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Nuclear DNA content in two Sphaeriidae species from Southern Chile (Bivalvia: Veneroidea)

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ABSTRACT

The family Sphaeriidae is a group of cosmopolitan freshwater clams that inhabit temporary and permanent fluvial and lacustrine environments around the world. In Chile, Sphaeriidae is represented by 11 species belonging to the genera *Musculium*, *Pisidium* and *Sphaerium*. In this study, the nuclear DNA content (or 2C-value) of *Musculium argentinum* (d'Orbigny, 1835) and *Pisidium llanquihuense* Ituarte, 1999 was estimated for the first time. *Musculium argentinum* showed a 2C-value of 3.7 ± 0.38 pg, whereas *P. llanquihuense* showed a 2C-value of 3.57 ± 0.36 pg. These 2C-values are within the range previously reported for Sphaeriidae (2.35–5.7 pg), which includes diploids and polyploids, amounting to 13 of the species examined around the world. Of the cytological data available for the family, the nuclear DNA content can have a unifying role in understanding the magnitude of the chromosome polymorphisms within this polyploid complex.

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2C-value; image microdensitometry; *Musculium*; *Pisidium*; polyploidy

Introduction

The family Sphaeriidae is a group of cosmopolitan freshwater clams that first appeared in the Cretaceous, and are presently ubiquitous inhabitants in temporary and permanent fluvial and lacustrine environments around the world. This family includes three genera: *Musculium* Link, 1807 and *Sphaerium* Scopoli, 1777 (considered as closely related); and *Pisidium* Pfeiffer, 1821 (Dreher-Mansur and Meier-Brook 2000). The sphaeriids are small hermaphrodite bivalves (< 30 mm in shell length; Piechocki 1989) with ovoviviparous or viviparous reproduction (Lee 1999; Guralnick 2004; Korniusshin and Glaubrecht 2006; Peredo et al. 2010). Although they are not economically important as an edible species, they play a significant role in the dynamics of nutrient and energy cycles in freshwater bodies (Peredo et al. 2007).

Chilean Sphaeriidae are represented by 11 species belonging to the genera *Musculium*, *Pisidium* and *Sphaerium* (Parada and Peredo 2002, 2006; Ituarte 2007) which are distributed from 18°S to 55°S. The biological framework for Chilean Sphaeriidae has increased during the last few years to include studies on taxonomy, biogeography, population dynamics and reproductive patterns (Ituarte 1999; Sobarzo et al. 2002; Parada and Peredo 2002, 2006; Peredo et al. 2007, 2009, 2010; Parada et al. 2009; Jara-Seguel et al. 2011). These data are additional to those reported for the northern hemisphere species which include a spectrum of biological data (Herrington 1962; Heard 1965; Holopainen and Hanski 1986; López and Holopainen 1987; Kuiper et al. 1989; Barsiené et al.

1996; Burch et al. 1998; Araujo and Ramos 1999; Guralnick 2004; Korniusshin and Glaubrecht 2006; Funk and Reckendorfer 2008). Despite all this knowledge, genome data are scarce for Sphaeriidae, and include only cytogenetic descriptions (for 27 species) and DNA sequencing used for phylogenetic reconstructions (approximately for 40 species) (Cooley and Ó Foighil 2000; Lee and Ó Foighil 2002, 2003). However, not all species of the family with available cytogenetic data have been included in phylogenetic studies, nor are their genomic sizes known. On the other hand, genetic studies based on electrophoretic analyses of enzymatic systems have been published previously in the 1970s and 1980s, and were focused on answering some questions related to environmental heterogeneity and its effects on levels of polymorphism in the populations (Hornbach et al. 1978, 1980a, 1980b; Way et al. 1980; McLeod et al. 1981).

Currently, data on chromosome numbers are available for 27 species of Sphaeriidae and estimations on nuclear DNA content (or 2C-values) have been reported for 11 of them (Lee 1999; Jara-Seguel et al. 2005, 2010; Petkeviciute et al. 2006; Kořínková and Morávková 2010; Kořínková and Král 2011). Interestingly, only four species belonging to the genus *Sphaerium* so far studied are diploids and their 2n number counts are 30 and 36 for *S. corneum* (Linnaeus, 1788), 30 for *S. nitidum* Westerlund, 1876 and *S. nucleus* (Studer, 1820), and 44 for *S. rhomboideum* (Say, 1822) (summarised by Kořínková and Morávková 2010). The other 22 species included within the genera *Sphaerium*,

Table 1. Collection sites and results of Feulgen microdensitometry on epithelial gill cell nuclei of *Musculium argentinum*, *Pisidium llanquihuense* and the standard *Oncorhynchus mykiss*.

Species	Collection sites	Mean IOD (au)	2C-value (pg)
<i>Musculium argentinum</i> (d'Orbigny, 1835)	Lateral pond to Cautín River, Lautaro locality, Southern Chile (38°32' S, 72°25' W)	7.7 ± 0.80	3.7 ± 0.38
<i>Pisidium llanquihuense</i> Ituarte, 1999	El Moro stream, tributary of the Llanquigüe Lake, Southern Chile (40°38' S, 72°31' W)	7.27 ± 0.74	3.57 ± 0.36
<i>Oncorhynchus mykiss</i> (Walbaum, 1792)	Culture hatchery, Universidad Católica de Temuco, Chile	11.3 ± 1.00	5.5

IOD—integrated optical density; au—arbitrary units; pg—picograms.

Musculium and *Pisidium* are polyploids with 2n numbers from c. 100 up to 247 (Lee 1999; Park et al. 2002; Jara-Seguel et al. 2005; Petkeviciute et al. 2006; Kořínková and Morávková 2010). For Chilean taxa, chromosome counts have been previously documented for *M. argentinum* (d'Orbigny, 1835) (2n = c. 130) (Jara-Seguel et al. 2005), whereas for *Pisidium chilense* (d'Orbigny, 1846) and *P. huillichum* Ituarte, 1999 the studies have focused on determining 2C-values. For these species, chromosomes have been preliminary counted with diploid (2n) numbers within the polyploid range (2n > 100; Jara-Seguel et al. 2010). Thus, cytogenetic data are available for three Sphaeriidae species from Chile, being the only records for taxa from the southern hemisphere. In general, the cytological data available for Sphaeriidae show a high intraspecific and interspecific variation in their 2n number and ploidy level. The 2n number varies approximately 8.2-fold among species (range of 2n between 30 and 247), whereas ploidy level varies from 2n to 13n. Intraspecific variation in 2n number has been described for *P. amnicum* (O.F. Müller, 1774) (2n = 214, 230), *P. casertanum* (Poli, 1791) (2n = 150, 180, 190, 200) and *P. obtusale* (Lamarck, 1818) (2n = 200, 204, 210, 220) for which the same cytological method has been performed. In these cases it is possible that the intraspecific variation is a result of technical artefacts due to incomplete counting or poorly spread mitotic plates, as discussed by Kořínková and Morávková (2010). On the other hand, at interspecific level, 2C-values do not vary in the same magnitude as 2n numbers, with a range between 2.35 and 5.7 pg (c. 2.4-fold) (Kořínková and Morávková 2010; Jara-Seguel et al. 2010).

The goal of this study was to estimate for the first time the nuclear DNA content of *M. argentinum* and *P. llanquihuense* Ituarte, 1999, thus testing if the uniformity in 2C-values so far described within Sphaeriidae is present in these other two species from Southern Chile. *Musculium argentinum* has a wide distribution inhabiting from 38°S to 41°S in Chile and part of Argentina, whereas *P. llanquihuense*, has a narrow distribution occurring from 40°S to 41°S in Southern Chile (Parada and Peredo 2002, 2006; Parada et al. 2009).

Materials and methods

Individuals of *M. argentinum* and *P. llanquihuense* were collected in freshwater bodies from Southern Chile

(Table 1). The shell length of the studied individuals was between 5 and 10 mm.

Measurements of nuclear DNA content (2C-values) were done by Feulgen image microdensitometry in epithelial gill cells obtained from adult specimens, using the software Image Pro-Plus 4.0 (© Media Cybernetic 1993–1998). To estimate DNA content, the cytological and microdensitometrical method described by Jara-Seguel et al. (2010) for *Pisidium* species was followed. The epithelial gill cells of *M. argentinum* and *P. llanquihuense* were spread on slides, air-dried, fixed in methanol–glacial acetic acid (3:1, v/v) at 4 °C for 24 h and stained with the Feulgen reaction (hydrolysis with 5N HCl for 60 min at room temperature, staining with Schiff's reagent for 60 min, followed by three washes of 5 min each in H₂SO₄). The software captures black and white images from the Nikon Eclipse 400 microscope connected to a CCD COHU camera, and analyses the different structures visible on the images. Nuclear optical density (OD) is calculated by the software according to the formula:

$$OD = \log_{10}(1/T) = -\log_{10}T$$

where T = intensity of transmitted light/intensity of incident light. From this estimation, the computer integrates the values of OD obtained for each one of the pixels and calculates the integrated optical density (IOD = ΣOD). For *M. argentinum* and *P. llanquihuense* IOD values of 100 individual nuclei of epithelial gill cells were determined by the software. The IOD values were converted to absolute mass of DNA by simultaneous comparison with erythrocyte smears of rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) (2C = 5.5 pg, 2n = 58–62; Hartley and Horne 1985). The standard erythrocyte smears of *O. mykiss* were included in the same staining runs and IOD estimations as the cells of *M. argentinum* and *P. llanquihuense*. The 2C-value was determined using the equation:

$$CV_u = CV_s \times (IOD_u/IOD_s)$$

where CV_u = 2C-value of *Musculium* or *Pisidium*, CV_s = 2C value of standard, IOD_u = mean IOD of *Musculium* or *Pisidium*, IOD_s = mean IOD of standard.

Results

Musculium argentinum showed a 2C-value of 3.7 ± 0.38 pg, whereas *P. llanquihuense* had a 2C-value of

3.57 ± 0.36 pg. The coefficient of variation in both cases was CV = 10% ($n = 100$ cells). In the case of the erythrocytes of *O. mykiss* used as standard, the coefficient of variation was CV = 8.4% ($n = 100$ cells). The 2C-values of DNA estimated in nuclei of gill epithelial cells for both species are shown in Table 1. These data on 2C-values increase to four the Chilean species studied, amounting to a total of 13 species of the family with known nuclear DNA content worldwide.

Discussion

The two Chilean Sphaeriidae species studied here showed 2C-values within the range previously documented for polyploid co-familial taxa of *Pisidium* from Chile (2C = 2.35 and 4.04 pg; $2n > 100$) using the same microdensitometry method (Jara-Seguel et al. 2010). The coefficient of variation was 10% in both species, which is acceptable for the cell type and for the microdensitometrical method used here (see Hardie et al. 2002). In the case of *P. llanquihuense* examined in this study, accurate chromosome counts to elucidate its ploidy level were not made because early embryos are required for that analysis (Jara-Seguel et al. 2005, 2010).

The cytogenetic data available for Chilean Sphaeriidae include 2C-values for four species and the chromosome number for three of them, covering 36% of the sphaeriids that inhabit Chile. This is the only cytogenetic data available for southern hemisphere taxa of the family. In the case of northern hemisphere sphaeriids, nine species from the Czech Republic have been studied (Korínková and Moránková 2010) estimating 2C-values from mean flow cytometry of cells obtained from the foot muscle. In this study, the range of the coefficient of variation for all species studied was wide, varying between 1.9% and 24%, using nuclei of human leukocytes as a standard. Both methods, image microdensitometry and flow cytometry, have been extensively used to estimate 2C-values in plants (Vilhar et al. 2001; Bennett and Leitch 2005) as well as in animals of different groups (e. g., bivalve and gastropod molluscs, crustaceans; Méndez et al. 2001; Gregory 2003; Pascoe et al. 2004; Jeffery and Gregory 2014). An advantage of the microdensitometry method used in this work is the ability to choose the nuclei using features such as shape, size and stain intensity (Méndez et al. 2001; Hardie et al. 2002) In bivalve molluscs, 50–160 cells have been found to be an adequate number to make measurements (Gregory 2003; Pascoe et al. 2004). The principal tissues used in aquatic animals have been gills and haemocytes, with chicken and trout erythrocytes the most used as standards (see Gregory 2003; Jeffery and Gregory 2014). On the basis of the results obtained using image microdensitometry and flow cytometry, and

to understand the variation, species of *Sphaerium*, *Musculium* and *Pisidium* can be segregated according to their ploidy levels. Thus, the two diploid species (*S. corneum* $2n = 30$, and *S. nucleus* $2n = 36$) showed 2C-values of 5.22 and 5.10 pg, respectively, whereas within the polyploids two groups of species were found: those whose 2C-values were higher than the diploids ranging from 5.30 to 5.70 pg (*P. obtusale*, *S. rivicola* [Lamarck, 1818] and *M. lacustre* [O.F. Müller, 1774]; $2n = 210$ – 240 chromosomes) and those whose 2C-values were lower than the diploids ranging from 4.0 to 5.10 pg (*P. amnicum*, *P. supinum* A. Schmidt, 1851, *P. casertanum* and *P. personatum* Malm, 1855; $2n = 160$ – 230 chromosomes) (Korínková and Moránková 2010). For the Chilean species so far studied, the estimated 2C-values (range between 2.35 to 3.7 pg) are lower than those described for species from the Czech Republic (Korínková and Moránková 2010), except *Pisidium chilense* (2C = 4.04 pg) (Jara-Seguel et al. 2010) which falls within the range of the last polyploid group mentioned above, that includes four *Pisidium* species. Genome mechanisms such as DNA elimination, chromosome packing and/or variation in content of constitutive heterochromatin have been previously discussed to explain the differences in 2C-values observed among Chilean species (Jara-Seguel et al. 2010), which may also explain the differences observed when compared with European species. In addition, lower 2C-values given for some Chilean species may be related to differences in chromosome number (or ploidy level) and size. For this reason accurate chromosome studies are required for Chilean species including those taxa for which 2C-values have been previously estimated, but $2n$ numbers have not been exactly counted. Furthermore, within this available dataset for Sphaeriidae it is remarkable that the 2C-values of the closely-related polyploid species *S. rivicola* (2C = 5.7 pg), *M. lacustre* (2C = 5.3 pg) and *M. argentinum* (2C = 3.7 pg) are close to the estimated value for the diploids *S. corneum* and *S. nucleus*, not showing a significant increment as may be expected according to evidence described for some taxa of plants and animals in which a polyploid complex has been described. In such cases, the polyploids have significantly increased the nuclear DNA content compared to their close diploid relatives, thus showing an evolutionary tendency in which the diploidy is the basal level to initiate higher ploidy levels with high DNA content in derived species (Gallardo et al. 1999; Gregory and Hebert 1999; Soltis and Soltis 2000; Leitch and Leitch 2008). Similarly, in induced polyploids of marine bivalves used in aquaculture production the new tetraploid cytotypes double in both $2n$ number and 2C-value compared to the original diploid cytotypes (Méndez et al. 2001; Thirirot-Quievréux 2002). Compared to the

diploids of the *Sphaerium* genus the *Pisidium* species do not show variation in 2C-values.

All the cytogenetic characteristics so far described for Sphaeriidae give evidence of the complexity and dynamics of their genomes, which may be the product of an evolutionary process that occurred over a long timespan (since the Cretaceous). Regarding the complexity of the genomes, the current evidence based upon molecular analysis suggests polyploidy (auto or allopolyploidy) as the evolutionary mechanism occurred in the different lineages within Sphaeriidae (Lee & Ó Foighil 2002). Besides, the chromosome rearrangements (centric fissions) described in some sphaeriid species may also be part of the process of adjustment of the genomes (Petkeviciute et al. 2006; Korínková and Moránková 2010), being accompanied by a drastic reduction of the chromosome sizes such as has been evidenced in the current polyploids where the largest pairs do not exceed 1–2 µm in length compared with the diploids in which the largest pairs are 10.6–13 µm in length (Petkeviciute et al. 2006; Jara-Seguel et al. 2010; Kořínková and Moránková 2010). The reduction in chromosome size in polyploids may involve DNA sequence elimination, thus resulting in 2C-values near to diploids as revealed by the available data for the 13 species so far studied (Jara-Seguel et al. 2010; Korínková and Moránková 2010). With regard to genome functionality, cytological observations have described strict bivalent formation and disomic segregation in meiosis of polyploid species showing evidence of genome stabilisation of the divisional dynamic in germ cells (Lee 1999; Park et al. 2002). In addition, the existence of disomic segregation of allozymic loci is also possible, based upon previous studies using allozyme electrophoresis carried out in *Sphaerium striatinum* (Hornbach et al. 1978), a species later described as polyploid ($2n = 152$; Lee 1999). Such structural and functional genome modifications (including loss of DNA) have been described in polyploid animals (allopolyploids) using cytological and molecular evidence (Köhler et al. 2000; Petrov 2002; Gallardo et al. 2006), and many cases have also been documented for various groups of plants (Adams et al. 2003; Soltis et al. 2004; Adams and Wendel 2005). However, many of the genomic mechanisms which occurred during the evolution of Sphaeriidae are still unknown and additional research is necessary, using conventional and/or molecular cytogenetic methods (e. g., C-banding heterochromatin, FISH, GISH, sequencing of satellite DNA), thus supplying important data to help solve this interesting enigma on the genome evolution of this diverse family. Therefore, the diploid species *S. corneum* and *S. nucleus* are nested within the otherwise polyploid species in molecular phylogenetic trees, which suggests either multiple origins of polyploidy or two, independent reversions to diploidy from polyploid

ancestors (Cummings and Graf 2010). Molecular phylogenetic studies have not, however, included all the species whose 2C-values have been described (e.g., the three *Pisidium* species from Chile, as well as *P. obtusale* and *S. rivicola* from the Czech Republic), so new insights on ancient or neopolyploidy, as well as tendencies in variation of genome size, cannot be robustly envisaged. Finally, due to its low variation at interspecific level compared to the high variation observed in 2n numbers, nuclear DNA C-values can be included in attempts to understand the magnitude of the chromosome changes (e.g., Robertsonian translocations, inversions, polyploidisation) which have occurred among species within this polyploid complex.

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Disclosure statement

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