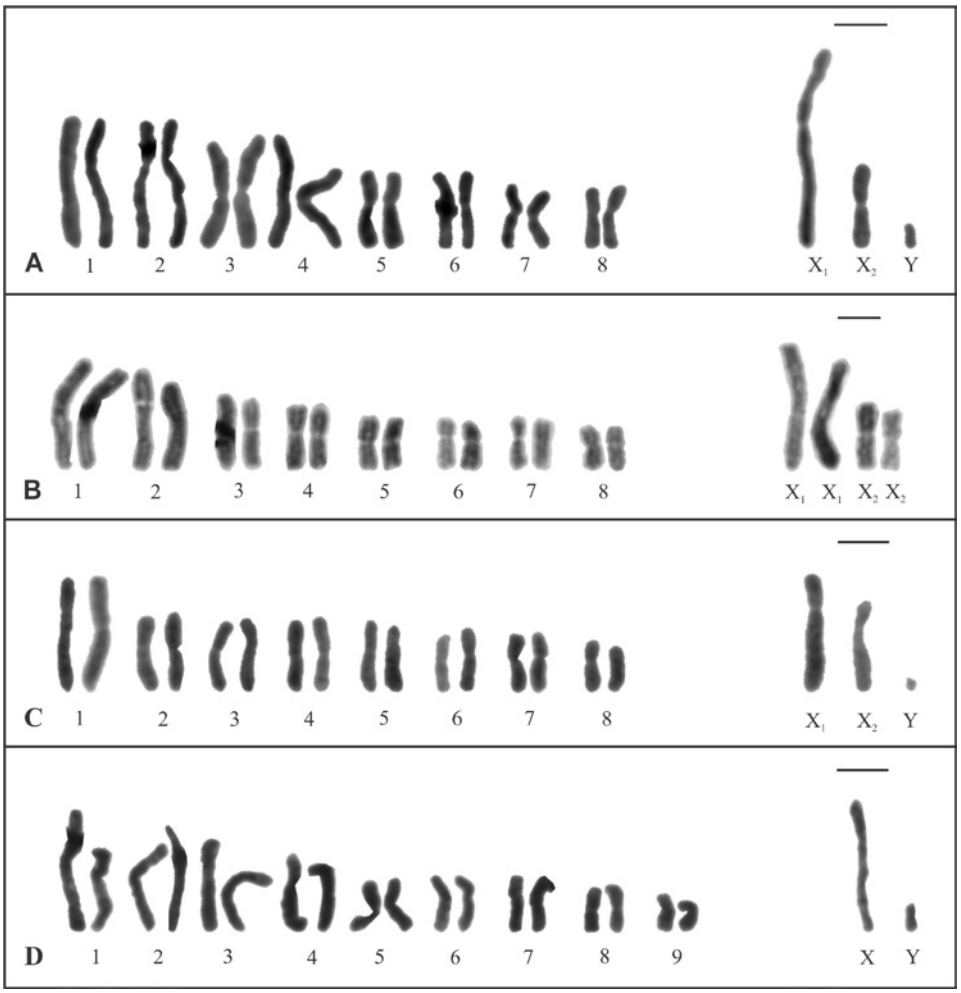

Figure 1. Karyotypes of three *Sicarius* species. **A-B**. *Sicarius* cariri with 2n♂=16+X_1X_2Y and 2n♀=16+$X_1X_1X_2X_2$, respectively. **C.** *Sicarius ornatus*, 2n♂=16+X_1X_2Y. **D.** *Sicarius tropicus*, 2n♂=18+XY.
In all species, the chromosomes were predominantly metacentrics. Scale bar=5μm.

large-sized, the pairs 2, 3 and 4 medium-sized and the pairs 5 to 9 small-sized. The X and Y sex chromosomes corresponded to the largest and the smallest elements of the karyotype, respectively (Fig. 1D).

The analysis of meiotic cells of *S. cariri* and *S. tropicus* revealed, in the pachytene, autosomal chromosomes completely paired and a single and very small element, interpreted as Y chromosome (Fig. 2A-B). For both species, the X sex chromosomes were not identified in this meiotic substage. Diplotene cells of *S. tropicus* presented 9 autosomal bivalents, with up to two interstitial or terminal chiasmata, and one heteromorphic bivalent, formed by the end-to-end paired XY chromosomes (Fig. 2C). Nuclei in metaphase II of this species showed the haploid sets n=9+X and n=9+Y (Fig. 2D).

Silver-impregnated mitotic metaphase nuclei of the three *Sicarius* species revealed active NORs on the long arm terminal region of pair 1 (Fig. 3A-F). In *S. cariri*, the 18S rDNA sites were located in the long arm terminal region of the X_1 sex chromosome (Fig. 4A). In this species, the incongruence between the results of Ag-NOR and FISH were observed among the cells of a same individual as well as in cells of different specimens. In *S. ornatus* and *S. tropicus*, the ribosomal cistrons occurred only in the long arm terminal region of the 1st autosomal pair (Fig. 4B-E), confirming the results of silver impregnation.

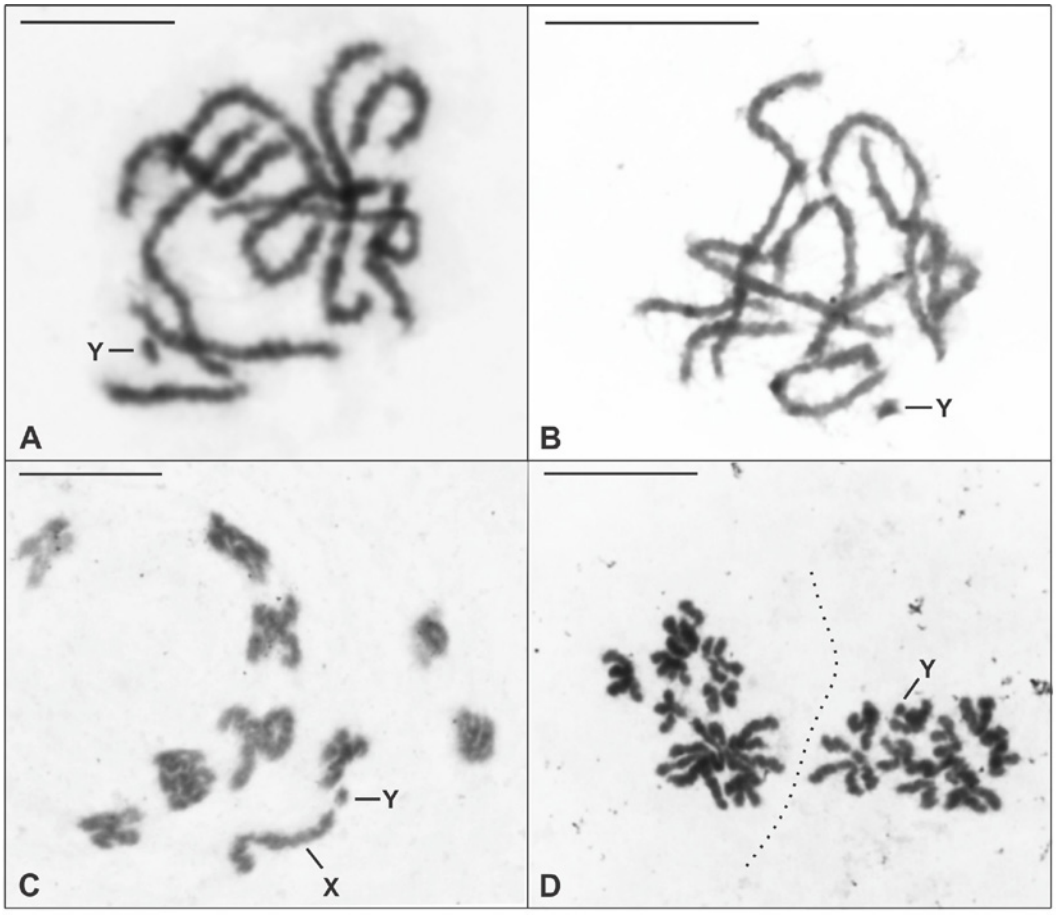

Figure 2. Testicular cells of *Sicarius cariri* (A) and *Sicarius tropicus* (B-D) stained with Giemsa. **A-B**. Pachytene nuclei, showing the univalent and very small Y chromosome. **C.** Diplotene with nine autosomal bivalents and one heteromorphic XY bivalent. Note the end-to-end association between the sex chromosomes. **D.** Metaphase II cells, with n=9+X (left) and n=9+Y (right). Scale bar=10µm.

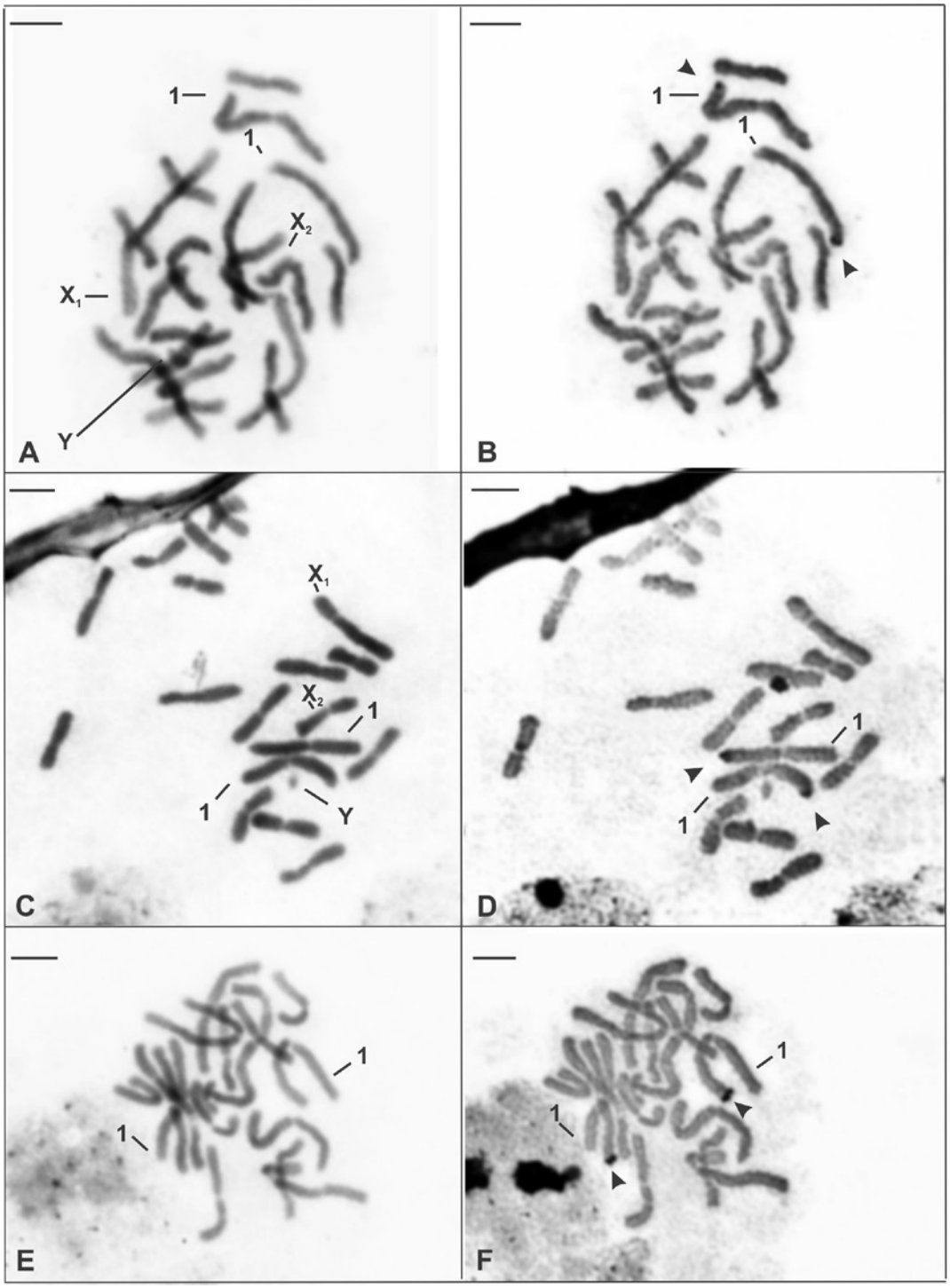

Figure 3. Mitotic metaphase cells of *Sicarius* species submitted to Giemsa-staining (A, C, E) and silver impregnation (B, D, F) to reveal the nucleolar organizer regions (arrowhead). **A-B**. *Sicarius cariri*. **C-D.** *Sicarius ornatus*. **E-F**. *Sicarius tropicus*. The cells showed in C and D are with incomplete diploid set. Scale bar=5µm.

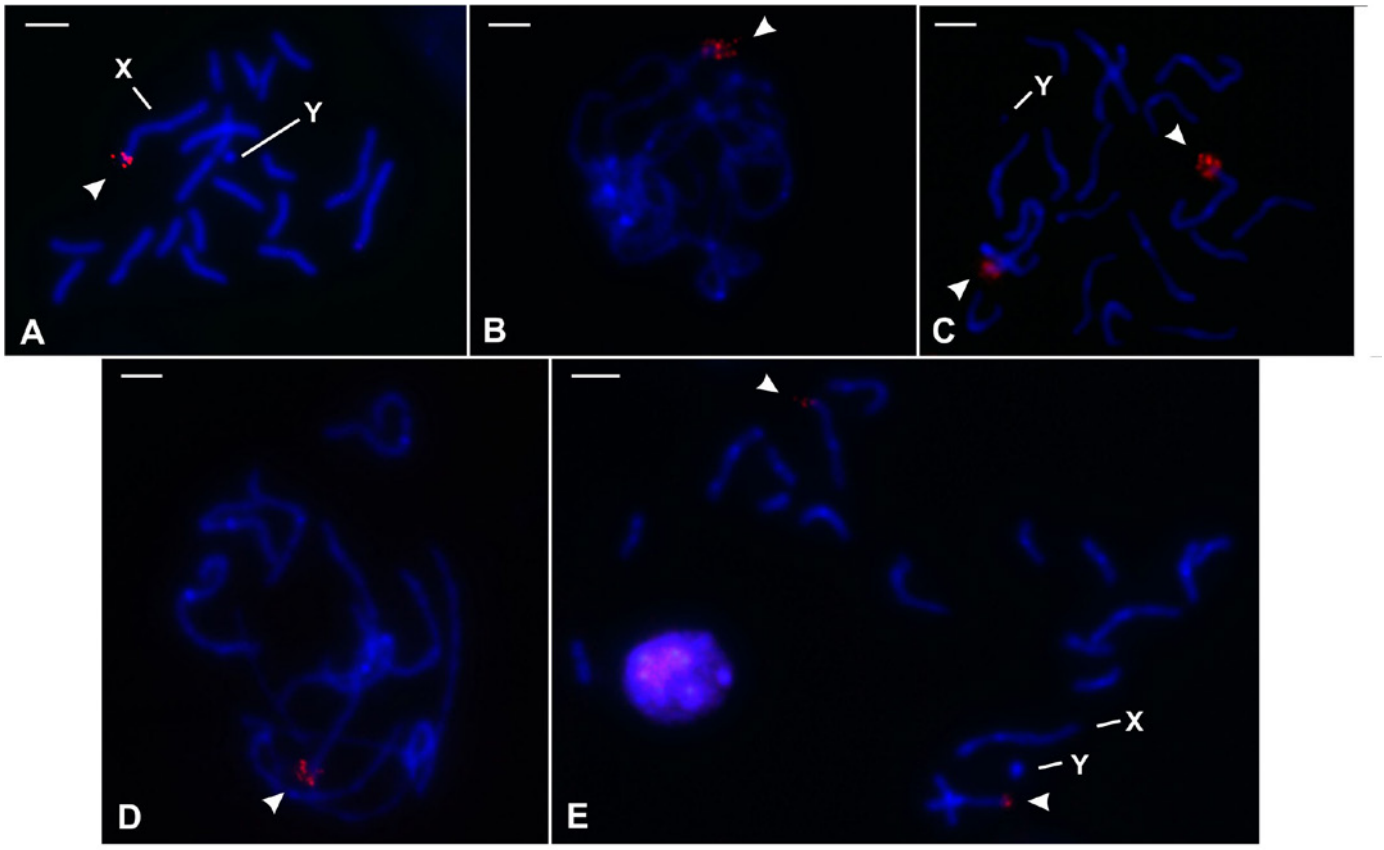

Figure 4. Spermatogonial cells of *Sicarius* species after fluorescent in situ hybridization with 18S rDNA probe. **A.** Mitotic metaphase of *Sicarius cariri,* indicating rDNA site (arrowhead) in the X_1 chromosome. **B-C.** Pachytene and mitotic metaphase of *Sicarius ornatus*. Note the bright signal (arrowhead) in the terminal region of one bivalent (B) and in pair 1 (C). **D-E.** Pachytene and mitotic metaphase of *Sicarius tropicus* exhibiting rDNA (arrowhead) in the terminal region of one bivalent (D) and in the 1st autosomal pair (E). Scale bar=5µm.

Chromosome evolution

The maximum parsimony analyses revealed 2n=18-22 as the ancestral autosomal number for Scytodoidea and Sicariidae (Fig. 5A). Overall, the karyotype evolution occurred through independent decreased in autosomal number, with the exception of two species of Scytodidae (*Scytodes fusca* - 30 autosomes and *Scytodes* sp. – 26 chromosomes, without description of the sex chromosome system) and Drymusidae (*Izithunzi capense*, 34 autosomes).

The presence of sex chromosome system including two X chromosome and one Y chromosome (X_1X_2Y) seems to be the ancestral state for Sicariidae and the clade composed by Sicariidae (Drymusidae + Periogopidae) (Fig. 5B, C). On the other hand, for Ochyroceratidae + Scytodidae, the X0 sex chromosome system is the shared character (Fig. 5B, C). The only exception is an unidentified species of the genus *Scytodes*, in which the sex chromosome system was not described. Within Sicariidae, the only change in the number of X sex chromosome was reported in *S. tropicus* with a XY sex chromosome system. The loss of Y chromosome was recorded only in *L. reclusa* and *L. rufipes*.

Despite the low number of species characterized, the presence of three or four rDNA sites seems to be the ancestral condition for Scytodoidea (Fig. 5D). However, this state was frequently changed during the evolution of this group. In *L. amazonica* and *L. puortoi*, these changes involved the increase of the number of major rDNA cistrons while in *Sicarius* species seems to be occurred a decrease in the number of these sites. The analyses also showed that in Ochyroceratidae + Scytodidae and *Sicarius* species, the ancestral rDNA number is lower than those observed in Scytodoidea.

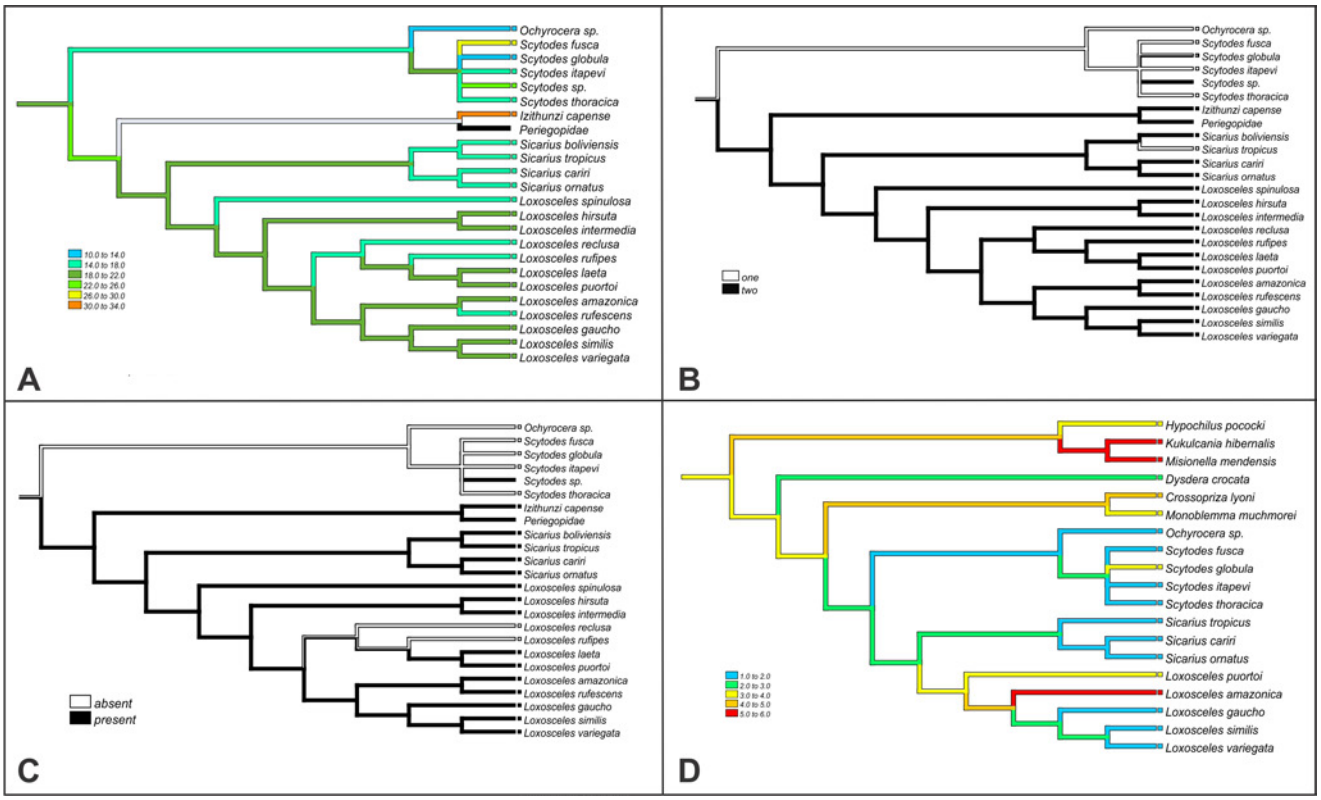

Figure 5. Chromosome evolution in Scytodoidea spiders obtained after Mesquite analysis. **A.** Autosomal number. **B.** Number of X sex chromosome. **C.** Presence of sex chromosome system including a Y chromosome. **D.** Number of chromosomes with NOR or rDNA sites.

DISCUSSION

The diploid number 2n=19, the X_1X_2Y sex chromosome system and the chromosomal morphology predominantly metacentric herein observed in *S. cariri* and *S. ornatus* are similar to those previously described for *S. tropicus* and only one species of the genus *Loxosceles*, *L. spinulosa* (Král *et al.*, 2006; Araujo *et al.*, 2020). Additionally, the X_1X_2Y sex chromosome system verified in *S. cariri* and *S. ornatus* is the most common in Sicariidae, occurring in 12 out of the 15 species cytogenetically characterized so far (Araujo *et al.*, 2020). The tendency of decreasing of the diploid number verified in some Scytodoidea species is the main mechanism of chromosome evolution for spiders and has been reported in many studies accomplished with related species (Stávale *et al.*, 2010; Araujo *et al.*, 2020; Ávila Herrera *et al.*, 2021). In an elegant cytogenetic work with many Pholcidae spiders, in which data of molecular and paleontological studies were discussed, Ávila Herrera *et al.* (2021) suggested that the X_1X_2Y sex chromosome system possesses an ancient origin in spiders and could have arise before the emergence of Araneomorphae lineage.

The karyotype found here for *S. tropicus* (2n=18+XY) differed from that registered for other population of this same species (2n=16+X_1X_2Y) (Araujo *et al.*, 2021), and the description of a simple sex chromosome system of the XY type is original for Sicariidae. The high similarity regarding to the size of the Y chromosome among the *Sicarius* species having the X_1X_2Y and XY systems indicates that the evolution of the XY system occurred through rearrangements involving only the X chromosome. The XY system probably had origin from the X_1X_2Y system, in which the ancestral and metacentric X_1 and X_2 chromosomes were pericentrically inverted, originating subtelo-acrocentric chromosomes, such as those verified in *Sicarius* sp. (Franco y Andía, 2013). In a subsequent event, the X_1 and X_2 chromosomes were fused, converting the X_1X_2Y into a XY system. This hypothesis regarding XY sex chromosome evolution was proposed by Král *et al.* (2006) and Ávila Herrera *et al.* (2021), analyzing the behavior of the XY sex chromosomes during the meiosis of *Diguetia albolineata* (O. Pickard-Cambridge, 1895) (Diguetidae) and *Wugigarra* sp., (Pholcidae) respectively. In these species as well as in *S. tropicus* analyzed here, the X and Y chromosomes exhibited only one end-to-end association during prophase I, without the presence of chiasma. The present study in *Sicarius* species filled in an important gap in the hypothesis of Král *et al.* (2006)

about the evolution of sex chromosomes systems in basal clades of Araneomorphae, taking into account that the hypothetic X_1X_2Y system with subtelo-acrocentric X_1 and X_2chromosomes was exclusively observed in *Sicarius* sp. (Franco y Andía, 2013).

The differences related to diploid number and sex chromosome system observed in *S. tropicus* (present study; Araujo *et al.*, 2021) may represent an interpopulational variation, indicating that the karyotype 2n=18+XY is not well established in all populations of this species or it had an independent origin in the populations analyzed by us. Magalhaes *et al.* (2014), performing a phylogeographic study in *S. cariri*, using sequence data of nuclear and mitochondrial genes, revealed highly structured populations, which might be evolving independently. It is possible that *S. tropicus* populations are also strongly structured geographically, which could explain the differences in the karyotypes. Alternatively, the specimens initially described by Araujo *et al.* (2021) as *S. tropicus* could correspond to another species of the genus *Sicarius*, considering that the cytogenetic study accomplished by Araujo preceded the taxonomical and systematic revision of the genus *Sicarius* (Magalhaes *et al.*, 2013, 2017).

The supposed stability of number and localization of NORs in spiders has knocked down with the increase of cytogenetic studies. In an analysis of NORs in 30 Pholcidae spiders, Ávila Herrera *et al.* (2021) revealed a great diversity of number of this site, which can occur in autosomes and/or X sex chromosome. The results obtained herein using FISH with rDNA probe only in three *Sicarius* species revealed the presence of ribosomal cistrons in autosomes (*S. ornatus* and *S. tropicus*) and X_1 sex chromosome (*S. cariri*). It is interesting to emphasize that this difference of localization of rDNA in autosome/sex chromosome occurs in species with similar karyotype characteristics, indicating that the changes involving the ribosomal genes can be independent of the differentiation of the sex chromosome system. In *S. cariri*, the localization of active NORs and 18S rDNA showed incongruous data, considering that the silver-impregnated regions were visualized on the terminal sites of the 1st autosomal pair, such as in *S. ornatus* and *S. tropicus*, but the FISH evidenced a bright signal in the terminal region of the X_1 sex chromosome. Therefore, in *S. cariri* the silver impregnation might have evidenced false Ag-NORs, taking into account that this technique reveals the NORs indirectly. This occurs due to the affinity of the silver nitrate by acidic proteins associated with the rRNAs or heterochromatic regions (Sanchez *et al.*, 1995; Lorite *et al.*, 1997; Dobigny *et al.*, 2002; Kasahara, 2009; Kavalco *et al.*, 2009; Reis *et al.*, 2012). On the other hand, the impregnation of the terminal region of pair 1 of *S. cariri*, which is certainly carrier of 18S rDNA genes in the two other closely related species, *S. ornatus* and *S. tropicus*, might suggest the presence of cryptic NORs in *S. cariri*, such as those reported by Cabrero y Camacho (2008) in some grasshopper species. The silver impregnation on pair 1 of *S. cariri* can represent a vestigial locus of rDNA gene for this species, which was translocated to the X_1 sex chromosome; this vestigial rDNA is very small to be detected by the FISH technique but it retains its transcriptional activity.

In conclusion, the data shown herein expanded the knowledge of the karyotype diversity already registered for sicariid spiders. Moreover, we identified an intriguing variation when the results of Ag-NOR and FISH were compared. Therefore, the Scytodoidea spiders are not only interesting for cytogenetic studies due to the variability in the sex chromosome system, but also because they are suitable for investigating karyotype evolution in spiders and its relationship to the distribution and activity of rDNA genes.

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EVOLUTIONARY DYNAMICS OF AUTOPOLYPLOIDS IN NATURAL POPULATIONS: THE CASE OF *TURNERA SIDOIDES* COMPLEX

DINÁMICA EVOLUTIVA DE AUTOPOLIPLOIDES EN POBLACIONES NATURALES: EL CASO DEL COMPLEJO *TURNERA SIDOIDES*

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ABSTRACT

Turnera sidoides (*x*=7) is one of the few well-studied South American autopolyploid complexes. Since polyploidy has played a prominent role within this complex, ongoing studies in *T. sidoides* focus on understanding the mechanisms involved in the origin and the establishment of polyploids using integrative approaches. This paper synthesises the results of more than 20 years of research on this topic. Cytogenetics analysis provided evidences for the production of unreduced male and female gametes, supporting the hypothesis of bilateral sexual polyploidization as the mechanism of origin of polyploids in *T. sidoides*. The finding of viable triploids suggested that unilateral sexual polyploidization could also be an important mechanism for the origin of tetraploids in *T. sidoides*. The occurrence of plants continuously forming many unreduced gametes would play a key role in the establishment of neopolyploids in natural populations. Also, the higher number of propagules that tetraploids contribute to subsequent generations, the ability to multiply asexually by rhizomes, and the occurrence of occasional cases of self-compatibility and successful illegitimate crosses in polyploids increase the likelihood that a low frequency of neopolyploids can be maintained in natural populations of *T. sidoides*. In addition, integration of cytogeographic and genetic divergence data together with past niche modelling provided further insights supporting the hypothesis that historical climatic and geomorphological events provided favourable conditions for the establishment of autopolyploids, with the wider distribution of tetraploids of *T. sidoides* being the result of their range expansion.

Key words: cytogeography, establishment, origin, polyploidy, unreduced gametes

RESUMEN

Turnera sidoides (*x*=7) es uno de los pocos complejos autopoliploides sudamericanos bien estudiados. Como la poliploidía ha tenido un papel destacado en el complejo, los estudios en curso en *T. sidoides* se centraron en la comprensión de los mecanismos implicados en el origen y el establecimiento de los poliploides mediante diferentes enfoques. En este trabajo se sintetizan los resultados de más de 20 años de investigación sobre este tema. El análisis citogenético proporcionó evidencias de la producción de gametos masculinos y femeninos no reducidos, sustentando la hipótesis de la poliploidización sexual bilateral como mecanismo de origen de los poliploides en *T. sidoides*. Sin embargo, el hallazgo de triploides fértiles sugirió que la poliploidización sexual unilateral también sería un mecanismo importante de origen de tetraploides en *T. sidoides*. La ocurrencia de plantas que forman continuamente gametos no reducidos desempeñaría un papel clave en el establecimiento de neopoliploides. Además, el mayor número de propágulos que los tetraploides aportan a las siguientes generaciones, la capacidad de multiplicación asexual por rizomas y los casos ocasionales de autocompatibilidad y cruzamientos ilegítimos exitosos aumentarían la probabilidad de que se mantenga una baja frecuencia de neopoliploides en las poblaciones naturales de *T. sidoides*. Asimismo, la integración de datos citogeográficos y de divergencia genética junto con el modelado de nicho en el pasado aportó información que sustenta la hipótesis de que los eventos climáticos y geomorfológicos históricos proporcionaron las condiciones favorables para el establecimiento y expansión de los tetraploides de *T. sidoides*.

Palabras clave: citogeografía, establecimiento, gametos no reducidos, origen, poliploidía

INTRODUCTION

Polyploidy is a common phenomenon across numerous eukaryotic taxa (Soltis *et al.*, 2014; Marchant *et al.*, 2016) and is believed to play a significant role in the evolution of vascular plants (Otto and Whitton, 2000; Levin, 2002). As many as 70–80% of Angiosperms, including many crop species, and up to 95% of Pteridophytes have a polyploid origin (Bennet, 2004; Gregory and Mable, 2005; Otto, 2007). In addition, owing to the role in the origin of evolutionary novelties and the maintenance of diversity in plant populations, polyploidy was recognized as an integral component of the ecological and evolutionary dynamics of plant species populations. In this regard, different theoretical and experimental efforts to examine the evolutionary significance of polyploidy have focused on the processes responsible for the origin of polyploid plants and the conditions favouring their establishment and persistence (Thompson and Lumaret, 1992).

Spontaneous doubling of somatic chromosomes (zygotic or meristematic) was long considered the predominant mode of polyploid origin in flowering plants (Winge, 1917). However, it became soon clear that 2*n* gametes detected in many plant' species (Karpechenko, 1927; Darlington, 1937) are involved in polyploid origin and, that sexual polyploidization is the driving force giving rise to polyploid plant species (Harlan and de Wet, 1975; Camadro, 1986; Thompson and Lumaret, 1992; Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998; Brownfield and Köhler, 2011). Particularly, autotetraploids may be formed in single steps through the union of two 2*n* gametes produced by diploids (bilateral polyploidization) or, alternatively, may arise in two steps from matings involving viable triploids (triploid bridge hypothesis), themselves the result of the union of *n* and 2*n* gametes produced by diploids (unilateral polyploidization) (Harlan and de Wet, 1975).

On the other hand, the process of neopolyploids establishment in diploid populations may be limited by the difficulty of mating with other plants of the same ploidy level, the unviability of triploid hybrids, the viability and fertility of polyploids relative to diploids, and the potential for genetic swamping of the more frequent cytotype (minority cytotype exclusion) (Levin, 1975; Fowler and Levin, 1984; Bever and Felber, 1998; Erilova *et al.*, 2009; Chrtek *et al.*, 2017). Self-pollinating plants and individuals with multiple breeding opportunities throughout life can more easily overcome the difficulties associated with intercytotypic mating (Rodriguez, 1996; Bever and Felber, 1998; Ramsey and Shemske, 1998; Husband, 2000). However, in outbreeding plants and if pollination is random, neopolyploids will be at a disadvantage, as most of their megaspores will be fertilised by *n* microspores of diploids forming triploids, whereas most of the relatively abundant diploid megaspores will be conveniently fertilised. Consequently, a neopolyploid is likely to be excluded from a diploid population (Levin, 1975).

Besides, polyploidy can potentially contribute to the acquisition of new morphological, genetic and/or physiological traits, which may improve the competitive ability, fitness or ecological tolerance of polyploids compared to the diploid parents. These events, which have occurred on time scales ranging from ancient to contemporary, are assumed to be of fundamental importance for plant adaptation and range expansion (Levin, 1983; Udall and Wendel, 2006; Van de Peer *et al.*, 2021). Thus, polyploids may have a distinct or peripheral distribution compared to the parental diploids along climatic or environmental gradients (Levin, 2002). Such differences in geographical ranges between cytotypes may reflect historical patterns of colonisation or genetic differentiation that have occurred in association with or subsequent to polyploid formation (Levin, 1983). Alternatively, the spatial patterns of cytotypes may be explained through frequency-dependent production of hybrids with low frequency (Levin, 1975). Despite the evolutionary significance of polyploidy, many important questions about the mechanisms by which polyploids are formed and become established in natural populations remain unanswered (Soltis *et al.*, 2010; Castro *et al.*, 2018).

Turnera sidoides L. (Passifloraceae, Turneroideae) is one of the few well-studied autopolyploid complexes in South America (Fernández, 1987; Solís Neffa, 2000; Solís Neffa and Fernández, 2001, 2002; Elías *et al.*, 2011, Kovalsky and Solís Neffa, 2012, 2015, 2016; Kovalsky *et al.*, 2014, 2018; Roggero Luque *et al.*, 2015, 2017; Solís Neffa *et al.*, 2022). Because of the prominent role played by polyploidy within *T. sidoides*, in order to understand the evolutionary significance of autopolyploidy, an investigation is in progress in this complex using integrative approaches combining cytogenetics, together with genetic, morphological, biogeographic and environmental data. As a part of this study, we selected this autopolyploid complex as an ideal biological model system to investigate the evolutionary dynamics of polyploids in natural populations. In this paper we synthesise the results of more than 20 years of research on this topic.

TURNERA SIDOIDES

Turnera sidoides (Figure 1) is a complex of perennial, rhizomatous herbs that is naturally distributed in southern Bolivia and Brazil, southwestern Paraguay, Uruguay and Argentina, reaching up to 39° S (Arbo, 1985; Solís Neffa, 2000). It grows in a wide variety of habitats ranging from mountain regions (up to 2700

Figure 1. *Turnera sidoides* complex. **A)** *T. sidoides* subsp. *carnea*. **B)** *T. sidoides* subsp. *holosericea*. **C)** *T. sidoides* subsp. *integrifolia*. **D)-E)** *T. sidoides* subsp. *pinnatifida* **D)** Andino morphotype. **E)** Serrano morphotype. **F)** *T. sidoides* subsp. *sidoides*.

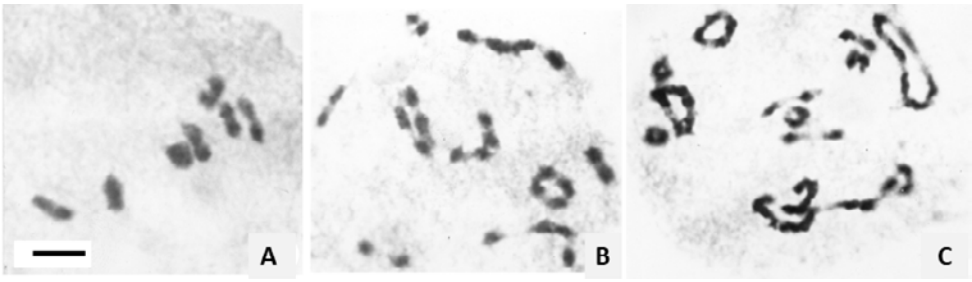

Figure 2. Meiotic chromosomes of *Turnera sidoides*. **A)** Diploid cytotype, 7 II. **B)** Tetraploid cytotype, 4II + 5IV. **C)** Hexaploid cytotype, 8 II + 5 IV + 1 VI. Bar= 5µm.

m a.s.l) to the sea level and encompassing different climatic regimes (Arbo, 1985; Solís Neffa, 2000, 2010).

Turnera sidoides is outbreeder because of distyly and genetic self-incompatibility, being legitimate crossings those between short-styled (S) and long-styled (L) morphs, S x L or L x S (Arbo, 1985; Solís Neffa, 2000). Pollination is carried out by butterflies, small wasps and bees, and seeds are dispersed by gravity and ants. Discrete populations, ranging from less than ten to hundreds of plants, are separated from each other by a few to several kilometres (Solís Neffa, 2000).

Five subspecies were recognized on the basis of geographical distribution and the variability of some morphological features (Arbo, 1985): *T. sidoides* subsp. *carnea* (Cambess.) Arbo, *T. sidoides* subsp. *holosericea* (Urb.) Arbo, *T. sidoides* subsp. *integrifolia* (Griseb.) Arbo, *T. sidoides* subsp. *pinnatifida* (Juss. ex Poir.) Arbo, and *T. sidoides* subsp. *sidoides*. Besides, populations of *T. sidoides* subsp. *carnea* were grouped into two morphotypes (*grandense* and *mercedeño*) according to the leaf consistency, the colour of the flowers and geographical distribution (Moreno *et al.*, 2021); while in *T. sidoides* subsp. *pinnatifida* five morphotypes (*andino*, *chaqueño*, *mesopotamico*, *pampeano* and *serrano*) have been distinguished based on the degree of incision of the leaf blade, the colour of the flowers and geographical distribution (Solís Neffa, 2010).

In addition to the morphological variability, polyploidy is the most outstanding feature of *T. sidoides* complex, with ploidy levels ranging from diploid ($2n=2x=14$) to octoploid ($2n=8x=56$), all based on $x=7$. Polyploid series within each subspecies/morphotype were reported (Fernández, 1987; Solís Neffa, 2000; Solís Neffa and Fernández, 2001; Solís Neffa *et al.*, 2004; Speranza *et al.*, 2007; Elías *et al.*, 2011; Moreno *et al.*, 2015, 2021; Solís Neffa *et al.*, 2022). From the study of meiosis (Figure 2) and pollen viability it was suggested that polyploids of *T. sidoides* would have an autopolyploid origin (Fernández, 1987; Solís Neffa, 2000). These observations were supported by the results obtained from the analysis of the effect of polyploidy in some morphological, cytological, and biochemical traits (Solís Neffa, 2000; Solís Neffa and Fernández, 2002; Roggero Luque *et al.*, 2022; Solís Neffa *et al.*, 2003).

MECHANISMS OF POLYPLOID ORIGIN IN *TURNERA SIDOIDES*

Evidence of 2n microspore production in natural diploid populations of T. sidoides

Aiming to contribute to the understanding of the mechanisms involved in the origin and the establishment of polyploids, we firstly investigate the occurrence and frequency of unreduced microspores in diploids of *T. sidoides*. Early experimental studies provided the first evidences for the production of 2*n* and 4*n* microspores by analysing the size range of pollen and the constitution of the sporads in diploid plants (Panseri *et al.*, 2008). Different facts supported that the giant pollen grains observed in the complex correspond to unreduced gametes. First, the volume of the giant grains relative to the mean pollen size of the population studied was comprised in the range proposed by different authors as typical of unreduced gametes (Darlington, 1965; Orjeda *et al.*, 1990; Ramsey and Schemske, 1998). The existence of unreduced microspores in some plants was further confirmed by a particular bimodal distribution of pollen volume. Additionally, the presence of triads and dyads in plants with giant pollen indicates that 2*n* gametes certainly occurred and that the formation of 2*n* gametes would be expected. Moreover, the finding of monads suggested that "jumbo" (4*n*) gametes may also be formed. The mean frequency of unreduced gametes observed in such populations was in agreement with the estimations of the production of unreduced gametes for non-hybrid plants (Ramsey and Schemske, 1998; Ramsey, 2007).

Then, to further contribute to the understanding of the mode of polyploid formation in *T. sidoides*, we selected natural diploid populations that had been experimentally identified for its high production of 2*n* gametes (Panseri *et al.*, 2008). In these natural populations we studied the occurrence of unreduced microspores and estimated the frequency of their production by analysing the pollen size distribution and the constitution of the sporads (Figure 3) from samples obtained in the field (Kovalsky, 2012; Kovalsky and Solís Neffa, 2012). Some of these plants were then grown under the same soil and irrigation conditions at the greenhouse of the Instituto de Botánica del Nordeste for, at least, one year. The exhaustive study on such natural populations of *T. sidoides* (Kovalsky and Solís Neffa, 2012) revealed that some plants were more capable of inducing unreduced microspores production than others. The percentage of 2*n* gamete producer plants detected (~26%) evidenced that the production of unreduced microspores was not uncommon in diploid populations of *T. sidoides*. These studies also showed that, within populations, S and L plants differed in their ability to produce 2*n* microspores and, that the frequency of 2*n* gametes production was highly variable. Such variation in the frequencies of unreduced microspores

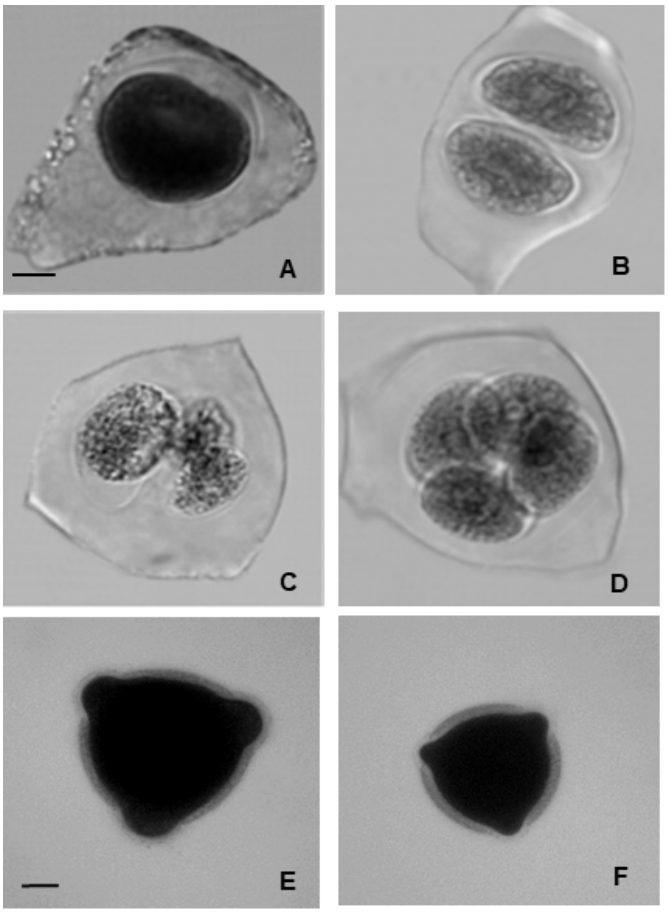

Figure 3. Sporads and pollen in *Turnera sidoides*. **A-D)** Sporads: **A)** Monad. **B)** Dyad. **C)** Tryad. **D)** Tetrad. **E-F)** Pollen grains. **E)** Unreduced pollen grain (2*n*). **F)** Reduced (*n*) pollen grain. Bar= 5 µm.

production observed in *T. sidoides* was attributed to different factors. Considering that only some individuals produced unreduced microspores, the frequency of 2*n* microspores production varied among producer plants and that, producer plants identified in the field were consistently classified as producers in the greenhouse we concluded that production of 2*n* microspores in this species is under genetic control. However, the variation in the frequency of 2*n* microspores production among producer plants and also among anthers of the same flower suggested differences in gene expression. On the other hand, although the flowering period of *T. sidoides* is extended throughout spring and summer (Solís Neffa, 2000), the frequency of 2*n* microspores yield by each producer plant cultivated in the greenhouse increased up to 22% in summer. Therefore, it was suggested that the higher production of unreduced microspores might be caused by the environmental stress due to the high temperatures to which plants of *T. sidoides* were exposed (Kovalsky and Solís Neffa, 2012).

Finally, in order to contribute to the understanding of the cytological mechanisms involved in the production of unreduced male gametes, we analysed the meiotic behaviour and pollen viability of 2*n* and 4*n* gamete

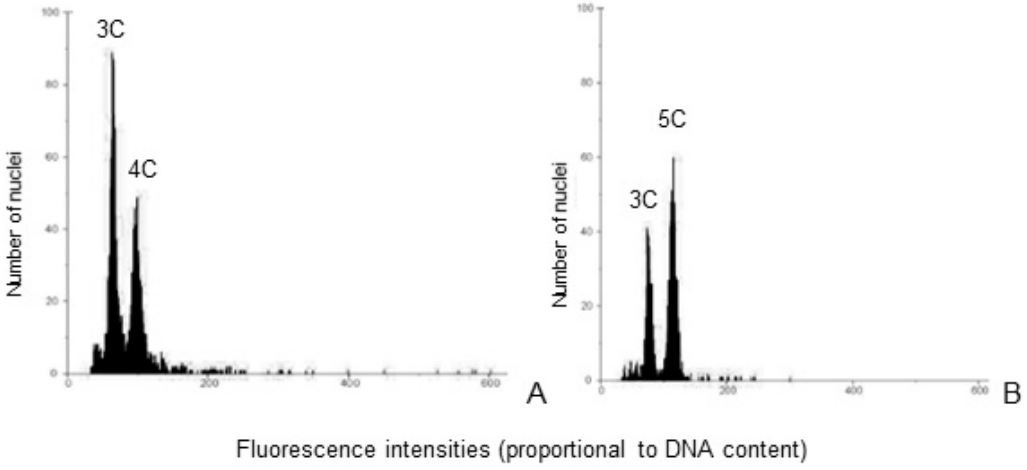

Figure 4. Flow cytometry histograms of triploid seeds of *Turnera sidoides*. **A)** Triploid seed originated from an 2*n* microspore showing and embryo with a peak at 3C and a peak at 4C corresponding to the endosperm. **B)** Flow cytometry histogram of a triploid seed originated from an 2*n* megaspore showing and embryo with a peak at 3C and a peak at 5C corresponding to the endosperm.

producers from diploid populations of *T. sidoides*. The results obtained showed that meiotic behaviour was mostly regular; however, some irregularities such as parallel spindles, fused spindles and nuclear restitution were also detected. Pollen viability varied from 44.75% to 95.82%. Overall, our results suggested that nuclear restitution at both the first and the second meiotic division were involved in the production of 2*n* male gametes in *T. sidoides* (Kovalsky *et al.*, 2014).

Evidence of the production of 2n eggs in diploid plants of T. sidoides complex

As a second step to understand how unreduced gametes may have contributed to the origin and establishment of polyploids in natural diploid populations of *T. sidoides*, we provide the first evidence of the production of 2*n* megaspores by progeny test and by flow cytometric analysis of seeds (Figure 4) collected in natural populations (Kovalsky and Solís Neffa, 2016). In spite of the low number of 2*n* megaspores detected, our results suggested that, in natural diploid populations of *T. sidoides*, some plants would be more likely to produce 2*n* megaspores than others. This data agrees with our previous findings in this species, which demonstrated that only some S and L plants (26%) were capable of producing 2*n* and 4*n* microspores and, that the capability of producing such unreduced gametes is under genetic control (Kovalsky and Solís Neffa, 2012, 2015). The finding of only some plants of *T. sidoides* that produced 2*n* megaspores suggests that their production could be under genetic control as well. Furthermore, although the frequency of plants producing 2*n* megaspores in *T. sidoides* was lower than that previously reported for plants producing 2*n* microspores (Kovalsky and Solís Neffa, 2012), all plants that produced 2*n* megaspores also produced 2*n* microspores. However, the fact that plants producing 2*n* microspores do not always produce 2*n* megaspores it was proposed that their simultaneous production may be independent from each other. Our results also suggested differences in the relative contributions of 2*n* microspores and 2*n* megaspores to polyploid formation. In diploid–tetraploid crosses, 2*n* megaspores are supposed to be more likely to generate viable seeds than 2*n* microspores (Thompson and Lumaret, 1992; Ramsey and Schemske, 1998) and it was suggested that formation of neopolyploids in natural populations would proceed in a similar way to these interploidy crosses (Ramsey, 2007). However, this would not be the case of *T. sidoides*, since the major frequency of triploid embryos from 2*n* microspores in seeds collected in natural populations suggested that 2*n* microspores would contribute more than 2*n* megaspores to the origin of neopolyploids. Although, this would not imply that triploid embryos generated by fusion of 2*n* microspores and *n* megaspores result in plants which will effectively establish in populations of *T. sidoides*. In this species, plants which produced 2*n* megaspores develop more viable seeds per fruit, and seeds have a higher germination rate than those plants that exclusively produce 2*n* microspores (Kovalsky, 2012). This suggested that, although in *T. sidoides* 2*n* microspores were involved in the origin of most triploid embryos found in natural populations, the ploidy ratios among embryo and endosperm and/or epigenetic processes might confer triploids originated from the fusion of 2*n* megaspores with *n* microspores an advantage during more advanced stages of their development and establishment, being effectively established in diploid populations (Kovalsky and Solís Neffa, 2016).

Unilateral vs bilateral sexual polyploidization

Both unilateral and bilateral sexual polyploidization may be involved in the origin of the polyploids of *T. sidoides* in natural populations. Because all polyploids of the complex so far analysed have an even ploidy level (Fernández, 1987; Solís Neffa and Fernández, 2001; Solís Neffa *et al.*, 2004), bilateral polyploidization was expected to be the most important mechanism of polyploid origin in the complex (Panseri *et al.*, 2008). However, taking into account that this species is dystilous and outbreeder (Solís Neffa, 2000), for bilateral sexual polyploidization to occur both, S and L individuals should produce 2*n* microspores and 2*n* megaspores. In this sense, our finding of 2*n* microspores (Kovalsky and Solís Neffa, 2012), together with the detection of 2*n* megaspores (Kovalsky and Solís Neffa, 2016) in non-hybrid diploid populations of *T. sidoides*, and the fact that both the L and S plants can produce both 2*n* microspores and 2*n* megaspores, suggested that bilateral sexual polyploidization can occur in natural populations of this species. However, owing to the limited chances of fertilization between simultaneously formed 2*n* microspores and 2*n* megaspores, bilateral polyploidization would occur less frequently than the unilateral sexual polyploidization. The finding of triploids in natural diploid populations of *T. sidoides* (Elías *et al.*, 2011; Kovalsky and Solís Neffa, 2012; Kovalsky *et al.*, 2018) may reflect triploid formation through the union of *n* and 2*n* gametes, suggesting that unilateral polyploidization by a triploid bridge may be an alternative mechanism of polyploid formation in this species complex.

Multiple origin of polyploids

The formation of autopolyploids was considered a rare event, but it was later recognised that multiple origins typify polyploid plant species (Segraves *et al.*, 1999). In *T. sidoides*, the multiple diploid–polyploid transitions revealed in a phylogenetic analysis based on the sequences of the plastid DNA regions supported a multiple origin of autopolyploids in each subspecies and morphotypes (Solís Neffa *et al.*, 2022), as previously suggested for the complex (Solís Neffa and Fernández, 2001). Also, the finding of lineage-exclusive haplotypes in diploid and polyploid populations were interpreted as independent polyploidization events. The occurrence of polyploids with unique haplotypes, not directly related to any of the haplotypes detected within the diploids analysed, suggested an additional independent origin of polyploids (Solís Neffa *et al.*, 2022). Likewise, analyses of the genetic variability and structure of the diploid and polyploid populations of *T. sidoides* using nuclear molecular markers, showed that the greater genetic similarity of tetraploids to diploids from the same geographic region than to diploids from other regions was consistent with the origin of polyploids in multiple polyploidization events from genetically differentiated diploid populations (Panseri, 2012; Dabrio *et al.*, 2020). Such multiple polyploidization events detected in *T. sidoides* complex were related to the capacity of many diploid populations to produce unreduced gametes (Panseri *et al.*, 2008; Kovalsky and Solís Neffa, 2012, 2016), but also to the ability of polyploids to survive and establish in nature.

POLYPLOID ESTABLISHMENT IN *T. SIDOIDES*

Owing to the lower rate of neopolyploid formation in *T. sidoides* complex, their establishment constitute a critical step. Thus, for a better understanding of the evolution of polyploidy in *T. sidoides* our next objective was to assess the possible factors influencing neotetraploids establishment in natural populations.

Patterns of cytotype variation of T. sidoides in a diploid-tetraploid contact zone

As a first step, we examined the cytotypes structure in a diploid-tetraploid contact zone of *T. sidoides* in the mountain ranges of central Argentina, aiming to analyse whether the frequency and distribution of cytotypes at microgeographical scale was explained by ecological sorting in heterogeneous environments or, alternatively, due to competitive cytotype exclusion (Elías, 2010; Elías *et al.*, 2011). The results evidenced that diploids and tetraploids were spatially segregated, although both cytotypes can occur in close proximity. Diploids grew in the piedmont of the hills, tetraploids in the adjacent valley, while patches of diploids and triploids plants were found, in the transition zone of both cytotypes. Since diploids and tetraploids occur under similar climatic conditions, it is unlikely that climate influences cytotype distribution in the contact zone. Also, the edaphic conditions would only partially contribute to the spatial segregation of cytotypes at local scale. The similar ecological preferences of both cytotypes and the lack of mixed diploid-tetraploid patches suggested that the separate distribution of cytotypes in the contact zone may be independent of the selective environment; diploids and tetraploids being unable to coexist as a result of reproductive exclusion (Elías, 2010; Elías *et al.*, 2011).

Additionally, to assess whether differences in the biological fitness between cytotypes would be influencing the cytotype distribution and frequency in the contact zone, we analysed the variation in reproductive and phenological traits of diploids and tetraploids (Elías, 2010; Panseri, 2012). From this study

it was evident that although the average number of seeds per fruit was similar in both cytotypes, the continuity of flowering and fruiting in tetraploids in relation to the discontinuity of diploids, the highest number of bloomed individuals per site as well as the greater number of fruits per individual in tetraploids result in an increase number of propagules that tetraploids contribute to the seed bank as well as to the next generation, increasing the frequency of tetraploids in each generation. This fact, together with the perennial condition of *T. sidoides* and its ability to multiply asexually by rhizomes (Solís Neffa, 2000) would favour the success and establishment of neotetraploids in natural populations. Also, the occurrence of occasional cases of self-compatibility and successful illegitimate crosses (S × S and L × L) in polyploids of *T. sidoides* suggests that such polyploids may produce seeds by selfing or from crosses between plants of the same floral type, further increasing their chances of establishment and expansion (Solís Neffa, 2000; Solís *et al.*, 2020).

The role of 2n gametes in polypoid establishment of the T. sidoides

Theoretical models of polyploid evolution suggest that 2*n* gamete production by diploids is an essential factor in the dynamics of mixed diploid-tetraploid populations since tetraploids are more likely to establish or to be maintained at a low frequency within diploid populations when they are formed recurrently through the union of 2*n* gametes (Levin, 1975; Felber and Bever, 1997). *Turnera sidoides* is an outbreeder, therefore, the occurrence of plants that continuously form many 2*n* microspores and 2*n* megaspores would play a key role in the establishment of neopolyploids in natural diploid populations. Taking into account that *T. sidoides* grow in discrete populations (mostly with fewer than 100 individuals, Solís Neffa, 2000), the occurrence of a low number of plants producing 2*n* gametes may be significant to polyploid dynamics in diploid populations. Moreover, since the capability to produce 2*n* microspores is a heritable trait in this species complex and, the frequency of production of 2*n* microspores was higher in the progeny of 2*n* microspores producers (Kovalsky and Solís Neffa, 2015), the frequency of 2*n* microspores and 2*n* megaspores and, consequently, the likelihood of origin of neopolyploids by sexual polyploidization would increase after successive generations. Additionally, considering that *T. sidoides* is a perennial species and, that its seeds are mostly dispersed by gravity in such a way that individuals concentrate in localized areas (Solís Neffa, 2000), the progeny of plants that produce 2*n* gametes would concentrate near the mother plant, increasing the likelihood of crosses between 2*n* microspores and 2*n* megaspores producers. The continuous formation of neopolyploids as a consequence of successive backcrosses between 2*n* gametes producers and their progeny would favour the establishment and persistence of such neopolyploids in diploid populations of *T. sidoides.* Furthermore, although diploid individuals produced low levels of 2*n* gametes but, under certain environmental conditions, such production may be increased, enhancing the likelihood that a low frequency of polyploids can be originated and maintained in this population (Kovalsky and Solís Neffa, 2015).

The role of triploids in the origin and evolution of polyploids of T. sidoides complex

Simulation models have demonstrated that the evolution of tetraploids in a diploid population depends on the reproductive efficiency of triploids and the ploidy level of functional gametes (*n*=*x*, *n*=2*x* and, *n*=3*x*) they produce, contributing to the formation of neopolyploids in each generation (Husband, 2004). Thus, aiming to account for the role of triploids in the origin and demographic establishment of tetraploids of *T. sidoides* in natural populations, we analysed the microsporogenesis of triploids as well as the crossabilty among diploid, triploid and tetraploid plants of the complex (Kovalsky *et al.*, 2018). Triploids exhibit irregular meiotic behaviour that results in unbalanced and/or non-viable gametes and, low pollen viability. Although, in spite of abnormalities in chromosome pairing and unequal chromosome segregation, triploids are not completely sterile and form viable gametes (pollen viability up to 67%). Triploids originated by the fusion of 2*n* × *n* gametes showed more regular meiotic behaviour and higher fertility than triploids from the contact zone among diploids and tetraploids (Elías, 2010) and, triploids with hybrid origin (Moreno *et al.*, 2015). Also, our results evidenced that reproductive isolation among cytotypes of *T. sidoides* was not so strong. In this species complex most populations are constituted by a single cytotype, even though some mixed diploid – triploid – tetraploid populations were also detected (Elías *et al.*, 2011; Kovalsky and Solís Neffa, 2012; Moreno *et al.*, 2015). This fact together with our finding of triploids in the progeny of 2*x* × 4*x* experimental crosses, evidence that triploid block may be frequently overcome, being not enough to prevent the regular occurrence of triploids in natural populations of *T. sidoides* (Kovalsky *et al.*, 2018). Moreover, although in this species the success of crosses involving triploids is generally low, their gametes can participate in fertilization, producing progeny of variable ploidy level. The diploid plants used for experimental crossings produced reduced gametes (n=x), therefore, the frequency of diploid and triploid progeny obtained from crosses 2*x* × 3*x* constituted an indirect measure of the frequency of *n* and 2*n* gamete production by triploids. The results of our studies evidenced that triploids of *T. sidoides* produce *n* and 2*n*

gametes, although *n* gametes were the most frequently produced. This finding was supported by the fact that the progeny of 3*x* × 4*x* crosses was 100% triploid, resulted of the fusion of *n* gametes of triploid and tetraploid parents. Besides, the experimental crosses performed evidenced that triploids produce diploid and, triploid progeny, suggesting that new generations of triploids and tetraploids would originate by crossings between triploids or by backcrosses with diploid progenitors that produce 2*n* microspores and 2*n* megaspores. Therefore, triploids of *T. sidoides* would contribute both to the origin of new tetraploids (Kovalsky *et al.*, 2018) and to gene flow among diploids and tetraploids in the contact zones (Moreno *et al.*, 2015).

POLYPLOIDY AND RANGE EXPANSION

Since the analysis of geographical differentiation of cytotypes provides useful insights into the evolutionary dynamics and ecological differentiation of polyploids, we employed a cytogeographical approach for *T. sidoides*. The results so far have revealed that, at a geographical scale and considering the complex as a whole, diploids have restricted and disjunct distributions, while tetraploids are the most widespread, being the cytotype that gives continuity to the complex throughout the distribution area. Likewise, populations with higher ploidy levels are rare and were found in disjunct, and usually marginal, areas of the complex. These patterns of cytotypes frequencies and distribution support the classification of *T. sidoides* as a mature polyploid complex as was initially proposed (Solís Neffa, 2000) following the criteria of Stebbins (1971).

Proximal and historical nonexclusive hypotheses were proposed to explain the spatial patterns of the cytotypes in this species (Solís Neffa, 2000; Solís Neffa and Fernández, 2001; Solís Neffa *et al.*, 2004). Given that the widespread latitudinal and longitudinal range of *T. sidoides* encompasses a wide diversity of climates and ecological regions with contrasting precipitation regimes, it was first hypothesised that the geographical separation of the cytotypes was due to differences in habitat requirements. However, taking into account the relative frequency and geographical distribution of the cytotypes, it was also hypothesised that the distribution of extant cytotypes might reflect the Quaternary geomorphologic and climatic changes that affected most of the current geographical range of *T. sidoides*. Consequently, restricted diploids may be relictual populations that have survived these periods of change, while such historical events could provide favourable conditions for the establishment of polyploids, with the wider distribution of tetraploids being the result of range expansion (Solís Neffa and Fernández, 2001; Solís Neffa *et al.*, 2004; Elías *et al.*, 2011).

Additionally, the integration of cytogeographic and genetic divergence data together with past niche modelling provided further insights into the geographic context of the processes driving divergence and range expansion in *T. sidoides* complex (Solís Neffa *et al.*, 2022). The projected model for the mid-Pliocene (~3.3–3.0 Myr), revealed four areas of highest climatic suitability for a possible ancestor of *T. sidoides*. The main one, in the NW of the current species range (following the direction of main orographic systems) were coincident with the proposed refugial centres for *T. sidoides* complex based on plastid genome diversity (Speranza *et al.*, 2007, Moreno *et al.*, 2018). This area was also proposed as the ancestral geographic area for *T. sidoides* (Moreno *et al.*, 2018). Additionally, three smaller suitable areas were located in the Pampean ranges and in NE Argentina as well as in the SE of the current range, along the Serranías del Este (Uruguay) (Solís Neffa *et al.*, 2022). The spreading of large areas of semi-arid conditions in the Chaquean Domain concomitantly with the final uplift of the Andes (Gregory-Wodzicki, 2000) would have acted as a barrier to gene flow among populations of *T. sidoides*, promoting the divergence between the two main lineages by the time of the Great Patagonian Glaciations (~1.17 Myr). Further diversification of lineages within the two main clades of *T. sidoides* complex mostly coincides with colder periods, suggesting that cooling and drying during Pleistocene (Villagrán and Hinojosa, 1997; Rabassa *et al.*, 2011) may have caused the isolation and divergence of ancestral *T. sidoides* complex populations (Moreno *et al.*, 2018).

The predicted distribution of diploids during interglacial periods suggests a more continuous area of diploids than at present, probably along the most elevated terrains (Moreno *et al.*, 2018; Solís Neffa *et al.*, 2022). The evolutionary history of the complex from the Last Glacial Maximum up to present day mostly involved the range expansion of the allopatrically differentiated populations to their current limits. During the Mid-Holocene, the warmer and wetter climate would have promoted the westward reduction of the area of diploids and the range expansion of tetraploids. Semiarid conditions during the Late Holocene (Iriondo and García, 1993) may have determined the progressive retraction of the suitable area for diploids, while the subhumid climate period would have favoured the persistence of diploids in the inter-Andean valleys (Solís Neffa *et al.*, 2022).

EVOLUTIONARY SIGNIFICANCE OF AUTOPOLYPLOIDY IN *T. SIDOIDES*

Cytogenetic data together with past niche modelling as well as cytogeographic and genetic divergence analyses provided strong evidence that *T. sidoides* complex is in an active process of intraspecific allopatric diversification

at diploid level since the early Pleistocene. Further diversification of the complex involved the emergence of independent polyploid series in each morphologically divergent lineage.

Polyploidy did not produce extensive morphological changes in *T. sidoides*, so diploids and polyploids within each subspecies and morphotypes can only be distinguished from each other on the basis of detailed analysis of microcharacters (Panseri, 2012) and quantitative traits (Solís Neffa, 2000). These results together with cytogenetic (Solís Neffa, 2000; Roggero Luque *et al.*, 2015) and molecular data (Moreno *et al.*, 2015) suggested that autopolyploidy did not contributed significantly to the diversification of *T. sidoides*. Instead, changes associated with genome size variation and the gigas effect were recorded in the polyploid series of the complex (Solís Neffa, 2000). All these chromosome doubling-induced changes may have had an adaptive significance that may promote an increase range of subspecies and morphotypes through the expansion of polyploids arising from multiple diversified diploid populations of *T. sidoides* (Solís Neffa *et al.*, 2022). In this sense, despite the fact that polyploid series of each subspecies and morphotype of *T. sidoides* inhabits the same ecoregion, the cytotypes tend to be spatially segregated at a more local scale (Solís Neffa and Fernández, 2001; Solís Neffa *et al.*, 2004; Elías *et al.*, 2011; Solís Neffa *et al.*, 2022). A clear example was demonstrated in the *serrano* morphotype of subspecies *pinnatifida* (Elías *et al.*, 2011), in which diploids and polyploids, although inhabiting the same ecoregion, are segregated along altitudinal and bioclimatic gradients at regional/local scales. The wider distribution of tetraploids compared to their related diploids in each polyploid series of *T. sidoides* complex supports the hypothesis that autopolyploids may have been more effective colonisers of new ecological niches.

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CHROMOSOMES OF TWO *OLYRA* L. SPECIES FROM MISIONES, ARGENTINA (POACEAE, BAMBUSOIDEAE, OLYREAE)

CROMOSOMAS DE DOS ESPECIES DE *OLYRA* L. DE MISIONES, ARGENTINA (POACEAE, BAMBUSOIDEAE, OLYREAE)

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ABSTRACT

The subfamily Bambusoideae comprises three monophyletic tribes, Arundinarieae, Bambuseae and Olyreae. Here we report the gametic number and the chromosomal meiotic behavior of two species belonging to the herbaceous tribe Olyreae, *Olyra latifolia* and *Olyra humilis*. Accessions were collected in Misiones, at Northeastern Argentina. We report a new gametic number for *O. humilis*, *n*=18, and we confirmed *n*=11 for *O. latifolia.* Chromosomal features, like the basic and gametic chromosome number, are important in understanding the evolution of the Poaceae family, especially in delimiting clades and elucidate inter and intra-clades relationships, and therefore it is necessary to continue producing this type of data.

Key words: bamboos, cytogenetics, meiosis, ploidy, Poaceae

RESUMEN

La subfamilia Bambusoideae comprende tres tribus monofiléticas, Arundinarieae, Bambuseae y Olyreae. Aquí reportamos el número gamético y el comportamiento meiótico de los cromosomas de dos especies pertenecientes a la tribu Olyreae de bambúes herbáceos, *Olyra latifolia* y *Olyra humilis*. Las introducciones se recolectaron en la provincia de Misiones, en el noreste argentino. Reportamos un nuevo número gamético para *O. humilis*, *n*=18, y confirmamos *n*=11 para *O. latifolia*. Los números cromosómicos básicos y gaméticos son importantes para comprender la evolución de la familia Poaceae, especialmente para delimitar sus clados y las relaciones existentes entre ellos, por lo que es necesario continuar produciendo este tipo de datos.

Palabras clave: bambúes, citogenética, meiosis, ploidía, Poaceae

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INTRODUCTION

The subfamily of bamboo grasses (subfamily Bambusoideae) comprises 1,700 species belonging to 128 genera which are distributed worldwide (Clark *et al.*, 2015; Zhang *et al.*, 2020). Bambusoideae comprises three monophyletic tribes, Arundinarieae, Bambuseae and Olyreae; the first two are represented by woody bamboos, whereas Olyreae species are herbaceous without woody characteristic (Ruiz-Sanchez *et al.*, 2021). Herbaceous bamboos have a wide distribution, inhabiting from sea level towards 2,200 m.a.s.l. (Clark *et al.*, 2015). Olyreae species are native to the New World with one exception, *i.e. Buergersiochloa* that is a monotypic endemic genus of New Guinea (Oliveira *et al.*, 2014; Clark *et al.*, 2018).

The tribe Olyreae comprises 22 genera and 124 species, and occurs widely in the Neotropical region, from Mexico and the Caribbean to North Argentina, and Paraguay (Oliveira *et al.*, 2014). Most genera in this tribe have species defined by vegetative characters. Generally, the spikelets exhibit little interspecific variation, with the exception of *Olyra* L., the most widespread and heterogeneous genus in the tribe (Judziewicz and Zuloaga, 1992). In addition, *Olyra* was defined as paraphyletic and polyphyletic, in order to explain diverse incongruences in phylogenetic trees based on plastid molecular markers (Judziewicz *et al.*, 1999; Ruiz-Sanchez *et al.*, 2019).

Olyra is the largest genus within the subtribe Olyrinae, with *ca.* 24 species. Probably this estimate will soon change since phylogenetic studies evidenced its non-monophyletic nature (Oliveira *et al.*, 2014, 2020). Currently, four species of *Olyra* habit in Argentina, *O. ciliatifolia* Raddi, *O. fasciculata* Trin., *O. humilis* Nees and *O. latifolia* L. and the only two chromosome counts known for Argentinian specimens are from *O. latifolia*. Chromosomal and ploidy data are useful for clarifying phylogenetic relationships and tracing evolutionary trajectories (Honfi *et al.*, 2021; Deanna *et al.*, 2022). For this reason, this work aims to contribute with meiotic studies of *O. latifolia* and *O. humilis*, two significant herbaceous species of the tropical forest in Misiones.

MATERIAL AND METHODS

Table 1 shows a complete list of the studied accessions, including their geographic origin, voucher specimens, and herbaria. Herbarium acronyms follow Thiers (2017 onward). Voucher herbarium specimens were deposited at Universidad Nacional de Misiones Herbarium (MNES) and duplicates at the following herbaria: Instituto de Botánica del Nordeste (CTES), Instituto de Botánica Darwinion (SI) and Conservatoire et Jardin Botaniques de la Ville de Gèneve (G).

The analyses of chromosome meiotic behavior were made according to Honfi *et al.* (2021) and Reutemann *et al.* (2021). Briefly, young inflorescences were fixed in 3:1 ethanol: acetic acid solution for at least 24 h at room temperature. Then, spikelets were transferred to 70% ethanol at 4ºC. Pollen mother cells (PMCs) were obtained from immature anthers and were stained with acetocarmine 2%. Permanent slides were made with Venetian Turpentine solution. At least 50 PMCs at diakinesis and/or metaphase I were analyzed for each accession of both species. Anaphase (I and II) and telophase (I and II) were analyzed to observe abnormal segregation. Microphotographs were taken with a Leica camera added to a Leica optical microscope.

Table 1. Chromosome gametic number (*n*) of *Olyra* species and studied accessions.

Species	*n*	Provenance
O. humilis Nees	18	Arg. Misiones, San Pedro, Parque Provincial Moconá. *Honfi* 500, 25/11/1993 (MNES, CTES, G). Arg. Misiones, San Pedro, Parque Provincial Moconá. *Honfi* 511, 25/11/1993 (MNES, CTES, G). Arg. Misiones, Cainguás, Campo Grande. *Honfi* 582, 19/2/1994 (MNES) Arg. Misiones, Parque Provincial Moconá. *Seijo* 856, 29/11/1993 (MNES). Arg. Misiones, Parque Provincial Moconá. *Daviña* 125, 18/12/1992 (MNES, CTES, SI)
O. latifolia L.	11	Arg. Misiones, Parque Provincial Moconá. *Seijo* 863, 29/11/1993 (MNES, CTES, G)

RESULTS AND DISCUSSION

Chromosome meiotic behavior of six *Olyra* South American accessions was studied (Table 1). The two *Olyra* species studied in this work exhibited a persistent nucleolus, which was clearly distinguishable up to (and including) diakinesis in agreement with Nicora and Rugolo de Agrasar (1987) karyosistematic definition of Bambusoideae familiy.

Chromosome gametic number, ploidy levels and meiotic behavior for *O. humilis* are reported for the first time. Persistent nucleoli, sometimes four small ones, were present in PMCs at diakinesis (Figure 1A). The studied accessions showed a regular meiotic behavior with 18 bivalents at diakinesis and metaphase I (Figure 1B). Few PMCs exhibited 17 bivalents and 2 univalents. Chromosome segregation was normal without laggards. The gametic chromosome number of n=18 is a new haploid chromosome number for the genus. Alternatively, there is a new derived basic chromosome number x=18, or a polyploid based on x=9, which is an uncommon basic chromosome number for *Olyra*. Previous reports of a tetraploid cytotype with x=9 were originally described as *Olyra* (Davidse and Pohl, 1978). Currently, these accessions belong to *Parodiolyra lateralis* (Presl. ex Nees von Essenbeck) Soderstrom & Zuloaga (Soderstrom and Zuloaga, 1989). Therefore, there is no basic number in *Olyra* to explain the gametic number n=18 found here.

In Olyreae, chromosome data suggest that *Olyra* species have a descending aneuploid series in basic numbers that started from an ancestral and original x=12, and nowadays is composed by x=11, 10 and 7 (Table 2). However, several authors considered x=12 as a secondary ancestral basic number (Hunziker *et al.*, 1982). At least two hypotheses can explain the chromosome number found in *O. humilis*. The first hypothesis is an allotetraploid origin based on x=9, with regular chromosome pairing in bivalents. In the second one, a new derived basic chromosome number of x=18 for *Olyra* results from an ascending aneuploid series. For example, a polyploid origin for n=18 found in *Anomochloa marantoideae* Brongn. (Anomochloaceae) was previously proposed, considering a primitive base number x=6 for bamboos and Poaceae in general (Hunziker *et al.*, 1989).

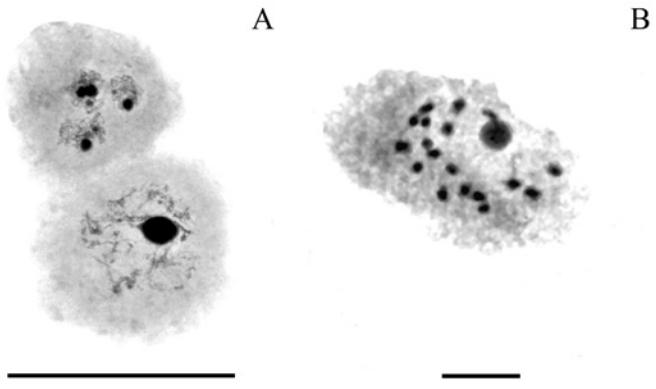

Figure 1. Pollen mother cells (PMCs) of *Olyra humilis* (Honfi 582) at meiosis. **A.** Prophase I; upper PMC with four small nucleoli, lower PMC with one unique and big nucleolus. **B.** Diakinesis with a persistent nucleolus and 18 bivalents. Scale [A]= 50 µm, [B]= 10 µm.

Judziewicz and Zuloaga (1992) suggested four morphological natural groups of species within Olyra, mainly based on the presence and distribution of trichomes on the female floret. The Glaberrima group of *Olyra* consists in species closely related to *O. glaberrima* Raddi, such as *O. humilis*, *O. maranonensis* Swallen and *O. davidseana* Judz. & Zuloaga (Judziewicz and Zuloaga, 1992). In addition, a close relationship between *O. glaberrima* and *O. humilis* is highly supported by recent phylogenetic analyses (Oliveira *et al.*, 2014; 2020). Furthermore, Oliveira *et al.* (2014) corroborated the monophyly of *Arberella* with species of *O. glaberrima* group. Cytological studies in all members of *Arberella* and Glaberrima group can shed light in the new chromosome number found in *O. humilis,* especially if they are multiple of n=18 or x=9 or 18.

In *O. latifolia*, a gametic chromosome number of n=11 was found. Meiotic behavior was regular with the presence of a persistent nucleolus and 11 bivalents or 10 bivalents and two univalent in PMCs at diakinesis and metaphase I (Figure 2 A, B, C). Chromosomes segregated normally at anaphase I and microsporogenesis resulted in an isobilateral tetrad of microspores (Figure 2 D, E). Our data agree with previous chromosome counts reported in several works (Tateoka, 1962; Pohl and Davidse, 1971; Davidse and Pohl, 1972a, b; 1974; Olorode, 1975; Dujardin, 1978; Hunziker *et al.*, 1989; Guo *et al.*, 2019). Besides, a tetraploid cytotype was registered in Bahia, Brazil (Hunziker *et al.*, 1982), Aragua and Bolivar, Venezuela (Davidse and Pohl, 1974; 1978) and Trinidad (Davidse and Pohl, 1972b). The basic chromosome number for this species is x=11 and the meiotic behavior indicates that the studied materials from Misiones are diploid accessions. *O. latifolia* is a robust herbaceous species widespread in the Neotropics, and the studied accessions are from the southernmost localities in South America. Diploids are the most frequent cytotype in Neotropics (America), and the only ploidy level found in Africa. Tetraploids are commonly detected in tropical areas (Table 2, Davidse and Pohl, 1974, 1978). Clearly, *O. latifolia* is a polymorphic complex with diploid and polyploid phenotypes. According to Davidse and Pohl (1972b), plants with very broad leaf blades, named *O. cordifolia* Kunth with 2n=ca. 30 (Gould and Soderstrom, 1967) are similar to tetraploid plants (n=22) of *O. latifolia*. Furthermore, in highly fertile diploid Mexican accessions of this species, meiotic unpaired chromosomes behaving as univalents or heterozygous translocation were also

Table 2. Records of chromosome numbers and ploidy level (*x*) of *Olyra* species.

Species	Chromosome data	x	Location	References
O. ciliatifolia Raddi	*n*=11	2*x*	Brazil: Rio de Janeiro	Hunziker *et al.*, 1982
O. fasciculata Trin.	2*n*=14, *n*=7	2*x*	Brazil: Rio de Janeiro, Guanabara, Estrada da Gávea.	Calderon and Soderstrom, 1973
			Brazil. Rio de Janeiro	Hunziker *et al.*, 1982
O. humilis Nees	*n*=18	4*x*		This work
O. latifolia L.	2*n*=22; *n*=11	2*x*	Costa Rica: San José, La Cisica between Alto de San Juan and La Alfombra, highway 223, 11 mi. SW of San Isidro de General	Reeder, 1969
			Costa Rica: Cartago IICA, Turrialba; Guanacaste: 6 km S of La Cruz; 1km W of Cafias Dulces; Puntarenas; 5 km SW of Rinc6n de Osa; 4km NE of Escuela Santa Constanza	Pohl and Davidse, 1971
			Mexico: Veracruz, near San Jose de Mata Clara; El Salvador: Chalatenango, near Chalatenango; Nicaragua: Zelaya, Lecus.	Davidse and Pohl, 1972a
			Costa Rica: Guanacaste: 30 km N of Caiias; El Salvador: Santa Ana: 2 km SE of Metapin; Trinidad: 1 mi W of Arirna	Davidse and Pohl, 1972b
			Venezuela.: bolivar, 17 km W of the Rio Caura	Davidse and Pohl, 1978
			Cote-d'Ivoire	Kamacher *et al.*, 1973
			Nigeria: University Campus, Ile-Ife; forest floor	Olorode, 1975
			Zaire: Kinshasa, Mont Ngafula, Lukaya Valley, 25 km S of Kinshasa; Bas-Zaire, Kasangulu, Kindongo, 15 krn N of Kasangulu	Dujardin, 1978
			Brazil. Bahia	Hunziker *et al.*, 1982
			Argentina: Misiones, Depto Iguazu, Cataratas del Iguazu.	Hunziker *et al.*, 1985
			Argentina: Misiones, Depto Gral. M. Belgrano, 5 km SW of Deseado.	Hunziker *et al.*, 1989
			Germplasm Bank of Wild Species in Kunming	Guo *et al.*, 2019
				This work
	2*n*=ca. 30		Brazil: Distrito Federal	Gould and Soderstrom, 1967
	n=22	4*x*	Venezuela. Aragua: Rancho Grande	Davidse and Pohl, 1974
	2*n*=48	4*x*	Colombia: Caqueta	Gould and Soderstrom, 1970
O. loretensis Mez	2*n*=22	2*x*	Colombia. Amazonas	Gould and Soderstrom, 1970
O. obliquifolia Steud.	2*n*=23, 44	2*x*, 4*x*	Brazil: Para. Suriname: Zuid Rivier	Gould and Soderstrom, 1967
O. taquara Swallen	2*n*=20	2*x*	Brazil: Distrito Federal	Gould and Soderstrom, 1967

No data available for the following species: *Olyra amapana* Soderstrom & Zuloaga; *O. bahiensis* R. P. Oliveira & Longhi-Wagner; *O. buchtienii* Hackel; *O. caudata* Trin.; *O. davidseana* Judz. & Zuloaga; *O. ecaudata* Doell.; *O. filiformis* Trin.; *O. glaberrima* Raddi; *O. holttumiana* Soderstrom & Zuloaga; *O. juruana* Mez; *O. latispicula* Soderstrom & Zuloaga; *O. longifolia* Kunth; *O. maranonensis* Swallen; *O. retrorsa* Soderstrom & Zuloaga; *O. standleyi* Hitchc.; *O. tamanquareana* Soderstr. & Zuloaga; *O. wurdackii* Swallen.

reported (Davidse and Pohl, 1972a). Extensive genetic system studies might be very helpful to understand the polymorphisms of *O. latifolia* complex across its range.

Olyra latifolia and *O. humilis* have distinct positions in the phylogenetical topology in highly supported trees (Oliveira *et al.*, 2014; 2020), and the basic and gametic chromosome numbers found here for both species agree with this result. Olyreae chromosome numbers are highly variable, and the tribe has the greatest variation in basic chromosome numbers and ploidy levels. In addition, polyploid complexes with 2*x* and 4*x* were described in *O. latifolia*. Intensive cytogenetical studies on Southamerican herbaceous bamboos are necessary to understand evolutionary trends in bambusoid subfamily, considering that all bamboos probably derived from herbaceous ancestors (Soderstrom and Calderon, 1974).

Recently, the ploidy levels of bamboo grasses were reviewed by Zhou *et al.* (2017). As a rule, the chromosome number in temperate woody bamboo species remains constant with 2*n*=48, instead the chromosome number of paleotropical woody bamboos, was variable with 2*n*=70 ± 2, and a few species having 2*n*=64, 80, 96, 98, and 104 (Zhou *et al.*, 2017 and references therein). The neotropical woody bamboos were also diverse but in a minor degree with 2*n*=40, 44 or 48 chromosomes (Zhou *et al.* 2017). A revision of chromosome data of herbaceous bamboo is not available yet. *Olyra* is the largest genus in Olyrae and has been poorly studied regarding its cytogenetics. Further cytological studies are needed to clarify the chromosome number evolution in this genus as a supporting contribution to understand phylogenetical and reproductive ongoing analyses.

Species of Olyreae are endangered due to the continuing loss of forests (Oliveira and Clark, 2009). Under the climatic change impact, is necessary to claim for conservation efforts of bamboo species because several species are endemic and poorly studied. Besides, we ignore if they are an important resource for other species as food, forage or grains in forest ecosystems. The conservation of natural populations in subtropical South America should be considered as the focus of future studies in this tribe.

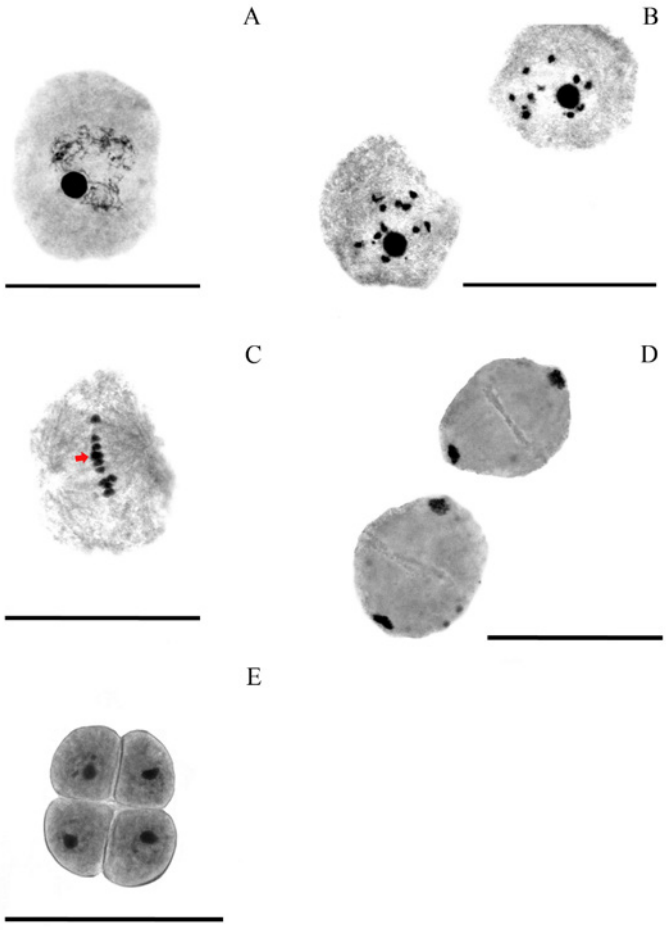

Figure 2. Pollen mother cells (PMCs) of *Olyra latifolia* (*Seijo* 872) at meiosis. **A.** Early prophase I with a unique prominent nucleolus. **B.** Two PMCs at diakinesis with 11 bivalents and a persistent nucleolus. **C.** Metaphase I with 11 II in the equatorial plate. Two overlapping bivalents are indicated with an arrow. **D.** Two PMCs in telophase I with regular behavior. **E.** Isobilateral tetrad of microspores. Scale = 50 µm.

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CHROMOSOMIC STUDIES IN *ZEPHYRANTHES CITRINA* BAKER (AMARYLLIDACEAE), A POLYPLOID ORNAMENTAL

ESTUDIOS CROMOSÓMICOS EN *ZEPHYRANTHES CITRINA* BAKER (AMARYLLIDACEAE), UN POLIPLOIDE ORNAMENTAL

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ABSTRACT

Zephyranthes citrina is an ornamental American bulbous plant used as an ornamental garden crop for the aesthetic qualities of its yellow perigonium. The objective of this work was to characterize the species by classical chromosome staining and fluorochrome banding. A sporophytic chromosome number of $2n=8x=48$ chromosomes was observed, being the karyotypic formula 20 m + 26 sm + 2 st. Satellites were detected in the short arm of metacentric chromosomes 8, 9, 11 and 12, which colocalized with constitutive heterochromatin $CMA^+/DAPI^{-/0}$ bands. The karyotype comprised chromosome pairs with terminal constitutive heterochromatin bands that included satellites and heteromorphic clusters indicating that it is an allooctoploid. These results will be used as a tool for monitoring genetic improvement, in interspecific crosses and its progenies and in biotechnological procedures by *in vitro* culture.

Key words: constitutive heterochromatin, chromosome banding, bulbous, plant genetic resources, karyotype

RESUMEN

Zephyranhtes citrina es una planta bulbosa americana, ornamental, utilizada en jardines por las cualidades estéticas de su perigonio amarillo. El objetivo de este trabajo fue caracterizar citogenéticamente la especie con tinción clásica convencional y bandeo cromosómico. Se observó un número cromosómico esporofítico de $2n=8x=48$, siendo la fórmula cariotípica 20 m + 26 sm + 2st. Se detectaron satélites en el brazo corto de los cromosomas metacéntricos 8, 9, 11 y 12, que co-localizaron con bandas de heterocromatina constitutiva $CMA^+/DAPI^-$. El cariotipo comprendió pares de cromosomas con bandas de heterocromatina constitutivas terminales que incluyeron satélites y grupos heteromórficos que indican que es un alooctoploide. Estos resultados serán usados como herramientas en el monitoreo del mejoramiento genético, en análisis de cruzamientos interespecíficos y progenies y en procedimientos biotecnológicos de cultivo *in vitro*.

Palabras clave: heterocromatina constitutiva, bandeo cromosómico, bulbosa, recursos fitogenéticos, cariotipo

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INTRODUCTION

Zephyranthes Herb. is a genus of perennial bulbous plants belonging to the Amaryllidaceae family, which stands out for its high ornamental potential and, at the same time, as a producer of phytochemicals. The species of this genus are of American origin but have been cultured and naturalized as ornamentals in various countries (Meerow *et al.*, 1999; Tapia-Campos *et al.*, 2012; Katoch and Singh, 2015). Phytochemical research of the genus began around 1940 with the report of the presence of alkaloids, such as lycorine, and is currently one of the areas of greatest scientific interest in these bulbous plants due to the pharmacological, antimicrobial, antifungal, acetylcholinesterase and cytotoxic properties of their active principles (Greahouse, 1941; Katoch and Singh, 2015). Taxonomically, *Zephyranthes* belongs to the tribe Hippeastreae (Amaryllidaceae) and its species inhabit tropical and subtropical regions of America (Meerow *et al.*, 2000; Tapia-Campos *et al.*, 2012), and although several efforts have been made to understand its evolutionary complexity, it is still a controversial clade, due to interspecific cross-linked hybridization revealed by molecular data and phylogenetic analyzes (García *et al.*, 2014, 2019).

Cytogenetically, *Zephyranthes* exhibits a wide range of chromosome numbers ranging from $2n=2x=10$ to $2n=96$, diploid and polyploid species, polyploid complexes, and the presence of aneuploid-polyploid polymorphisms with varied karyotypic formulas (Raina and Khoshoo, 1971; Bhattacharyya, 1972; Greizerstein and Naranjo, 1987; Daviña and Fernandez, 1989; Daviña, 2001; Felix *et al.*, 2011a; Daviña and Honfi, 2018). Furthermore, there are at least three basic numbers $x=5$, 6 and 7 whose diploids are found in the subtropical zone of South America (Daviña *et al.*, 2019). Fluorescent chromosome banding techniques allow longitudinal differentiation of chromosomal regions (Honfi *et al.*, 2017). In plants, the specific identification of constitutive heterochromatin regions with a sequential triple staining with chromomycin, distamycin and 4'-6-diamidino-2-phenylindole (CMA/DA/DAPI) (Daviña, 2001) has been used infrequently in the clade Hippeastreae and there are some antecedents in the genera *Zephyranthes* (Daviña, 2001; Felix *et al.*, 2011b) and *Habranthus* Herb. (Barros e Silva and Guerra, 2010).

Zephyranthes citrina Baker is a species native to the Gulf of Mexico, described for the first time in 1882, when it was spread to South America and is currently used ornamentally for the aesthetic qualities of its perigonium, particularly for the intense yellow coloration of its tepals (Hume, 1935; Tapia-Campos *et al.*, 2012). In a genus where white and pink shades are the most widespread, the intense yellow tepals are of great interest and value in breeding. Likewise, various phytochemicals have been found in this species, some of them of pharmacological importance (Boit *et al.*, 1957; Herrera *et al.*, 2001; Kohelova *et al.*, 2021). Recently, 27 different alkaloids have been detected in this species, among them, seven were unknown to science. Some of these alkaloids have shown biological activity associated with Alzheimer's disease and cytotoxic activity linked to oncological diseases (Prakash and Vedanayaki, 2019; Kohelova, 2021; Kohelova *et al.*, 2021). Within the framework of the characterization of the phytogenetic resources of the Amaryllidaceae family of ornamental and phytochemical interest, the objective of this work was to describe the species chromosomally and to detect karyotypic markers that easily identify it.

MATERIALS AND METHODS

Within the framework of scientific cooperation between CIATEJ (Mexico) and UNaM (Argentina), we studied individuals from a population of *Zephyranthes citrina* (Daviña 681) cultivated in Posadas, Misiones, Argentina, whose control specimen is deposited in the herbarium of the Universidad Nacional de Misiones (MNES) (Figure 1).

Figure 1. *Zephyranthes citrina* (D 681) in bloom visited by pollinators.

Standard cytological techniques

The protocols used by Daviña (2001) were applied and the number of chromosomes in mitotic cells was determined using the meristems of the root tips pretreated with a 0.002M saturated solution of 8-hydroxyquinoline for 8 h at room temperature. They were fixed in absolute ethanol:glacial acetic acid in a 3:1 ratio and stored in the same fixative at about 4° C. The conventional Feulgen

staining was then performed, which consists of an acid hydrolysis of the rootlets in 1N HCl for 10 min at 60° C and a subsequent staining with basic fuchsin (Schiff's reagent) in a dark chamber for at least 20 min. The meristematic zones were macerated in 2% acetic orcein and subsequently squashed.

Molecular chromosome techniques

Pretreated and fixed roots were used as described above in standard staining techniques and also following the protocol suggested by Schwarzacher *et al.* (1980), which consists of macerating the root tips in an enzymatic solution (2% cellulase, 1% pectinase, in 0.01 M citrate buffer, pH 4.8) and squashing in 45% acetic acid. The coverslip was removed with liquid nitrogen and air dried for 1 d at room temperature before use.

A triple sequential CMA/DA/DAPI staining was performed. For the CMA (chromomycin A3) bands, the procedure developed by Schweizer (1976) was followed. The slides were incubated in CMA staining solution (McIlvaine buffer pH 7, 10 mM Cl_2Mg, 0.12 mg/ml chromomycin A3) for 2 h in the dark at room temperature, washed and air dried, and mounted in a solution 1:1 glycerol:McIlvaine buffer with 5 mM Cl_2Mg. Next, they were stained with distamycin A (DA) drops dissolved in McIlvaine buffer pH 7. They were incubated in DA solution at room temperature in the dark in a humid chamber for 15 to 30 min. Subsequently, they were washed and dried. Finally, for the DAPI (4'-6-diamidino-2-phenylindole) bands, the method suggested by Schweizer (1976) was used. The slides were incubated in DAPI staining solution (McIlvaine buffer pH 7, 1-2 µg/ml DAPI) for 30-45 min in the dark at room temperature, washed and air dried, and mounted in the same solution as above.

Karyotype analysis

Chromosomes were observed and photographed with a Leica DML binocular epifluorescence microscope equipped with a DF C310 FX video equipment. 10 optimal metaphases were analyzed, and the nomenclature proposed by Levan *et al.* (1964) was used to classify chromosomes according to the centromeric index (i=s*100/c, where s=length of the short arm and c=total length of the chromosome). In addition, the total length of the chromosome complement (TCL) and the arm ratio (r=l/s) were calculated. In the idiograms, the chromosomes were grouped according to their morphology and within each group they were ordered by decreasing size. As it is a polyploid species, the idiogram was made considering all the chromosomes. The satellites were classified according to the nomenclature suggested by Battaglia (1955, 1999) with which microsatellites were distinguished from macrosatellites, since the former have diameters less than half the diameter of the chromosome. The value of the length of the satellites was included within the total length of the arm to which they were associated.

RESULTS

Mitotic metaphases revealed the octoploid condition of *Z. citrina* with $2n=8x=48$ chromosomes and a basic number of $x=6$ (Figure 2A). The karyotypic formula was 20 m + 26 sm + 2 st, (Figure 3) and the total complement length was 271.31 µm (Table 1). Satellites were observed in the short arm of metacentric chromosomes 8, 9, 11 and 12, all located terminally. In the case of chromosomes 8 and 9 they were macrosatellites, while those of chromosomes 11 and 12 were microsatellites. The mean centromeric index (i) was 36.04 and the mean chromosome length was 5.65 *µm*. The CMA/DA/DAPI triple fluorescent staining pattern showed constitutive GC (guanine-cytosine)-rich heterochromatin bands (Figure 2B, C). The terminal bands located on the short arm of chromosomes 8 and 9 (m) revealed the presence of a type of constitutive heterochromatin $CMA^+/DAPI^0$, whose size includes the satellite and was 1.6 *µm*. On chromosomes 11 and 12 (m), a $CMA^+/DAPI^-$ fluorescent band, rich in GC, 0.3 *µm* long, was identified on the short arm. The amount of constitutive GC-rich heterochromatin corresponded to 0.7% of the polyploid genome. The karyotype comprised chromosome pairs with terminal constitutive heterochromatin bands that included satellites and heteromorphic clusters indicating that it was an allooctoploid (Figure 3).

DISCUSSION

Zephyranhtes citrina is an octoploid species that belongs to the group of species with a basic number $x=6$, which is the most frequent of the genus; this group contains, in addition to diploids, the largest number of polyploid and aneuploid species?. The detected number agrees with those reported by Soontornchainaksang and Chaiyasut (1996), Bobby *et al.* (2003) and Raina and Khoshoo (1972a) (as *Z. sulphurea*) and differs from $2n=47$ registered by Gonzalez *et al.* (1980) in provenances from Cuba. This is the first description of constitutive heterochromatin for the species. So far, there are only two antecedents in the Hippeastreae clade on the presence of $DAPI^+$ bands, which correspond to *Habranthus robustus* Herb., a diploid with $2n=2x=12$ (= *Zephyranthes robustus, sensu* García *et al.*, 2019) and *Habranthus brachyandrus* (Baker) Seally, a tetraploid with $2n=4x=24$ (=*Zephyranhtes brachyandra, sensu* García *et al.*, 2019) (Barros e Silva and Guerra, 2010; Felix *et al.*, 2011b). The other species of *Habranthus* and

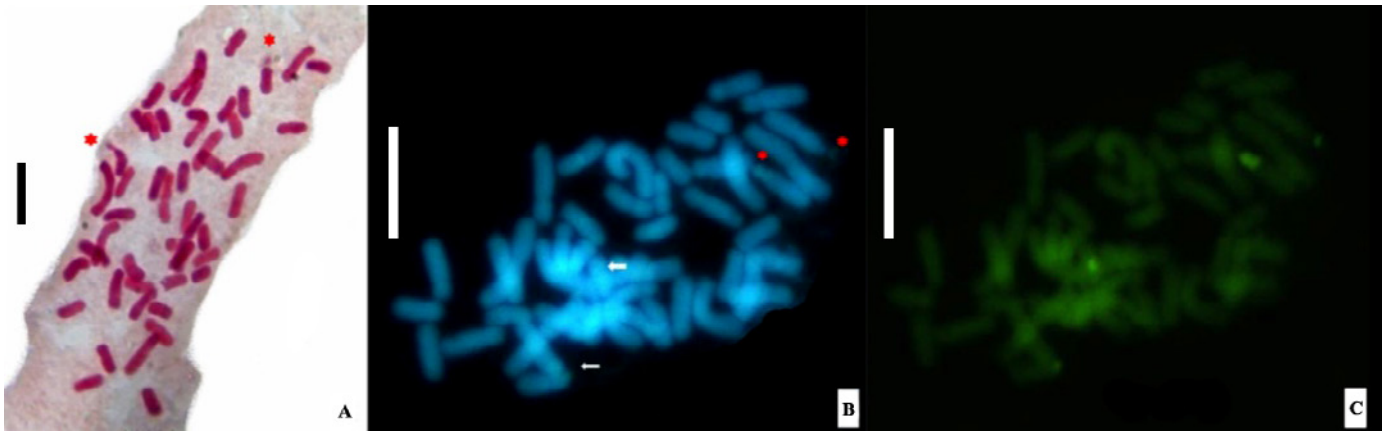

Figure 2. Mitotic metaphase of *Z. citrina*: **A-** conventional staining, 2*n*=8x=48, asterisks indicate the satellites of chromosomes 8 and 9 (m). **B-C-** Sequential banding CMA/DA/DAPI; arrows indicate sites DAPI – (bands CMA+/DAPIo) on chromosomes 8 and 9 (m); asterisk bands indicate CMA+/DAPI- on metacentric chromosomes 11 and 12 (m). Bars =10 µm.

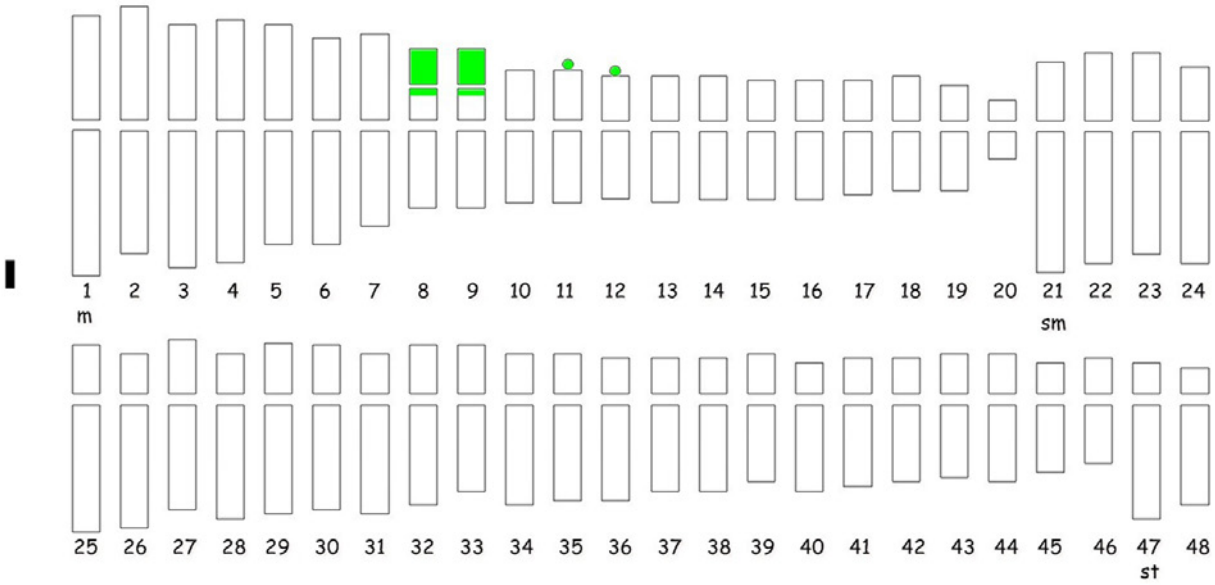

Figure 3. Idiogram of the complete chromosome set of *Z. citrina* 2*n*=48 (20 m + 26 sm + 2 st). Heterochromatic bands (in green) CMA+/DAPIo on chromosomes 8, 9, CMA+/DAPI- on chromosomes 11 and 12 (m), in the short arm. Bars =1 µm.

Zephyranthes, with known constitutive heterochromatin patterns, present banding patterns with regions rich in GC (Daviña, 2001; Barros e Silva and Guerra, 2010; Felix *et al.*, 2011b), as well as the type of pattern detected for *Z. citrina* in this work.

The origin of the polyploids in the Hippeastreae clade remains uncertain in many cases. The main reason is due to the few registered meiotic studies, since both microsporogenesis and megasporogenesis are processes that occur when the flower bud is still inside the bulb without showing external signs of such events and therefore, numerous bulbs must be sacrificed with no guarantees of finding the coveted meiotic stages. Reported male meiosis in both cultivars and natural species have revealed high percentages of bivalents and regular meiosis; multivalent and irregular meiosis to meiotic aberrations such as bridges, lagging chromosomes, micronuclei, among others (Coe, 1953; Sharma and Ghosh, 1954; Tandom and Mathur, 1965; Yokouchi, 1965; Raina and Khoshoo, 1972b; Daviña and Fernandez, 1989; Thoibi Devi and Borua, 1997; Daviña, 2001). For these reasons and based on the described characteristics of the karyotype, *Z. citrina* is considered to be allopolyploid, which may be clarified with future meiotic studies.

Natural and synthetic hybrids of *Zephyranthes* have been reported, resulting from intra- and inter-specific crosses, designed to expand options for growers (Raina and Khoshoo, 1972a; Chowdhury and Hubstenberger, 2006; David, 2011). Chowdhury and Hubstenberger

Table 1. Average of the morphometric parameters of the chromosomal set of *Z. citrina*.

Cromosoma	Long Total	Brazo Corto	Brazo Largo	Indice	Tipo	r
1	9,5	4	5,5	42,1052631579	m	1,375
2	9	4,3333333333	4,6666666667	48,1481481481	m	1,0769230769
3	8,8333333333	3,6666666667	5,1666666667	41,5094339623	m	1,4090909091
4	8,8333333333	3,8333333333	5	43,3962264151	m	1,3043478261
5	8	3,6666666667	4,3333333333	45,8333333333	m	1,1818181818
6	7,5	3,1666666667	4,3333333333	42,2222222222	m	1,3684210526
7	7	3,3333333333	3,6666666667	47,619047619	m	1,1
8	5,6666666667	2,6666666667	3	47,0588235294	m	1,125
9	4,8333333333	2	2,8333333333	41,3793103448	m	1,4166666667
10	4,8333333333	2	2,8333333333	41,3793103448	m	1,4166666667
11	4,8333333333	2,1666666667	2,6666666667	44,8275862069	m	1,2307692308
12	4,5	1,8333333333	2,6666666667	40,7407407407	m	1,4545454545
13	4,5	1,8333333333	2,6666666667	40,7407407407	m	1,4545454545
14	4,3333333333	1,6666666667	2,6666666667	38,4615384615	m	1,6
15	4,3333333333	1,6666666667	2,6666666667	38,4615384615	m	1,6
16	4,1666666667	1,6666666667	2,5	40	m	1,5
17	4,1666666667	1,8333333333	2,3333333333	44	m	1,2727272727
18	3,8333333333	1,5	2,3333333333	39,1304347826	m	1,5555555556
19	2,15	0,95	1,2	44,1860465116	m	1,2631578947
20	7,6666666667	2,3333333333	5,3333333333	30,4347826087	sm	2,2857142857
21	7,6666666667	2,6666666667	5	34,7826086957	sm	1,875
22	7,3333333333	2,6666666667	4,6666666667	36,3636363636	sm	1,75
23	7,1666666667	2,1666666667	5	30,2325581395	sm	2,3076923077
24	6,8333333333	2	4,8333333333	29,2682926829	sm	2,4166666667
25	6,3333333333	1,6666666667	4,6666666667	26,3157894737	sm	2,8
26	6,1666666667	2,1666666667	4	35,1351351351	sm	1,8461538462
27	6	1,6666666667	4,3333333333	27,7777777778	sm	2,6
28	6	1,8333333333	4,1666666667	30,5555555556	sm	2,2727272727
29	6	2	4	33,3333333333	sm	2
30	5,8333333333	1,6666666667	4,1666666667	28,5714285714	sm	2,5
31	5,8333333333	2	3,8333333333	34,2857142857	sm	1,9166666667
32	5,5	2	3,3333333333	36,3636363636	sm	1,6666666667
33	5,5	1,6666666667	3,8333333333	30,303030303	sm	2,3
34	5,3333333333	1,6666666667	3,6666666667	31,25	sm	2,2
35	5,1666666667	1,5	3,6666666667	29,0322580645	sm	2,4444444444
36	4,8333333333	1,5	3,3333333333	31,0344827586	sm	2,2222222222
37	4,8333333333	1,5	3,3333333333	31,0344827586	sm	2,2222222222
38	4,6666666667	1,6666666667	3	35,7142857143	sm	1,8
39	4,6666666667	1,3333333333	3,3333333333	28,5714285714	sm	2,5
40	4,6666666667	1,5	3,1666666667	32,1428571429	sm	2,1111111111
41	4,5	1,5	3	33,3333333333	sm	2
42	4,5	1,6666666667	2,8333333333	37,037037037	sm	1,7
43	4,6666666667	1,6666666667	3	35,7142857143	sm	1,8
44	4,33333333	1,33333333	3	30,769230716	sm	2,2500000056
45	4	1,3333333333	2,6666666667	33,3333333333	sm	2
46	3,8333333333	1,5	2,3333333333	39,1304347826	sm	1,5555555556
47	5,6666666667	1,3333333333	4,3333333333	23,5294117647	st	3,25
48	5	1,1666666667	3,8333333333	23,3333333333	st	3,2857142857

Abbreviations: **Nº**=chromosome number; **c**=total length of the chromosome in *µm* (c=s+l); **l**=long arm length in µm; **s**=short arm length in *µm*; i=centromeric index (i=sx100/c); **type**=chromosomal morphology according to Levan *et al.*, 1964: **m**=metacentric, **sm**=submetacentric, **st**=subtelocentric; **r**=arms ratio (r=l/s).

(2006) highlight seven barriers to the formation of hybrids in *Zephyranthes*, among which chromosome number and ploidy level are preponderant due to the existing chromosome variety in the genus. Another crucial aspect is the existence of reproduction by apomixis and pseudogamy in species of the genus, reproductive events that constitute barriers to obtain simple hybrids (Raina and Khoshoo, 1972a; Chowdhury and Hubstenberger, 2006; Crane, 2019). Obtaining hybrids implies the identification of possible progenitors suitable for crossbreeding plans, both to increase aesthetic and ornamental varieties and to obtain new phytochemical combinations.

At least two successful hybrid lineages are known from crosses with *Z. citrina*. One of them is of interspecific origin and the other is intergeneric. Among the interspecific hybrids with fertile progeny, the tri-hybrid "Best Pink Trihybrid" stands out, product of the cross [(*Z. candida* x *Z. citrina*) x *Z. macrosiphon*] (Chowdhury and Hubstenberger, 2006), where *Z. citrina* was used as a pollen donor because it is an apomictic species (Howard, 1996). On the other hand, *Zephyranthes ajax* is a commercial hybrid with pale yellow tepals resulting from a cross between *Z. citrina* x *Z. candida*, a somatic chromosome number of 2*n*=42 and variable ploidy in the endosperm of its progeny (Tandon and Kapoor, 1962). These endosperm characteristics with mitotic aberrations and variable ploidy was also observed in *Z. citrina* (Bobby *et al.*, 2003). In the lineage of hybrids of intergeneric origin, there is a hybrid known as *Cooperanthes* "*Percy*" (also *Zephyranthes x Percyi*) that was introduced by Traub in 1954 by crossing *Z. citrina* and *Cooperia drummondii* Herb. (David, 2011).

Having cytogenetic markers provides a useful tool to detect in early stages if the hybridization was successful, before the first flowering period of the obtained progeny. It is evident that *Z. citrina* is a species of high value as a parent, of interest in crossbreeding plans due to the qualities of its corolla and the fact that cytological markers for the species have been detected in this work. These results characterize *Z. citrina* as an octoploid and contribute to the knowledge of its cytogenomic structure. Future crosses using this species as a male parent will allow the initiation of new hybrid lineages of ornamental and/or phytochemical interest, which will be able to multiply massively. Protocols for mass multiplication of bulbs (Rodriguez Mata *et al.*, 2018) and *in vitro* culture protocols, adjusted to obtain plants without ploidy alteration and with an efficiency of 85% in the acclimatization stage, are available (Syeed *et al.*, 2021).

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WHAT DO NEOTROPICAL PRIMATES TELL US UNDER THE LOOK OF CYTOGENETICS?

¿QUÉ NOS DICEN LOS PRIMATES NEOTROPICALES BAJO LA MIRADA DE LA CITOGENÉTICA?

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ABSTRACT

Cytogenetics studies in Neotropical Primates (Primates: Platyrrhini) have shown that these mammals comprise a heterogeneous group at the chromosomal level. The remarkable variety of karyotypes described provides significant evidence on the possible role of chromosomal rearrangements in their evolution. In the Grupo de Investigación en Biología Evolutiva (GIBE), the line of research on the evolutionary divergence process in Platyrrhini considering different aspects of the organization of the genome has been established and developed uninterruptedly for more than 30 years. Among the advances made in recent years is the quantification of the genome size in six species of caí monkeys (*Cebus* sp.) and two species of howler monkeys (*Alouatta* sp.) and the description of the composition of base pairs in the constitutive heterochromatin regions in the genera *Cebus* and *Ateles*. The first descriptions were made of the karyotype and meiotic behavior in early prophase I of two species of howler monkeys, *Alouatta caraya* and *A. guariba clamitans*. In this last species, the first pentavalent-type sexual system $X_1X_2X_3Y_1Y_2$ was identified in a primate species. The organization of euchromatin was characterized in terms of the content and distribution of AT and GC nucleotide bases in three species of howlers and in two species of caí monkeys. These, among other investigations, allowed contributing in an original way to the knowledge about speciation at different levels, as well as about the architecture and dynamics of the genome of these primates.

Key words: Neotropical Primates, cytogenetics and taxonomy, chromosome evolution, sex chromosomes.

RESUMEN

Los estudios de citogenética en Primates Neotropicales (Primates: Platyrrhini) han demostrado que estos mamíferos comprenden un grupo heterogéneo a nivel cromosómico. La notable variedad de cariotipos descriptos provee evidencia significativa sobre el posible papel de los reordenamientos cromosómicos en su evolución. En el Grupo de Investigación en Biología Evolutiva (GIBE), la línea de investigación sobre el proceso de divergencia evolutiva en Platyrrhini considerando distintos aspectos de la organización del genoma se ha establecido y desarrollado de manera ininterrumpida desde hace más de 30 años. Entre los avances realizados en los últimos años se encuentra la cuantificación del tamaño del genoma en seis especies de monos caí (*Cebus* sp.) y dos especies de monos aulladores (*Alouatta* sp.) y la descripción de la composición de pares de bases en las regiones de heterocromatina constitutiva en los géneros *Cebus* y *Ateles*. Se concretaron las primeras descripciones del cariotipo y comportamiento meiótico en profase I temprana de dos especies de monos aulladores, *Alouatta caraya* y *A. guariba clamitans*. En esta última especie se identificó el primer sistema sexual de tipo pentavalente $X_1X_2X_3Y_1Y_2$ en una especie de primate. Se caracterizó la organización de la eucromatina en términos del contenido y distribución de bases nucleotídicas AT y GC en tres especies de aulladores y en dos especies de monos caí. Estas investigaciones, entre otras, permitieron contribuir de forma original al conocimiento sobre la especiación en distintos niveles, así como sobre la arquitectura y dinámica del genoma de estos primates.

Palabras clave: Primates Neotropicales, citogenética y taxonomía, evolución cromosómica, cromosomas sexuales.

Teniendo en consideración las distintas revisiones taxonómicas podemos hoy decir que los primates comprenden dos grandes grupos: Strepsirrhini y Haplorrhini. Los Haplorrhini reúnen a los Primates del Viejo Mundo (Catarrhini) y a los del Nuevo Mundo (Platyrrhini o Ceboidea) (Rowe y Myers, 2017). Múltiples son las fuentes de conocimiento para abordar la caracterización de un grupo de mamíferos tan amplio y complejo. En particular, en lo que a caracterización genética se refiere, durante los últimos 20 años diversos autores han "diseccionado" el genoma de los Platyrrhini a través de enfoques metodológicos diversos y, a la vez, con niveles de análisis diferentes y complementarios. Los estudios de citogenética en Platyrrhini han demostrado, en las últimas décadas, que estos mamíferos comprenden un grupo heterogéneo a nivel cromosómico (Stanyon *et al.*, 2008, 2012; de Oliveira *et al.*, 2012). La especiación en este grupo pareciera haberse desarrollado bajo muy diversos procesos que involucraron reordenamientos cromosómicos estructurales, mayoritariamente fusiones, fisiones, inversiones y translocaciones, variaciones importantes en las regiones heterocromáticas y la llamativa presencia de sistemas sexuales múltiples (Seuánez *et al.*, 1986, 2005; Armada *et al.*, 1987; Matayoshi *et al.*, 1987; Ponsá *et al.*, 1995; Mudry *et al.*, 2001; Steinberg *et al.*, 2008; de Oliveira *et al.*, 2012). La notable variedad de cariotipos descriptos provee evidencia significativa sobre el posible papel de los reordenamientos cromosómicos en los cambios evolutivos experimentados por los primates hasta llegar a las formas actuales.

En el Grupo de Investigación en Biología Evolutiva (GIBE), la línea de investigación sobre el proceso de divergencia evolutiva de especies de Platyrrhini, considerando distintos aspectos de la organización del genoma, se ha establecido y desarrollado de manera ininterrumpida desde hace más de 30 años. Los estudios en primates se desarrollaron dentro de un marco teórico taxonómico-evolutivo, aplicando metodologías de análisis clásicas de la citogenética, que aportaron al esclarecimiento de la compleja sistemática de los primates de distribución marginal sur con representantes de dos familias de Ceboidea: Atelidae (*Alouatta* y *Ateles*) y Cebidae (*Cebus*, *Saimiri* y *Aotus*). Para concretar los estudios genético-evolutivos se relacionaron los datos de variabilidad genética con características geográficas regionales, disponibilidad y uso de recursos, demografía, estudios parasitológicos de tipo relación parásito-hospedador y particularidades sociales y comportamentales, entre otros aspectos de las especies referidas procedentes principalmente de las provincias argentinas de Corrientes, Chaco y Misiones (Giudice, 1997; Ascunce, 2002; Martinez *et al.*, 2002; Bruno *et al.*, 2005; Ascunce *et al.*, 2007; Arístide *et al.*, 2013; Nieves *et al.*, 2021). La citogenética clásica tomó valor diagnóstico y por ello se determinó el cariotipo que permitió identificar cromosomas marcadores en distintas especies, así como también diferentes reordenamientos involucrados en el proceso evolutivo en diversos géneros (Mudry, 1982, 1990; Mudry de Pargament *et al.*, 1984, 1985; Mudry *et al.*, 1990, 1992, 2001, 2007, 2015; Nieves y Mudry, 2016). Asimismo, se avanzó en el conocimiento de los reordenamientos cromosómicos de valor evolutivo mediante la aplicación de la hibridación *in situ* fluorescente o *FISH* (por su sigla en inglés *Fluorescence in situ Hybridization*) y sus variantes con el fin de identificar con mayor precisión las homeologías interespecíficas, investigar la relación entre las proporciones de eucromatina y heterocromatina y las diferencias cariotípicas intraespecíficas, y analizar el grado de diferenciación molecular, en especial a nivel de los cromosomas sexuales (Mudry y Nieves, 2010; Steinberg *et al.*, 2014a; Nieves *et al.*, 2018).

Así desde el GIBE podemos considerar que se contribuyó de forma original al conocimiento sobre la especiación en distintos niveles, arquitectura y dinámica del genoma, y en diferentes modelos biológicos, con especial énfasis en aquellos procesos ocurridos en el linaje de los primates. A continuación, se describirán los hallazgos más relevantes de los últimos 12 años de investigación ininterrumpida desde la última revisión de nuestros aportes publicada en esta misma revista (Mudry y Nieves, 2010), siempre dentro del marco de "Evidencia Total" (Kluge, 1989), i.e. analizando la mayor cantidad de tipos de evidencia posible para lograr importantes avances en el conocimiento y aportar contribuciones novedosas a la sistemática de una especie y/o género.

¿CON QUÉ CONTRIBUIMOS AL MÁS AMPLIO CONOCIMIENTO DE LA HETEROCROMATINA CONSTITUTIVA?

Un componente que aporta variabilidad a los genomas es la heterocromatina constitutiva, dependiendo su biología tanto de las secuencias de ADN repetitivas que la caracterizan como de las proteínas que se unen específicamente a ella. La mayoría de estas regiones contienen una alta densidad de secuencias de ADN satélite y elementos transponibles, que se encuentra sólo en niveles bajos o no se encuentra en absoluto en la eucromatina (Trojer y Reinberg, 2007; Sumner, 2008). En la heterocromatina constitutiva, dentro del genoma de una especie o dentro de un mismo cromosoma, puede haber diferentes tipos de ADN. Además, la longitud puede variar desde 2 pares de bases (pb) hasta unidades repetitivas de cientos y miles de pb. Asimismo, puede variar entre distintas especies, entre los cromosomas de una misma especie y entre distintas regiones de un mismo cromosoma (Sumner, 2008).

Por ello, la caracterización de la heterocromatina

constitutiva en función del contenido, localización, distribución y composición de secuencias de bases específicas es un aspecto que contribuye a la caracterización del cariotipo de cada especie. Si bien sus características estructurales y funcionales no se conocen profundamente, se ha visto que su presencia afecta la replicación del ADN y modula la estructura cromosómica, la expresión génica, la recombinación, la sinapsis y la segregación de los cromosomas, así como la organización y evolución del genoma. A su vez, se ha postulado que las diferencias en el contenido y la distribución de la heterocromatina entre especies relacionadas estarían involucradas en la evolución cariotípica y la diferenciación genética (Redi *et al.*, 2001; Grewal y Jia, 2007; Hughes y Hawley, 2009).

El estudio de la distribución y composición de la heterocromatina constitutiva en nuestro equipo de trabajo se remonta a la década de 1980 cuando empezamos a trabajar en citogenética clásica de primates (Mudry, 1982; Mudry de Pargament *et al.*, 1985; Mudry de Pargament y Labal de Vinuesa, 1988). Distintas experiencias con otros grupos biológicos ampliaron el espectro de nuestros intereses en la comprensión de la heterocromatina constitutiva como moduladora del genoma (Mudry, 1990; Ponsà *et al.*, 1995; García *et al.*, 1995; Nieves *et al.*, 2005; Garcia-Cruz *et al.*, 2009, 2011). La aplicación de diversas metodologías de tinción cromosómica diferencial puso en evidencia muy tempranamente las diferentes topologías, polimorfismos y variantes que se podían encontrar tanto en invertebrados como en vertebrados y, en particular, en el cariotipo de los primates. Con el advenimiento y el avance de la citogenética molecular se obtuvieron sondas específicas de heterocromatina de *Cebus* que permitieron analizar las homeologías interespecíficas, comparando con el ser humano y con distintas especies de primates; dado que no se observó la hibridación de esta sonda de heterocromatina en ninguna otra especie de primates, se demostró que la composición de la heterocromatina constitutiva de *Cebus* es específica de este género (Nieves *et al.*, 2011).

Considerando la diversidad cariológica referida anteriormente, cabía esperar cierta diversidad en otras variables nucleares asociadas, entre las cuales elegimos estudiar el tamaño del genoma. Desde que Thomas (1971) formuló la paradoja del valor C (tamaño *vs.* complejidad del genoma), se han realizado numerosos estudios sobre el tamaño del genoma de los vertebrados y su correlato con determinados parámetros fenotípicos. El contenido de ADN nuclear es una constante específica de especie considerada un parámetro citológico en el análisis e interpretación de los procesos evolutivos (Cavalier-Smith, 1982; Pellicciari *et al.*, 1982; Redi *et al.*, 2005). Como variable cuantitativa, el tamaño del genoma muestra un amplio rango de variabilidad entre los diferentes grupos de organismos vivos, si bien no se distribuye aleatoriamente dentro ni entre los grupos. A pesar de que los genomas de los Platyrrhini muestran una gran diversidad cariotípica, numérica, estructural y/o heterocromática, la variación del contenido de ADN entre las especies es menor que la observada en otros vertebrados. Sin embargo, al mismo tiempo se ha sugerido que la especiación en Platyrrhini habría ocurrido acompañada de alteraciones o cambios cuantitativos en el genoma (Pellicciari *et al.*, 1982; Ronchetti *et al.*, 1993; Morand y Ricklefs, 2005; Redi *et al.*, 2005). Asimismo, en primates se ha descripto una correlación positiva entre el contenido de ADN nuclear y la presencia de secuencias repetidas evidenciadas como heterocromatina constitutiva. Dentro de los primates, en Platyrrhini en particular, se ha observado la mayor proporción de heterocromatina constitutiva de localización extracentromérica. Entre los platirrinos existen géneros, como por ejemplo *Cebus* (=*Sapajus*), en los que se observa un alto contenido de secuencias altamente repetidas en el genoma, característica distintiva que nos llevó a plantear la existencia de variabilidad en su tamaño genómico asociado a esta particularidad (Nieves y Mudry, 2016; Nieves *et al.*, 2018). El género *Alouatta*, por el contrario, muestra la menor proporción de secuencias mediana y altamente repetidas a nivel extracentromérico, detectable por bandas C, descripta hasta hoy en Platyrrhini (Mudry *et al.* 1984, 1990, 1992, 2015). En este contexto, podría proponerse que los cambios en tamaño del genoma estarían relacionados no sólo con modificaciones en distintas regiones de la cromatina sino también con la posible interacción entre ellas. Ante esta premisa se cuantificó el tamaño del genoma en las especies de monos capuchinos *Cebus olivaceus*, *C. albifrons*, *C. cay*, *C. nigritus*, *C. xanthosternos* y *C. libidinosus*, y de monos aulladores *Alouatta caraya y A. guariba clamitans*. Cabe destacar que estos estudios nos permitieron describir por primera vez el tamaño del genoma de las especies de ambos géneros que habitan en la Argentina, *C. cay, C. nigritus, A. caraya y A. guariba clamitans* (Fantini, 2015). La variabilidad en el tamaño del genoma no fue estadísticamente significativa entre las especies de *Cebus* analizadas, si bien fue factible dar fundamento a la hipótesis de su posible relación con la proporción de heterocromatina constitutiva detectada por distintas metodologías citogenéticas. Asimismo, nos permitió afirmar que en las especies de *Alouatta* los cariotipos presentan una muy baja proporción de heterocromatina constitutiva extracentromérica y similar en todas ellas, contrario a lo que ocurre en *Cebus*. En los monos aulladores analizados no se observó una relación entre las diferencias de tamaño del genoma y la proporción de heterocromatina constitutiva.

Para identificar y determinar la localización cromosómica de las posibles diferencias en el tamaño del genoma en especies de *Cebus*, se consideró que las diferencias cuantitativas interespecíficas se deberían

a la distribución y posición extracentromérica del ADN satélite. Con este marco teórico se realizó la comparación de tres especies de *Cebus* elegidas específicamente por ser una de ellas, *C. olivaceus*, la de mayor tamaño de genoma y mayor proporción de heterocromatina constitutiva y las otras dos, *C. nigritus* y *C. xanthosternos*, las de menor tamaño de genoma y menor proporción de heterocromatina. Del análisis comparativo entre *C. nigritus* y *C. olivaceus* se hallaron diferencias cuantitativas en el tamaño del genoma sólo atribuibles al cromosoma sexual Y, dado que *C. nigritus* mostró regiones de ganancia relativa de ADN que no se evidenciaron en el cromosoma Y de *C. olivaceus.* Del análisis comparativo entre *C. nigritus* y *C. xanthosternos*, no se observaron estas diferencias cuantitativas en el cromosoma Y de *C. nigritus*, posiblemente debido a la mayor cercanía filogenética entre estas dos especies que entre *C. nigritus* y *C. olivaceus* (Nieves *et al.*, 2018). Estos hallazgos juntamente con los antecedentes disponibles en la literatura permitieron proponer como hipótesis que las secuencias repetidas modularían el genoma en estos mamíferos, constituyendo un campo de la investigación genética que aún presenta mucho por delante para seguir trabajando.

En los últimos años, profundizamos el estudio de la heterocromatina constitutiva en función de los patrones de distribución y composición de secuencias de bases específicas, siempre tomando especies de monos neotropicales como material del análisis citogenético. Estas investigaciones abarcaron el análisis del contenido y composición nucleotídica mediante patrones de tinción secuenciales con los fluorocromos DAPI (*4',6-diamidino-2-phenylindole*) para detectar zonas ricas en pares de bases A-T, y CMA_3 (cromomicina A_3) para zonas ricas en pares de bases G-C. Se observaron diferencias entre los géneros *Cebus* y *Ateles* respecto de la composición de pares de bases en las regiones de heterocromatina constitutiva, evidenciadas por bandas C. En las especies *C. cay*, *C. nigritus*, *C. xanthosternos*, *C. apella*, *C. olivaceus*, *C. libidinosus* y *C. albifrons*, los bloques de heterocromatina C-positiva resultaron ser CMA_3-positivos, demostrando que en el género *Cebus* la heterocromatina constitutiva está enriquecida en pares de bases G-C. Sin embargo, en *Ateles* las regiones de heterocromatina constitutiva resultaron ser DAPI-positivas, presentando entonces una composición nucleotídica rica en pares de bases A-T (Nieves *et al.*, 2018).

Por otro lado, se llevó a cabo un intenso trabajo analizando el material cariológico disponible en el GIBE para la caracterización cuantitativa de la distribución de heterocromatina en dos especies de *Cebus* cuya distribución geográfica llega al extremo norte de nuestro país, *C. cay* y *C. nigritus*. A su vez se realizó un análisis comparativo cariológico con material de archivo procedente de distintos centros y museos gracias a diferentes intercambios de pasantías de investigadores y convenios. Para ello, en el marco del Proyecto Primates, el material biológico (cultivos en monocapa de distintas líneas celulares establecidas tales como Gep2 y *CHO*, linfocitos de sangre periférica y extensiones de células espermatogénicas, entre otras) de ejemplares de Ceboidea de diferentes Zoológicos y Centros de Cría de la Argentina, fueron procesados con distintos abordajes citogenéticos, citogenético-moleculares y moleculares propiamente dichos (Steinberg *et al.*, 2014b; Nieves y Mudry, 2016). En distintos materiales se evaluó la variabilidad en los patrones de bandas C y la frecuencia de sus polimorfismos (Nieves y Mudry, 2016). A partir de este estudio se observó un patrón de bandas C especie-específico y se identificaron tres morfotipos en los patrones de distribución de la heterocromatina constitutiva, dos correspondientes a *C. cay* y uno a *C. nigritus*. En *C. cay* los morfotipos estaban relacionados con el origen geográfico de los ejemplares, uno presente en los ejemplares de Paraguay y el otro en los ejemplares de la provincia de Salta (Argentina), Bolivia y Perú. A su vez, se observó una variabilidad significativa en el tamaño del genoma en los ejemplares de *C. cay*, en concordancia con los análisis genético-moleculares y morfométricos realizados en forma paralela (Arístide *et al.*, 2013). Un estudio posterior, incorporando datos moleculares de la región control del ADN mitocondrial de estas especies, mostró tres morfotipos para *C. cay*, identificando dos grupos genéticamente divergentes en esta especie y distinguiendo los linajes correspondientes al noroeste y al noreste de la Argentina (Nieves *et al.*, 2021). Todas estas evidencias apoyan el marco teórico en el que nos propusimos trabajar que es el de "Evidencia Total" que implica la aplicación de un enfoque holístico, incorporando distintas fuentes de evidencias, para tratar de esclarecer cómo se mantiene la diversidad y la variabilidad en estas especies de primates.

¿QUÉ NOS ENSEÑÓ HASTA HOY EL ESTUDIO DE LA MEIOSIS MASCULINA?

Durante los últimos 20 años debemos reconocer una etapa en la que se realizó un intensivo estudio citogenético y citogenético-molecular en especies de monos aulladores del género *Alouatta*. En esa etapa se contó con las colaboraciones de grupos de investigación internacionales que permitieron avanzar en las caracterizaciones cariotípicas de especies poco estudiadas de este género. La colaboración de la Dra. Liliana Cortés-Ortiz (Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, Michigan, USA) y el Dr. Domingo Canales Espinosa (Instituto de Neuroetología, Universidad Veracruzana, Xalapa, México) ilustran más adelante estos destacados avances. En *Alouatta* se había descripto la presencia

de sistemas cromosómicos de determinación sexual múltiple, mediante estudios meióticos, originados por translocaciones Y-autosomas: de tipo trivalente X_1X_2Y en machos de *A. belzebul* (Armada *et al.*, 1987) y *A. palliata* (Solari y Rahn, 2005), de tipo cuadrivalente $X_1X_2Y_1Y_2$ en *A. seniculus stramineus* (Lima y Seuánez, 1991), *A. caraya* (Rahn *et al.*, 1996; Mudry *et al.*, 1998) y *A. pigra* (Steinberg *et al.*, 2008), y de tipo pentavalente $X_1X_2X_3Y_1Y_2$ en *A. guariba clamitans* (Steinberg *et al.*, 2017). Del trabajo conjunto con el equipo mexicano se logró la primera descripción del cariotipo del mono aullador negro *A. pigra,* caracterizando un número diploide 2n=58, con un sistema sexual en machos $X_1X_2Y_1Y_2$ (Steinberg *et al.*, 2008). Posteriormente, gracias a una colaboración con la Dra. Vanessa Fortes (Laboratorio de Primatología de la Universidad Federal de Santa María, RS, Brasil), se concretó la caracterización genética del mono aullador marrón *A. guariba clamitans,* que llega en su distribución a la Argentina. El muestreo de esta especie se efectuó en Brasil dado que las poblaciones en Argentina fueran diezmadas por la epidemia de Fiebre Amarilla de 2008, dificultando o incluso impidiendo el acceso a ejemplares en este país. Como resultado de este trabajo colaborativo se logró la primera caracterización mitótica y meiótica de un sistema de determinación sexual masculino de tipo pentavalente $X_1X_2X_3Y_1Y_2$ en una especie de primate (Steinberg *et al.*, 2017).

La realización de estudios comparativos analizando los cariotipos de las especies de aulladores nos permitió proponer hipótesis sobre el posible origen de los sistemas sexuales múltiples en las especies sudamericanas (*A. caraya*, *A. guariba*, *A. sara, A. belzebul*, *A. macconelli*, *A. seniculus, A. arctoidea*) y mesoamericanas (*A. pigra* y *A. palliata*), así como determinar los autosomas involucrados en los reordenamientos que habrían dado origen a los multivalentes sexuales en los aulladores de ambos orígenes americanos (Steinberg *et al.*, 2014a). En las especies sudamericanas, el autosoma involucrado en los sistemas sexuales múltiples posee homeología con los cromosomas humanos 3 y 15, por lo que esta sintenia 3/15 está asociada a los sistemas sexuales en estas especies. El estudio de conservación genómica mediante *FISH* evidenció que la sintenia 3/15 no estaría conservada en las especies mesoamericanas *A. pigra* y *A. palliata,* sino que el par autosómico que dio origen a los sistemas sexuales en estas dos especies posee homeología con el cromosoma humano 7. Este trabajo, junto a un estudio más exhaustivo de las homeologías cromosómicas interespecíficas, nos permitió proponer que los autosomas involucrados en las translocaciones que darían origen a los multivalentes sexuales en las especies sudamericanas y mesoamericanas serían distintos. Del análisis de los datos obtenidos, se postuló un origen independiente para los sistemas sexuales múltiples de estos dos grupos que involucraría dos translocaciones Y-autosoma diferentes. Estos hallazgos brindaron información original sobre la cariología de Ceboidea. En el marco conceptual de "Evidencia Total", el análisis combinado de variables moleculares y cromosómicas resolvió las relaciones de parentesco entre las especies de aulladores de ambos orígenes americanos, demostrando su utilidad en el esclarecimiento de controversias que relacionan la taxonomía y la evolución de los primates ceboideos (Steinberg *et al.*, 2014a).

Otro aporte original al conocimiento resultante de estos estudios comparativos se refiere al estudio meiótico del comportamiento y la dinámica cromosómica en profase I temprana de los sistemas sexuales múltiples en dos especies de monos aulladores, *A. caraya* (2n=52, $X_1X_1X_2X_2/X_1X_2Y_1Y_2$, hembra/macho) y *A. guariba clamitans* (2n=46, $X_1X_1X_2X_2X_3X_3$, hembra/2n=45, $X_1X_2X_3Y_1Y_2$, macho). Del análisis de espermatocitos en los monos aulladores, se observó que los multivalentes sexuales están completamente sinapsados en la profase meiótica I, incluso antes de que los bivalentes autosómicos lo estén; por ello, los cromosomas sexuales muestran un comportamiento meiótico diferencial. En *A. guariba clamitans* se observó al pentavalente sexual totalmente extendido en 70% de los espermatocitos en paquitene temprana, mientras que en *A. caraya* se observó al cuadrivalente sexual extendido en sólo 20% de los espermatocitos (Steinberg *et al.*, 2018a). El cuadrivalente sexual de *A. caraya* muestra un plegamiento sobre sí mismo mucho mayor que el pentavalente de *A. guariba clamitans* (Figura 1A, B). Este patrón estructural podría estar relacionado con el número y localización de los quiasmas, o el número de cromosomas involucrados en el multivalente. En *Cebus cay* (2n=54, XY, macho) se observó una sinapsis incompleta del bivalente correspondiente al par autosómico 11 (que posee una banda de heterocromatina extracentromérica que ocupa 70% de la longitud del brazo q) aún después de la sinapsis total del par sexual XY; este par autósomico resultó ser el último del complemento cromosómico en sinapsar. Estas diferencias entre las dos especies de monos aulladores y el mono caí *C. cay* podrían explicarse por la escasa heterocromatina constitutiva que posee el cariotipo de los aulladores en comparación con la del mono caí. Considerando que se ha propuesto que la heterocromatina constitutiva tiene un papel importante en el emparejamiento de cromosomas homólogos en la meiosis y algunos efectos negativos en el emparejamiento meiótico y entrecruzamiento (Sumner, 2008), la heterocromatina podría retrasar la sinapsis en *C. cay* (Garcia-Cruz *et al.*, 2009, 2011). El estudio del comportamiento meiótico en primates no humanos resulta muy valioso para el avance del conocimiento acerca de las características conservadas a nivel evolutivo interespecífico en primates en general, así como para todo lo referente al manejo y mantenimiento de la biodiversidad en los ambientes que son propios de su distribución geográfica natural. Otro aspecto que

no se puede evitar comentar como faceta de aplicación futura es que, dada la íntima relación evolutiva existente, los conocimientos obtenidos con el desarrollo de trabajos en estos primates tendrán un impacto sobre el conocimiento científico actual y contribuirán a una mejor comprensión del proceso meiótico en humanos. Los resultados obtenidos de las actividades de investigación, junto con los estudios de dinámica meiótica, contribuyen a una mejor comprensión de la compleja evolución de los cromosomas sexuales. La integración de estos conocimientos junto a los logros producto de la aplicación de nuevos abordajes experimentales permite expandir el campo de investigación poco explorado en nuestro país, así como ampliar el conocimiento del proceso meiótico en células eucariontes y aportar información para caracterizar una parte de la variabilidad generada por este proceso de división de las células germinales en mamíferos.

¿QUÉ NUEVOS HALLAZGOS OBTUVIMOS ACERCA DE LA COMPOSICIÓN NUCLEOTÍDICA?

El estudio de la dinámica del genoma, principalmente en especies de los géneros *Alouatta* y *Cebus,* ha permitido avanzar notablemente en los últimos años sobre el conocimiento de la estructura básica del genoma de estos platirrinos, tanto a nivel mitótico como meiótico. Los estudios de composición nucleotídica como aporte de genética citomolecular al más amplio conocimiento de los primates americanos recurren a la aplicación de fluorocromos para analizar contenido, tipificación y distribución de secuencias de pares de bases. De esta manera, el estudio integrando la citogenética clásica y la citogenética molecular brinda nueva información sobre la reorganización de la arquitectura cromosómica y la restructuración cariotípica en las especies de *Cebus* y *Alouatta*, y el papel potencial que podría jugar en la dinámica de los genomas. De la caracterización detallada de la organización de los cromosomas en términos del contenido, distribución y localización de bases nucleotídicas específicas mediante bandas secuenciales fluorescentes (DAPI-CMA_3) en tres especies de aulladores (*Alouatta caraya, A. guariba clamitans y A. pigra*) y en dos especies de monos caí (*Cebus cay* y *C. nigritus*, Figura 1C, D, E) se observó la presencia de bandas cromosómicas ricas en pares de bases G-C y A-T, de diversa localización, telomérica, pericentromérica e intersticial, en cada una de estas especies. Además, se demostró la colocalización de estas bandas fluorescentes con regiones heterocromáticas y regiones organizadoras nucleolares. Estos hallazgos fueron recientemente difundidos en distintos eventos científicos nacionales e internacionales (Steinberg *et al.*, 2018b, 2019, 2020a, 2020b; Nieves *et al.*, 2019). Para los monos caí se propuso la existencia de un patrón general específico para el género con respecto al contenido, distribución y localización de las bases nucleotídicas en los cariotipos de estas especies, mostrando un alto grado de conservación genómica. En los monos aulladores se observó una distribución diferencial de secuencias enriquecidas en G-C (DAPI-negativas/CMA_3-positivas) y en A-T (DAPI-positivas/CMA_3-negativas). El cariotipo del mono aullador negro, *A. pigra*, presentó un mayor número de bandas teloméricas enriquecidas en G-C, mientras que el cariotipo del mono aullador marrón, *A. guariba clamitans*, presentó una proporción mayor de estas bandas en posición pericentromérica. En tanto el cariotipo del mono aullador negro y dorado, *A. caraya*, presentó la mayor proporción de bandas enriquecidas en A-T en posición intersticial. Todos estos resultados nos permiten inferir la posible existencia de patrones de bandas cromosómicas específicos de cada especie. Las diferencias observadas en los patrones de bandas fluorescentes podrían deberse a los reordenamientos cromosómicos ocurridos en los linajes de estas especies de aulladores y a una posterior diversificación en la composición nucleotídica.

Como hemos mencionado en este trabajo, en los últimos años se estudiaron especies de ceboideos en estado silvestre, en cautiverio y en semicautiverio, contrastando la hipótesis de evolución cromosómica como mecanismo explicativo más probable del proceso especiogénico de las formas actuales de Primates Neotropicales. En esta misma línea de pensamiento, en el GIBE se fueron implementando y aplicando nuevos abordajes metodológicos citogenético-moleculares (e.g., *FISH*) para confirmar el estatus de especie relacionando vida silvestre y cautiverio, junto a otros estudios cromosómicos (e.g., hibridación genómica comparativa (*HGC o CGH,* por sus siglas en inglés *Comparative Genomic Hybridization*) orientados a mejorar el conocimiento genético de cada especie caracterizando la plasticidad genómica. En los años más recientes, los trabajos de investigación se centraron en caracterizar la arquitectura genómica somática y de determinación sexual en Ceboidea, tomando la evolución cromosómica como potencial modelo de estudio a nivel de mecanismos involucrados en el proceso especiogénico. Las investigaciones aquí planteadas permitieron mejorar el conocimiento científico actual de la arquitectura cromosómica y la dinámica del genoma en Primates Neotropicales y dilucidar el papel putativo de los cambios cromosómicos (i.e. reordenamientos cromosómicos, diferencias en el número, localización y distribución de secuencias de ADN repetitivas) en el proceso de especiación de este grupo.

De este modo, los estudios citogenético-moleculares ampliaron el espectro de posibles aplicaciones destinadas a profundizar las ya conocidas caracterizaciones de citogenética clásica. En el caso de los primates, donde

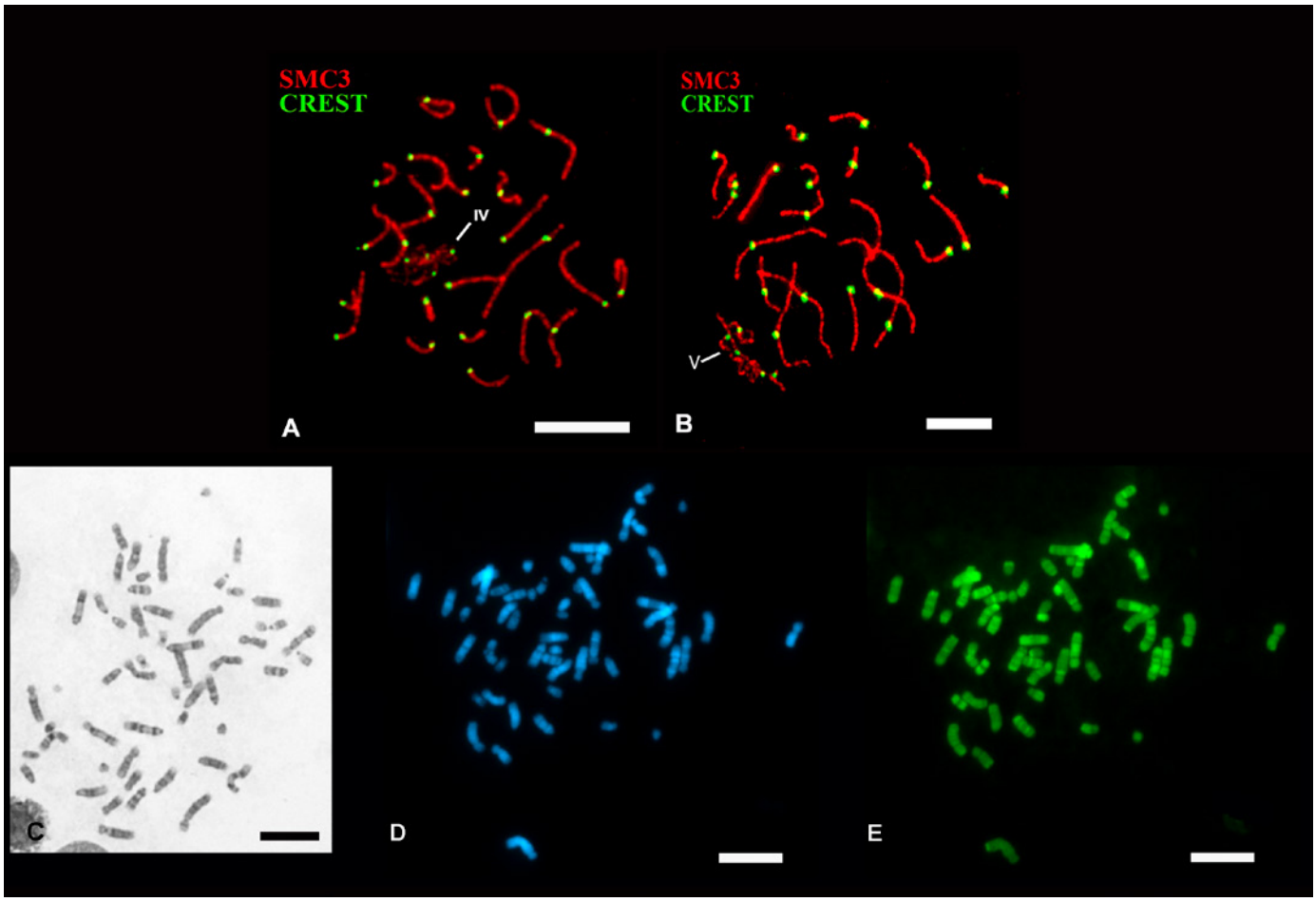

Figura 1. A, B: Inmunodetección en espermatocitos en paquitene tardío en *Alouatta caraya* (A) y *A. guariba clamitans* (B). Rojo: ejes del complejo sinaptonémico (SMC3). Verde: centrómeros (CREST). IV=cuadrivalente, V= pentavalente. Barra= 5 µm. **C, D, E:** Patrones de bandas cromosómicas en preparaciones mitóticas de *Cebus nigritus*. C. Bandas G en blanco y negro. D. DAPI (4',6-diamidino-2-phenylindole) en azul. E. CMA_3 (cromomicina A_3) en verde. Barra= 10 µm.

aún queda mucho camino por recorrer, sigue siendo todo un desafío continuar con las investigaciones sobre el genoma y sus particularidades y con los estudios comparativos a nivel de ambos sexos y de su descendencia, accediendo día a día a nuevos ejemplares ya sea en sus lugares de origen como a ejemplares de exhibición.

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