ANALYSIS OF THE EXTRANUCLEOLAR RIBONUCLEOPROTEIN PARTICLES OF CYCAS REVOLUTA THUNB. (CYCADACEAE) AND CERATOZAMIA MEXICANA BRONGN. (ZAMIACEAE)

Abstract

Background: Nuclear ribonucleoprotein particles play a key role in RNA processing and in the gene expression pathway. Interchromatin granules (IGCs) involved in the metabolism of pre-messenger RNA (pre-mRNA) were described in Allium cepa and Chiranthodendron pentadactylon. Other particles as Lacandona granules (LGs) were found in Lacandona 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 Cyas 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. Some particles are equivalent ultrastructurally to LGs. Ribonucleoprotein particles present in the cell nuclei of C. mexicana and C. revoluta are ultrastructurally equivalent to LGs.

Keywords: Atomic force microscopy, cell nucleus, C. revoluta, C. mexicana, PCGs, BRGs, LGs, GICs, DNA, RNA.
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 mm³) 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 form-var 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](image_url) **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).
Nuclear particles of *Cycas revoluta* and *Ceratozamia mexicana*

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 $32.20 \pm 1.25$ nm and $32.5 \pm 1.55$ 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.](#)
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. schsimatorca, 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).

Acknowledgments

This work was partially supported by Dirección General de Asuntos del Personal Académico-Universidad Nacional Autónoma de México-Programas institucionales de apoyo e impulso a la investigación y a la docencia (DGAPA-UNAM-PAPIIT IN217917, PAPIME PE213916).

Literature cited

Agredano-Moreno LT, Jiménez-García LF. 2000. New evidence that Lacandonia granules are ultrastructurally related to perichromatin and Balbiani ring granules. Biology of the Cell 92:71-78. DOI: https://doi.org/10.1016/S0248-4900(00)88765-1

Agredano-Moreno LT, Jiménez-García LF, Echeverría OM, Martínez E, Ramos CH, Vázquez-Nin GH. 1994. Cytochemical and immunocytochemical study of nuclear structures of Lacandonia schismatica. Biology of the Cell 82:177-184. DOI: https://doi.org/10.1016/S0248-4900(94)80020-0

Agredano-Moreno LT, Segura-Valdez ML, Jiménez-Ramírez J, Jiménez-García LF. 2018. Lacandonia granules are present in the cell nucleus of the gymnosperm Welwitschia mirabilis. Botanical Sciences 96: 678-683. DOI: https://doi.org/10.17129/botsci.1924

Alonso-Murillo CD, Jiménez-García LF. 2015. Plants related to early evolutionary events (Bryophytes) contain Lacandonia granules previously discovered in flowering plants. Acta Microscopica 24: 152-158

Bernhard W. 1969. A new staining procedure for electron microscopical cytology. Journal of Ultrastructural Research 27: 250-26. DOI: https://doi.org/10.1016/S0022-5320(69)80016-X

Bao-Yu Z, Quan Z, Gui-Zhen J, Chia-Jui C. 2004. The response of ultrastructure and function of chloroplasts from cycads to doubled CO₂ concentration. The Botanical Review 70: 72-78.

Dehgan B, Schutzman B. 1989. Embryo development and germination of Cycas seeds. Journal of the American Society for Horticultural Science 114:125-129

Delevoryas T. 1982. Perspectives on the origin of cycads and cycadeoids. Review of Paleobotany and Palynology 37: 115-132. DOI: https://doi.org/10.1016/0034-6667(82)90040-9

Echeverría O, Moreno Díaz de la Espina S, Jiménez-García LF, Vázquez-Nin GH. 1999. Supramolecular organization of a chromocentric plant nucleus. Biology of the Cell 91: 209-219. DOI: https://doi.org/10.1016/S0248-4900(99)80043-4

Forest F, Moat J, Baloch E, Brummitt NA, Bachman SP, Ickert-Bond S, Hollingsworth PM, Liston A, Little DP, Mathews S, Rai H, Rydin C, Stevenson DW, Thomas P, Buerki S. 2018. Gymnosperms on the EDGE. Scientific Reports 8: 6053. DOI: https://doi.org/10.1038/s41598-018-24365-4

Jiménez-García LF, Agredano-Moreno LT, Segura-Valdez M de L, Echeverría O, Martínez E, Ramos CH, Vázquez-Nin GH. 1992. The ultrastructural study of the interphase cell nucleus of Lacandonia schismatica (Lacandoniaceae:Triuridales) reveals a non-typical extranucleolar particle. Biology of the Cell 75: 101-110. DOI: https://doi.org/10.1016/0248-4900(92)90129-O

Jiménez-García LF, Elizundia JM, López-Zamorano B, Maciel A, Zavala G, Echeverría O, Vázquez-Nin GH. 1989. Implications for evolution of nuclear structures of animal, plant, fungi and protostists. Biosystems 22: 103-116. DOI: https://doi.org/10.1016/0006-3207(89)80039-7

Jiménez-García LF, Segura-Valdez M de L. 2004. Visualizing nuclear structure in situ by atomic force microscopy. In: Braga PC, Ricci D. eds. Atomic Force Microscopy: Methods in Molecular Biology. USA: New Jersey, DOI: https://doi.org/10.1385/1-59259-647-9:191
Jiménez-Ramírez J, Agredano-Moreno LT, Segura-Valdez ML, Jiménez-García LF. 2002. Lacandonia granules are present in Ginkgo biloba cell nuclei. *Biology of the Cell* 94: 511-518. DOI: https://doi.org/10.1016/S0248-4900(02)0019-9

Mamay SH. 1976. Paleozoic origin of the cycads. Geological Survey U.S. Washington, DC, USA: Professional Paper 934.

Medina MA, Moreno Diaz de la Espina S, Martin M, Fernández-Gómez, ME. 1989. Interchromatin granules in plant nuclei. *Biology of the Cell* 67: 331-339.

Monneron A, Bernhard W. 1969. Fine structural organization of the interphase nucleus of some mammalian cells. *Journal of Ultrastructural Research* 27: 266-288. DOI: https://doi.org/10.1016/S0022-5320(69)80017-1

Morassi-Bonzi L, Medeghini-Bonatti P, Marini C, Baroni-Fornasiero R, Paoletti C. 1992. Ultrastructural Studies on differentiating chloroplasts in the ‘Forma fuscoviridis’ of Ceratozamia mexicana Brongn. *The New Phytologist* 120: 427-434. DOI: https://doi.org/10.1111/j.1469-8137.1992.tb01083.x

Norstog KJ, Nicholls TJ. 1997. The biology of the cycads. Ithaca, New York: Cornell University Press. ISBN-10: 080143033X

Segura-Valdez ML, Mendoza-Sánchez AC, García-Mauleón PMR, Agredano-Moreno LT, Jiménez-García LF. 2020. Electron microscopy of nuclear nanoribonucleoproteins (nanoRNPs). *MOJ Anatomy & Physiology* 7: 15-17. DOI: https://doi.org/10.15406/mojap.2020.07.00282

Segura-Valdez ML, Zamora-Cura A, Gutiérrez-Quintanar N, Villalobos Nájera E, Rodríguez-Vázquez JB, Galván-Arrieta TC, Jiménez-Rodriguez D, Agredano-Moreno LT, Lara-Martinez R, Jiménez-Garcia LF. 2010. In: Méndez-Vilas A, Díaz J, eds. *Visualization of Cell Structure in Situ by Atomic Force Microscopy*. Badajoz, Spain: Microscopy: Science, Technology, Applications and Education. Formatex. pp. 441-448. ISBN-13: 978-84-614-6191-2

Spector DL. 1993. Macromolecular domains within the cell nucleus. *Annual Reviews in Cell Biology* 9: 265-315. DOI: https://doi.org/10.1146/annurev.cb.09.110193.001405

Sun CR. 1963. Submicroscopic structure and development of chloroplasts of Cycas revoluta. *Protoplasma* 56: 661-669.

Swift H. 1959. Studies on nuclear fine structure. *Brookhaven Symposium in Biology*. 12: 134-152

Tomlinson PB, Magellan TM, Griffith MP. 2014. Root contraction in Cycas and Zamia (Cycadales) determined by gelatinous fibers. *American Journal of Botany* 101: 1275-1285. DOI: https://doi.org/10.3732/ajb.1400170

Vázquez-Nin GH, Bernhard W. 1971. Comparative ultrastructural study of perichromatin and Balbiani ring granules. *Journal of Ultrastructural Research* 36: 842-860. DOI: https://doi.org/10.1016/s0022-5320(71)90034-7

Vázquez-Nin GH, Biggiogera M, Echeverría OM. 1995. Activation of osmium ammine by SO2-generating chemicals for EM Feulgen-type staining of DNA. *European Journal of Histochemistry* 39: 101-106

Vovides AP, Pérez-Farrera MA, González-Astorga J, González D, Gregory T, Chemnick J, Iglesias C, Octavio-Aguilar P, Avendaño S, Bárdenas C, Salas-Morales S. 2003. An outline of our current knowledge on Mexican cycads (Zamiaceae, Cycadales). *Current Topics in Plant Biology* 4: 159-174

WhitelyJM. 1985. Chromoplasts in some cycads. *New Phytologist* 101: 595-604. DOI: https://doi.org/10.1111/j.1469-8137.1985.tb02865.x

Woodenberg WR, Berjak P, Pammenter NW. 2010. Development of cycad ovules and seeds. Implication of the ER in primary cellularisation of the megagametophyte in Encephalartos natalensis. *Plant Growth Regulation* 62: 265-278 DOI: https://doi.org/10.1007/s10725-010-9469-6

Associate editor: Silvia Aguilar

**Author Contributions:** LTAM participated in the development of the methodologies and the writing of the article and contributed with the major ideas, planning, interpretation, and main work in producing results. MLSV contributed with planning and ideas, the methodology and results for atomic force microscopy, as well as interpretation and writing. JJR contributed with planning and ideas, as well as interpretation and LFJG participated in proposing the main ideas and coordinating the work, and in the writing of the article and interpretation.