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Analysis of the extranucleolar ribonucleoprotein particles of *Cycas revoluta* Thunb. (Cycadaceae) and *Ceratozamia mexicana* Brongn. (Zamiaceae)

Análisis de las partículas ribonucleoproteicas extranucleolares de *Cycas revoluta* Thunb. (Cycadaceae) y *Ceratozamia mexicana* Brongn. (Zamiaceae)

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Abstract

Background: Nuclear ribonucleoprotein particles play a key role in RNA processing and in the gene expression pathway. Interchromatin granules (GICs) involved in the metabolism of pre-messenger RNA (pre-mRNA) were described in *Allium cepa* and *Chiranthodendron pentadactylon*. Other particles as *Lacandonia* granules (LGs) were found in *Lacandonia schismatica* as well as *Ginkgo biloba* and *Welwitschia mirabilis*. LGs are structures equivalent to perichromatin granules (PCGs) described in mammals and to Balbiani ring granules (BRGs) described in the midge *Chironomus tentans*. PCGs and BRGs are involved in the metabolism of messenger RNA (mRNA). Here, we analyze the extranucleolar particles from *Cycas revoluta* and *Ceratozamia mexicana* and compare them to GICs and LGs using conventional electron microscopy and atomic force microscopy.
Species study: *Cycas revoluta* (Cycadaceae) and *Ceratozamia mexicana* (Zamiaceae)
Hypothesis: The extranucleolar ribonucleoprotein particles in the nuclei of *C. revoluta* and *C. mexicana* are equivalent to GICs or GLs.
Methods: Fragments of young leaves of *C. revoluta* and *C. mexicana* were processed for standard transmission electron microscopy. Thin sections were stained with the EDTA technique preferential for ribonucleoproteins and osmium amine specific for DNA. From the semithin sections the samples were studied with the AFM and images of them were obtained.
Results: Ribonucleoprotein particles 32 nm in diameter are present in the interchromatin and perichromatin space in *C. revoluta* and *C. mexicana*.
Conclusion: Ribonucleoprotein particles present in the cell nuclei of *C. mexicana* and *C. revoluta* are ultrastructurally equivalent to *LGs*.
Keywords: Atomic force microscopy, cell nucleus, Cycads, ribonucleoprotein particles, *Lacandonia* granules.

Resumen

Antecedentes: Las partículas ribonucleoproteicas nucleares desempeñan un papel clave en el procesamiento del RNA en la vía de la expresión génica. En *Allium cepa* y *Chiranthodendron pentadactylon* se describieron los gránulos intercromatinianos (GICs) involucrados en el metabolismo del RNA pre-mensajero (pre-mRNA). En *Lacandonia schismatica*, *Ginkgo biloba* y *Welwitschia mirabilis* se encontraron partículas conocidas como gránulos de *Lacandonia* (LGs), equivalentes a los gránulos pericromatinianos (PCGs) de mamíferos y del mosquito *Chironomus tentans* (BRGs). Los PCGs y los BRGs están involucrados en el metabolismo del RNA mensajero (mRNA). En este trabajo analizamos las partículas extranucleolares de *Cycas revoluta* y *Ceratozamia mexicana* y las comparamos con los GICs y LGs usando microscopía electrónica y de fuerza atómica.
Especies de estudio: *Cycas revoluta* (Cycadaceae) y *Ceratozamia mexicana* (Zamiaceae)
Hipótesis: Las partículas ribonucleoproteicas extranucleolares presentes en los núcleos de *C. revoluta* y *C. mexicana* son equivalentes a los GICs o a los GLs.
Métodos: Se procesaron fragmentos de hojas jóvenes de *C. revoluta* y *C. mexicana* para microscopía electrónica. Algunos cortes se contrastaron con la técnica de EDTA para ribonucleoproteínas y otros con la técnica de amina osmio para DNA. Algunos cortes semifinos se observaron con el microscopio de fuerza atómica y otros con el microscopio óptico.
Resultados: En *C. revoluta* y *C. mexicana* se presentan partículas ribonucleoproteicas de 32 nm de diámetro en el espacio intercromatiniano y periocromatiniano.
Conclusión: Las ribonucleoproteínas presentes en el núcleo celular de *C. mexicana* y *C. revoluta* son equivalentes ultraestructuralmente a los gránulos de *Lacandonia*.
Palabras clave: microscopía de fuerza atómica, núcleo celular, Cicadas, partículas ribonucleproteicas, gránulos de *Lacandonia.*

Living gymnosperms comprise four distinct lineages, Ginkgo, Gnetophytes, Cycads, and conifers (Forest *et al*. 2018). Cycads (Gymnospermeae) are among the most ancient dioecious seed plants on Earth, with a fossil history dating back to the Permian and perhaps the Carboniferous (Mamay 1976, Delevoryas 1982, Norstog & Nicholls 1997, Vovides *et al*. 2003). To date, some efforts to characterize the cell biology of this group have been made. For example, the ovule and seed development of *Encephalartos natalensis* and the involvement of the endoplasmic reticulum in this process have been analyzed using light and electron microscopy (Woodenberg *et al*. 2010). Embryo development and germination of *Cycas* were also studied (Dehgan & Schutzman 1989). Some ultrastructural studies have been carried out on the chloroplasts and chromoplasts of some Cycads (Sun 1964, Whatley 1985, Morassi-Bonzi *et al*. 1992, Zuo *et al*. 2004), and roots contraction in *Cycas* and *Zamia* has also been studied (Tomlinson *et al*. 2014). However, to our knowledge, no studies of the extranucleolar ribonucleoprotein (RNPs) particles of the cell nucleus of cycads have been conducted.

Ultrastructural studies using standard electron microscopy have described that other gymnosperms, like *G. biloba* and *W. mirabilis*, contain *Lacandonia* granules (LGs) previously described and characterized in some angiosperms, like *L. schismatica* and *T. brevistylis*. LGs in all these species are ribonucleoprotein particles that share the same pattern of distribution, the same diameter (~32 nm), and all of them maintain a physical connection with fibers forming a fibrogranular arrangement (Jiménez-García *et al*. 1992, Agredano-Moreno *et al.* 1994, 2000, 2018, Jiménez-Ramírez *et al.* 2002). In the present work, we analyze the extranucleolar particles of two species of cycads: *Cycas revoluta* Thunb. and *Ceratozamia mexicana* Brongn. and compare them to interchromatin granules (GICs) and *Lacandonia* granules (LGs). GICs are 20–25 nm in diameter ribonucleoprotein particles located in the interchromatin space of cell nuclei forming clumps. They were first described in the mammal cell nucleus (Swift 1959, Monneron & Bernhard 1969) and later in *Allium cepa* and *Chiranthodendron pentadactylon*, where they are scattered and disperse (Medina *et al.* 1989, Echeverría *et al*. 1999). These particles are involved in the metabolism of pre-messenger RNA (pre-mRNA) and are proposed as reservoir sites of splicing factors (Spector 1993). LGs are 32 nm ribonucleoprotein particles intermediate in size to GICs and perichromatin granules (PCGs) and Balbiani ring granules (BRGs) present in mammals and in the salivary glands of *Chironomus tentans*, respectively. PCGs (30-50 nm in diameter) and BRGs (40-50 nm) are involved in the metabolism of messenger RNA (mRNA). LGs contain SR proteins and poly(A)$^+$RNA and are equivalent structures to PCGs (Agredano-Moreno & Jiménez-García 2000). These granules were first described in *Lacandonia schismatica* and *Triuris brevistylis* (Jiménez-García *et al*. 1992), and later they were found in some ancient gymnosperms, such as *G. biloba* and *W. mirabilis*, as well as in some bryophytes (Jiménez-Ramírez *et al*. 2002, Alonso-Murillo & Jiménez-García 2015, Agredano-Moreno *et al*. 2018).

Materials and methods

Transmission Electron Microscopy. Samples (1 mm^3) of *C. revoluta* and *C. mexicana* young leaves were processed for standard electron microscopy (Jiménez-García & Segura-Valdez 2004). Briefly, fragments of leaves were fixed overnight at room temperature in a mixture of 6 % glutaraldehyde and 4 % paraformaldehyde in PBS (pH 7.2). Samples were post-fixed with 2 % osmic acid overnight and dehydrated in a graded series of ethanol, and finally embedded in an epoxy resin at 60 °C for 48 h. Sections (40-50 nm) were placed on copper grids covered with formvar. The contrast was conducted with 5 % uranyl acetate and 0.5 % lead citrate. Grids were observed with a transmission electron microscope (JEOL 1010 model: JEOL, Peabody, MA, USA) operating at 80 kV. The images were captured with a CCD300-RC camera (DAGE-MTI, Michigan City, IN, USA) coupled to the microscope. The electron micrographs of sections stained with the EDTA technique were taken at 100,000X.

EDTA staining for RNPs. Sections of 40-60 nm were stained with Bernhard's EDTA technique, preferential for ribonucleoproteins (RNPs) (Bernhard 1969). Briefly, 5 % uranyl acetate was used for 3 min, followed by treatment with EDTA for 13 min and 0.5 % lead citrate for 3 min.

Osmium amine. Specific staining of DNA in the cell nucleus of *C. revoluta* and *C. mexicana* was performed according to Vázquez-Nin *et al*. (1995) with modifications. Briefly, 60-90 nm sections mounted in gold grids without formvar were floated on a drop of 5 N HCl for 1 h at room temperature (acid hydrolysis). Grids were rinsed with deionized water and incubated in a wet chamber containing a drop of osmium amine solution. Finally, grids were washed and observed with an electron microscope without additional staining.

Light microscopy. Sections (300-350 nm) were stained with toluidine blue, and 100X pictures were taken in brightfield illumination with an optical microscope (Nikon, Eclipse E800). Images were taken with a digital camera CCD (3CCD, MTI) attached to the microscope and analyzed with the FlashPoint 3D FPJ program.

Atomic force microscopy. Atomic force microscopy was conducted as previously described (Jiménez-García & Segura-Valdez 2004, Segura-Valdez *et al*. 2010). Briefly, semithin sections about 300 nm thickness were mounted on glass slides and observed with an atomic force microscope (model BioScope, Digital Instruments, Santa Barbara CA, USA) working in contact mode. The scan size was from 30 to 100 µm at a scan rate of 2.1 Hz. Images from the microscope were produced with the software NanoScope IIIa control system. The AFM tips were silicon nitride tips with a curvature radius of 20-60 nm (model NP).

Results

The cell nucleus of *C. revoluta* and *C. mexicana* was analyzed with light, atomic force, and transmission electron microscopy (Figure 1A-F).

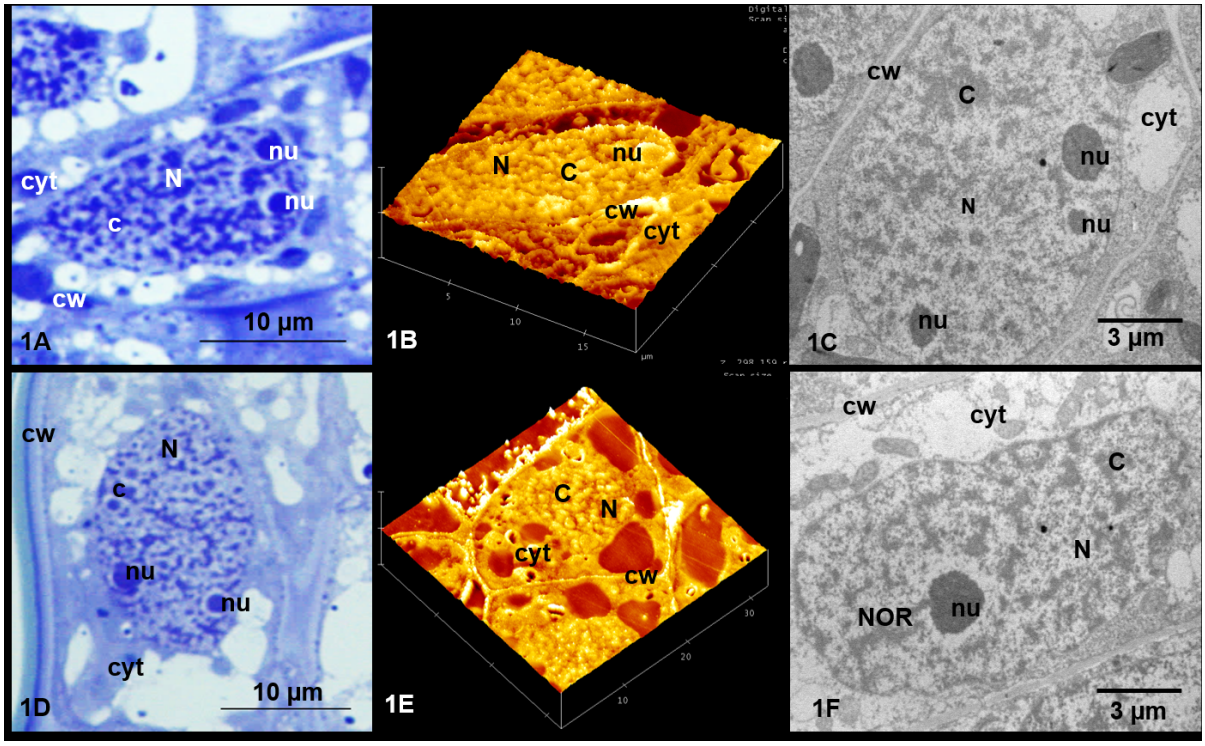

Figure 1. *C. revoluta* (1A-C) and *C. mexicana* (1D-F) cell nuclei. Light micrographs of a cell nucleus of *C. revoluta* and *C. mexicana*, respectively (1A, 1D), stained with toluidine blue. Three-dimensional arrangement of strands inside the nuclei of both species observed with atomic force microscopy (1B, 1E). Conventional staining for TEM showing the strands densely stained (1c, 1f). Nucleus (N), strands of chromatin (C), cytoplasm (cyt), and cell wall (cw).

Cell nucleus structure. In both species of cycads it is observed that the nuclei are elongated, also containing two compact nucleoli (Figure 1A, C, D). Toluidine blue staining showed strands of dense material extended inside the nucleus of *C. revoluta* and *C. mexicana* (Figure 1A, D). The atomic force microscope confirmed the presence of these strands and showed their three-dimensional arrangement (Figure 1B, E). Conventional staining for transmission electron microscopy demonstrated the reticulated arrangement of the strands heavily stained with uranyl acetate and lead citrate in both species (Figures 1C, F). To determine whether the reticulated strands observed in the nuclei of *C. revoluta* and *C. mexicana* correspond to DNA, the osmium amine technique specific for DNA was applied. This compound densely stained the strands present in the nuclei of *C. revoluta* and *C. mexicana* showing that they correspond to DNA (Figure 2A, B). Little or no DNA was present in the nucleoplasm of these species. Although the osmium ammine staining allowed the specific detection of DNA, we noted that the nucleolus of both species showed a slight contrast. However, the staining density was much less compared to chromatin strands and the clumps of chromatin in the nucleolus of *C. revoluta* (Figure 2A).

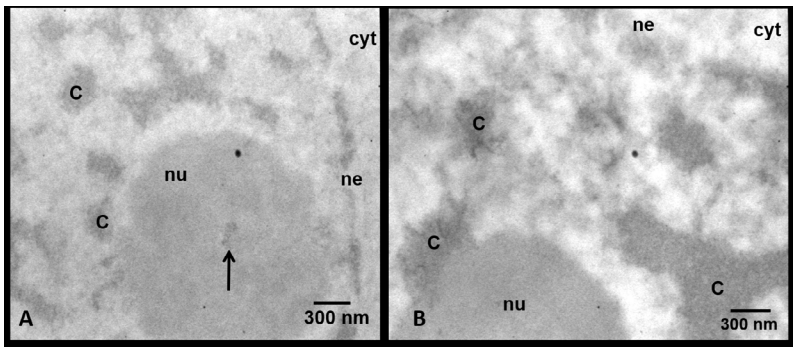

Figure 2. Nuclei of *C. revoluta* (A) and *C. mexicana* (B) stained with osmium amine specific for DNA. In both species, dense strands stained with osmium amine are observed (C). A small clump of DNA is observed in the nucleolus of *C. revoluta* (arrow). Nucleolus (nu), cytoplasm (cyt). Magnification, 30,000X.

Extranucleolar ribonucleoprotein particles. In the cell nuclei of *C. revoluta* and *C. mexicana*, we observed abundant extranucleolar particles between DNA fibers (Figure 3A, B). Different fields of the perichromatin and interchromatin space were observed at high magnification showing that these particles are almost round structures higher in size than ribosomes in the cytoplasm (Figure 4A, B). The distribution and abundance of these granules in *C. revoluta* and *C. mexicana* were similar to that of LGs. When we applied the EDTA technique preferential for RNPs to thin sections of leaves of *C. revoluta* and *C. mexicana*, chromatin strands were observed with low contrast, and the granules and fibers were observed heavily stained, indicating that the particles of *C. revoluta* and *C. mexicana* (Fig 5A, B) are ribonucleoproteins. Inside the granules, slight differences of staining intensity were observed. Most of the granules showed physical association with less contrasted fibers of different thickness (Figures 5A-B). The measurement of *C. revoluta* and *C. mexicana* particles showed that they are 32.20 ± 1.25 nm and 32.5 ± 1.55 nm in diameter, respectively.

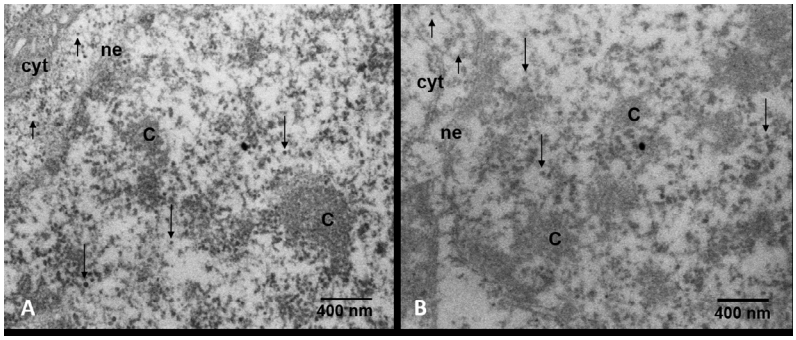

Figure 3. Cell nucleus of *C. revoluta* (A) and *C. mexicana* (B), stained with uranyl acetate and lead citrate. A fibrogranular arrangement is observed in the interchromatin space (large arrows). In the cytoplasm (cyt), ribosomes are observed (small arrows). Chromatin (C), nuclear envelope (ne). Magnification, 30,000X.

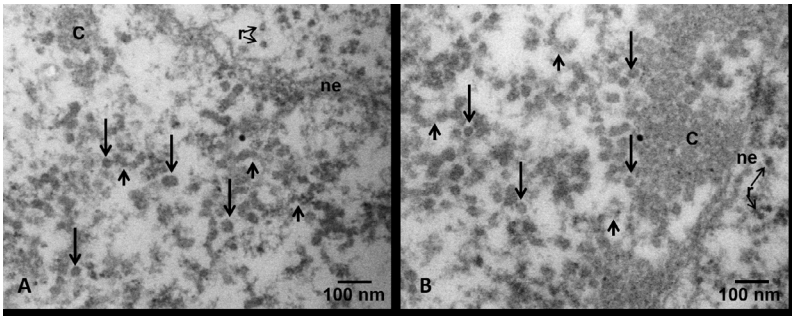

Figure 4. Cell nucleus of *C. revoluta* (A) and *C. mexicana* (B), stained with uranyl acetate and lead citrate. Electron dense granules (large arrows) in the nucleus of *C. revoluta* (A) and *C. mexicana* (B) are higher than ribosomes (signaled with r and small arrows) in the cytoplasm. Granules are in close association with fibers (small arrows). Nuclear envelope (ne). Magnification, 80,000X.

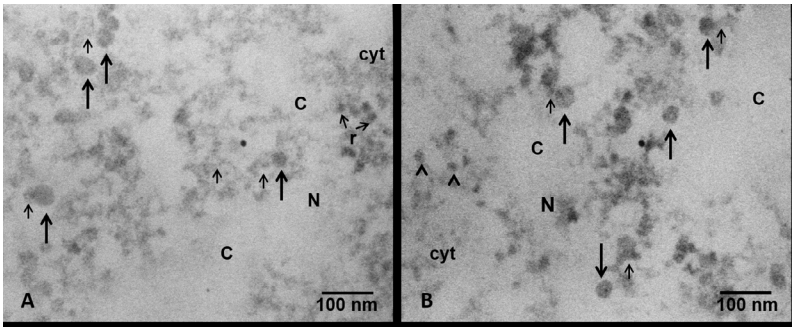

Figure 5. *Lacandonia* granules (large arrows) in a cell nucleus of *C. revoluta* (A) and *C. mexicana* (B), stained with the EDTA technique preferential for RNPs. Granules are in close association with fibers (small and thin arrows). Chromatin (C) is bleached. Ribosomes are marked with arrows in the cytoplasm (cyt). Magnification, 120,000X.

Discussion

The cytochemical staining methods for electron microscopy have been a powerful tool for studying the ultrastructure of ribonucleoprotein particles in animal and plant cell nuclei (Bernhard 1969, Monneron & Bernhard 1969, Vázquez-Nin & Bernhard 1971, Echeverría *et al*. 1999, Segura-Valdez *et al*. 2020). The use of these approaches has allowed us to determine that the fine structure and cytochemical features of RNPs in the interphase nucleus of animal and plant cells are well conserved (Jiménez-García *et al*. 1989).

To date, two types of RNPs particles, have been identified in plants using cytochemical techniques for electron microscopy: ICGs described in some angiosperms (Medina *et al*. 1989, Echeverría *et al*. 1999) and LGs present in the cell nuclei of *L. schsimatica*, *G. biloba*, *W. mirabilis* and some bryophytes (Jiménez-García *et al*. 1992, Alonso-Murillo & Jiménez-García 2015, Jiménez-Ramírez *et al*. 2002, Agredano-Moreno *et al*. 2018). In the present work we found that extranucleolar particles in the cell nuclei of *C. revoluta* and *C. mexicana* (32.20 ± 1.25 nm and 32.5 ± 1.55 nm in diameter respectively) are equivalent to LGs.

The finding of LGs in *C. revoluta* and *C. mexicana* coupled with the discovery of these RNPs in *G. biloba* and *W. mirabilis*, suggest that the presence and ultrastructural features of these extranucleolar particles such as size, distribution, physical association with fibers and ribonucleoprotein content is well conserved in these ancient species, representative of three of the four major groups of gymnosperms: Cycadophyta, Ginkophyta and Gnetophyta. However, further studies are required to know if LGs are also present in species of the fourth member of the gymnosperms, it is to say Coniferophyta. The knowledge of the of extranucleolar ribonucleoprotein particles ultrastructure of representative species of gymnosperms will contribute to a better understanding of these particles in this group.

Cytochemical techniques are an excellent approach to evaluate ultrastructural relationships between the cell nucleus of some angiosperms (Medina *et al*. 1989, Jiménez-García *et al.* 1992, Echeverría *et al*. 1999) and ancient species of gymnosperms and some bryophytes (Jiménez-García *et al*. 1992, Jiménez-Ramírez *et al*. 2002, Alonso-Murillo & Jiménez-García 2015, Agredano-Moreno *et al*. 2018).

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