

Genome sizes of all 19 *Araucaria* species are correlated with their geographical distribution

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Abstract Nuclear genome size was determined to investigate the relationships between all 19 species of *Araucaria* de Jussieu. Species from the two other genera of Araucariaceae, *Wollemia* and *Agathis*, were also studied. The genome size of 17 out of the 19 species of *Araucaria* are reported here for the first time. All Araucariaceae have the same chromosome number $2n = 26$. However, the nuclear DNA contents (2C value) for *Araucaria* range from 31.3 to 45.4 pg. There is a good correlation between genome size and division in sections, and geographical distribution. The two species from South America have 44.7 and 45.4 pg, the two species from Australia have 35.7 and 44.4 pg and the two species from New Guinea 34.7 and 40.4 pg. All 13 species of New Caledonia and the one from Norfolk Island have a similar, if not identical, amount of nuclear DNA of, on average, 31.9 pg. This corroborates the identical DNA *rbcL* sequences found for the New Caledonian araucarias. It suggests that the species from New Caledonia diversified more recently and it questions their status as separate species. Compared with this 31.9 pg a strong increase seems to have occurred in the genome size of the “mainland” araucarias. Genome sizes are evaluated and compared with available taxonomic treatments and extant geographic spreading. The nuclear DNA contents found within the sections are close, making it possible to assign an unknown plant to a section. A difference of 1 pg, which amounts to a difference of 978 Mbp, far exceeds a single character. Nuclear DNA content, as measured by flow cytometry, may conveniently be used to produce

systematic data. It is applicable even with young plants or seeds for monitoring the trade in endangered species.

Keywords *Araucaria* · Genome size · DNA 2C value · Taxonomy · Geography

Introduction

Conifers are economically very important. They are not only sources of timber but also of wood pulp and resins. Both *Araucaria* and the genus *Pinus* L. also provide edible seeds (Zonneveld 2011).

The conifers were divided by Pilger (1926) into seven families. However Eckenwalder (1976) argued that the Taxodiaceae should be merged with Cupressaceae and that *Sciadopitys* Siebold and Zucc. should be placed in a separate family. One of the now eight families is the Araucariaceae with two genera: *Araucaria* de Jussieu and *Agathis* Salisbury. Recently (1994) a third genus in the Araucariaceae, *Wollemia* Jones, Hill and Allen was found in Australia. Two valuable books covering all extant conifers are now available: that of Eckenwalder (2009) and that of Farjon (2010). In these books the number of *Araucaria* species is set at 19, the number of *Agathis* species at 15 or 17, and there is one species of *Wollemia*.

The Araucariaceae have an ancient origin and are a distinctive component of southern hemisphere forests. From an origin in the Triassic the family expanded in both hemispheres and remained an important component of the vegetation until the late Cenozoic. Seedcone scales indistinguishable from those of *A. heterophylla* have recently been found in the Upper Maastrichtian, ca 65 MYBP old (van der Ham et al. 2010). Continental separation and climatic drying reduced the ranges of conifers to moist

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mesothermal climates (Kershaw and Wagstaff 2001). Thirteen of the 19 species of *Araucaria* are found on New Caledonia that separated from Australia approximately 65 MYBP. New Caledonia is located approximately 1,200 km from Australia and 1,500 km from New Zealand. The flora of New Caledonia is characterized by the presence of a large number of lineages that seem to represent remnants of the Gondwana flora once covering a large part of Australasia.

During the Tertiary (40–45 MYBP) New Caledonia underwent a series of submersions, covering the island with peridotites, formed from ocean crust, and to a lesser extent with serpentine soil. These soils are ultrabasic and have a high content of heavy metals, for example iron and nickel, making them rather toxic to most plants. The ultrabasic soils enabled the older lineages to adapt and survive in a sort of local refugium (Thorne 1965). So it comes as no surprise that, among the 1,176 species in New Caledonia that occur exclusively on ultrabasic soils, 98 % are endemic (Lowry 1998). These ultrabasic soils presented the initial vegetation with ecological conditions that were favourable for subsequent speciation, as seen in the now 13 species of *Araucaria*. This is also born out by the fact that 43 of the 44 native species of gymnosperms are endemic (de Laubenfels 1972).

To elucidate the relationships between *Araucaria* species further, the taxonomic traits based on morphological characters, geographical distribution, and molecular data are here supplemented with data on nuclear DNA content. Earlier, *Agathis australis* (D.Don) Lindl. was measured with $2C = 31.6$ pg (Davies et al. 1997). Three araucarias were also measured: *A. cookii* R.Br. ex Lindl.(=*A. cunninghamii* Aiton ex A.Cunn.) with 19.1 pg, *Araucaria robusta* (*Agathis robusta*?) with 21.6 pg, and *A. cunninghamii* with 21.8 pg (Ohri and Khoshoo 1986). These araucarias were measured with Feulgen densitometry, a method that is not very reliable if not properly handled (Greilhuber and Tamsch 2001).

Nuclear DNA content can conveniently be measured by flow cytometry using propidium iodide, a stoichiometric DNA stain that intercalates in the double helix. Where all species in a genus have the same chromosome number as in the Araucariaceae (Khoshoo 1961), differences in genome size have proved to be very effective in delimiting infrageneric subdivisions (Ohri 1998). Greilhuber (2005) clearly showed that there is, in general, much less intra-specific variation of genome size than previously expected. Evolution of genome size has received increased attention in recent years. The smallest angiosperm genome size reported so far is for *Genlisia margarethae* Hutch. (Lentibulariaceae) with $2C = 0.13$ pg (Greilhuber et al. 2006) (now renamed *G. aurea*—the true *G. margarethae* has $2C = 0.36$ pg, Greilhuber personal communication). The

record holders for maximum genome size were, for eudicots, *Viscum album* L. with $2C = 205.8$ pg and, for monocots, *Trillium hageae* Miyabe and Tatew. (Melanthiaceae) with 264.9 pg (Zonneveld 2010a, b). The latter value was, a few months later, superseded by the octoploid *Paris japonica* (Melanthiaceae) with $2C = 304.5$ pg (Pellicer et al. 2010). Flow cytometry has been successfully used to measure genome size for the genera *Hosta* Tratt., *Helleborus* L., *Galanthus* L., *Narcissus* L., *Tulipa* L., *Eucomis* L'Hér., and *Hepatica* Mill, etc. by Zonneveld (2001, 2008, 2009, 2010a, b, 2011), Zonneveld and Van Iren (2001), Zonneveld and Duncan (2010), and Zonneveld et al. (2003). In the work discussed in this paper all 19 species of *Araucaria* were measured and the correlation of their genome size with geographical spreading is demonstrated. Genome size alone is sufficient to ascribe a species of *Araucaria* to its section and in most cases to its geographic origin.

Materials and methods

Plant material

Plant material was obtained from the collections of the Botanical Garden of Bochum Germany, the Arboretum Trompenburg Rotterdam, the Pinetum Blijdenstein Hilversum, the Botanical Garden of Leiden, and from commerce, all from the Netherlands. Care was taken to ensure correct identification of all material.

Flow cytometric measurement of DNA $2C$ value

Conifer needles are relatively difficult to chop. So, if possible, young leaves or buds were used to isolate nuclei. These were chopped together with a piece of *Agave americana* L. “Aureomarginata” as internal standard (see below). The material was chopped with a new razor blade in a Petri dish in 0.25 ml nuclei-isolation buffer to which 0.25 mg RNase/ml had been added (Zonneveld and van Iren 2001). After adding 1.75 ml propidium iodide solution (50 mg PI/l in isolation buffer) the suspension with nuclei was filtered through a 30- μ m nylon filter. The fluorescence of the nuclei was measured half an hour and one hour after addition of propidium iodide, by use of a Partec CA-II flow cytometer. The optical path contained a HBO mercury lamp, filters KG1 and BG12, dichroic mirror TK500, filter OG570, and a Leitz 50 \times 1 water-immersion objective. Data were analysed by means of DPAC software (Partec). The $2C$ DNA content of the sample was calculated as the sample peak mean, divided by the *Agave* peak mean, and multiplied with the amount of DNA of the *Agave* standard. Usually two different samples, with each at least 5,000

nuclei, were measured twice for each accession. Most histograms revealed a coefficient of variation of less than 5 %.

Internal standard and absolute DNA content

When measuring nuclear DNA content by flow cytometry it is necessary to chop tissue from the plant of interest together with an internal standard. This standard must be as close as possible to the plants of interest. In this way, variation in signal intensities because of staining kinetics, light absorption, quenching by sample components, and instrumental and other variables, is reduced to a minimum. *Agave americana* was chosen as internal standard for *Araucaria*. They are available year-round, keep fresh for several weeks without water, and, because they are large plants, a single specimen can serve a lifetime, thereby further reducing variation in readings. They also have a low background in propidium iodide measurements, a single G_0 peak, and are almost lacking G_2 arrest.

Fresh male human leucocytes ($2C = 7.0$ pg; $1\text{ pg} = 10^{-12}\text{ g} = 0.978 \times 10^9$ base pairs; Doležel et al. 2003) were chosen as primary standard (Tiersch et al. 1989). This yields $2C = 15.9$ pg for nuclei of *Agave americana* L. On the basis of a published male human genome size of 6.294×10^9 base pairs the nucleus was calculated as containing 6.436 pg (Doležel et al. 2003). However this is based on a human sequence for which the size of the very large repeat sequences could not accurately be determined, so the genome size could be closer to 7 pg than now envisaged.

Results and discussion

Araucariaceae

According to DNA evidence (Quinn et al. 2002; Kunzmann 2007; Rai et al. 2008; Liu et al. 2009) Podocarpaceae are close to Araucariaceae with Pinaceae at the base of the Coniferales. However the fossil record (Farjon 2010) points to a basal position of Podocarpaceae + Araucariaceae. Assuming an increase in DNA with time, the small genome sizes for the Podocarpaceae, with, on average, 13 pg (Zonneveld 2012, in press) (but not for the Araucariaceae) points indeed to an ancient position for the Podocarpaceae.

With three genera and 35 species, Araucariaceae are one of the smaller families of conifers. Molecular phylogeny (Gilmore and Hill 1997; Quinn et al. 2002; Rai et al. 2008; Liu et al. 2009) found *Wollemia* to be the sister group of *Agathis*, and *Araucaria* sister to both. This suggests that both increases and decreases in nuclear DNA content have

occurred in the long history of the Araucariaceae. This placement was not found by Setoguchi et al. (1998), the only publication on DNA sequencing of all 19 species. He found *Wollemia* basal to *Agathis* and *Araucaria*, probably because of their different choice of outgroups. The three genera of Araucariaceae are readily distinguished by their leaf arrangement on side branches—in *Araucaria* de Jussieu the leaves are spirally placed, in *Agathis* Salisburi the leaves are opposite and distichously placed, and in *Wollemia* Jones, Hill and Allen the leaves are opposite and placed in four rows.

Nuclear DNA content ($2C$ value; genome size = $1C$ value) was measured for all 19 species of *Araucaria*. For comparison, *Wollemia nobilis* and *Agathis dammara* (Lamb.) Rich. and A.Rich. were also measured. Genome sizes of only 2 of the 19 species of *Araucaria* were reported before (Murray et al. 2010). Genome size as investigated here (Table 1), complements work based on morphological characters and evidence from DNA sequencing. Despite the same chromosome number for all Araucariaceae the nuclear amount of DNA ranges from 31.3 to 45.4 pg. This difference of 14 pg is equivalent to approximately 1.4×10^{10} base pairs. The species are arranged in Fig. 1 and Table 1 according to their systematic classification and geographic distribution. This reveals the relationship between genome size, sectional division, and geographic spreading.

Araucaria de Jussieu

Araucaria fossils from more than 200 million years ago have been found, and the genus had worldwide tropical distribution in Gondwana. They became extinct in the northern hemisphere after the Uppermost Cretaceous (van der Ham et al. 2010) and became restricted to their present southern distribution in the Tertiary (Eckenwalder 2009). *Araucaria* has a disjunct distribution with two species in South America and the other 17 species in Australasia, a characteristic feature of Gondwanan relict species. They are usually divided in four sections that are distributed as follows: Section Eutacta New Caledonia (13 sp.), Norfolk Island (1 sp.), and Australia/New Guinea (1 sp.); Section Araucaria: Brazil (1 sp.), Chile (1 sp.); section Bunya: Australia (1 sp.), and section Intermedia: New Guinea (1 sp.). In other words, New Caledonia has 13 species, South America, Australia and New Guinea two species each, and Norfolk Island one species.

Were it not for the fossil record, three of the four sections with, in total, four species might well be merged in a single section *Araucaria* s.l (Eckenwalder 2009). However the same fossil record found that section Bunya was the oldest and this is not supported by DNA analyses. The fossil record also showed that of seven Tertiary fossil

Table 1 Nuclear DNA content (2C) of all species of *Araucaria* with standard deviation, origin, and the source of the material

Species of Araucariaceae	pg DNA (2C) per nucleus	SD	Origin	Source
<i>Araucaria</i>				
Section Araucaria				
<i>A. angustifolia</i> (Bertol.) Kuntze	44.7	0.9	Brazil/Argentina	Ex commerce
<i>A. araucana</i> (Molina) K.Koch	45.5	1.1	Chile/Argentina	Ex commerce
Section Bunya				
<i>A. bidwillii</i> Hook.	44.4	0.8	Australia	BG Barcelona
Section Intermedia				
<i>A. hunsteinii</i> K. Schum.	40.4	0.7	New Guinea	BG Trompenburg
Section Eutacta				
<i>A. cunninghamii</i> Aiton ex A.Cunn.	35.7	0.6	NE Australia	BG Leiden
<i>A. cunninghamii</i> var. <i>papuana</i>	34.7	0.4	McAdams N. Park New Guinea	BG Leiden
<i>A. heterophylla</i> (Salisb.) Franco	32.5	0.5	Norfolk Island	Ex commerce
<i>A. bernieri</i> J.T.Buchholz	32.2	0.6	New Caledonia	BG Bochum
<i>A. biramulata</i> J.T.Buchholz	32.0	0.7	New Caledonia	BG Bochum
<i>A. columnaris</i> (J.R.Forst.) Hook.	31.5	0.4	New Caledonia	BG Bochum
<i>A. humboldtensis</i> J.T.Buchholz	32.2	0.5	New Caledonia	BG Bochum
<i>A. laubenfelsii</i> Corbasson	31.9	0.0	New Caledonia	Pin. Blijdenstein
<i>A. luxurians</i> (Brongn.&Gris) de Laub.	31.8	0.9	New Caledonia	BG Bochum
<i>A. montana</i> Brongn. & Gris	32.1	0.7	New Caledonia	Pin. Blijdenstein
<i>A. muelleri</i> (Carriere) Brongn. & Gris	31.3	1.2	New Caledonia	BG Bochum
<i>A. nemorosa</i> de Laub.	31.7	0.2	New Caledonia	Pin. Blijdenstein
<i>A. rulei</i> F.Muell. 'Elegans'	32.3	0.5	New Caledonia	Pin. Blijdenstein
<i>A. schmidii</i> de Laub.	32.0	0.7	New Caledonia	BG Bochum
<i>A. scopulorum</i> de Laub.	31.7	0.4	New Caledonia	BG Bochum
<i>A. subulata</i> Vieill.	31.9	0.2	New Caledonia	Pin. Blijdenstein
<i>Agathis</i>				
<i>Agathis dammara</i> (Lamb.) Rich.	26.9	0.7	Indonesia/Malaysia	BG Leiden
<i>Wollemia</i>				
<i>Wollemia nobilis</i> W.G.Jones et al.	28.4	0.1	E. Australia	BG Leiden

species of Australia five could be placed in section Eutacta and two in section Araucaria, seemingly obviating the need for extra sections (Hill 1990).

If *Araucaria* is divided into two sections only, as already done by de Laubenfels (1972), they could be morphologically characterized as a section Araucaria s.l. with flat and straight leaves, pollen and seed cones axillary, four cotyledons, and mainly hypogeal germination (four species, type *A. araucana*) and section Eutacta (Link) Endl. with scaly or needle-like and curled leaves, pollen and seed cones terminally, two cotyledons, and epigeal germination (15 species, type *A. cunninghamii*) (de Laubenfels 1972). This division into two sections would not only be in accordance with earlier cladograms (Gilmore and Hill 1997; Quinn et al. 2002; Rai et al. 2008; Liu et al. 2009) but also with genome sizes, with 31.3–35.2 pg for section

Eutacta and 40.4–45.4 pg for section Araucaria s.l. For ease of discussion the division into four sections is used here.

Section Eutacta

This section has 13 species from New Caledonia with 31.3–32.3 pg (average 31.9 pg). These values, lower than for any of the other araucarias, make it unlikely they were derived after recolonization from Australia. Basal to this group is *A. heterophylla* (Salisb.) Franco, used worldwide as a house plant, from Norfolk Island with 32.5 pg, indicating a close relationship. These in turn have *A. cunninghamii* Aiton ex A.Cunn. as a basal species with 35.2 pg (Setoguchi et al. 1998). *A. cunninghamii* is divided in two varieties: var. *cunninghamii* from Australia with 35.7 pg

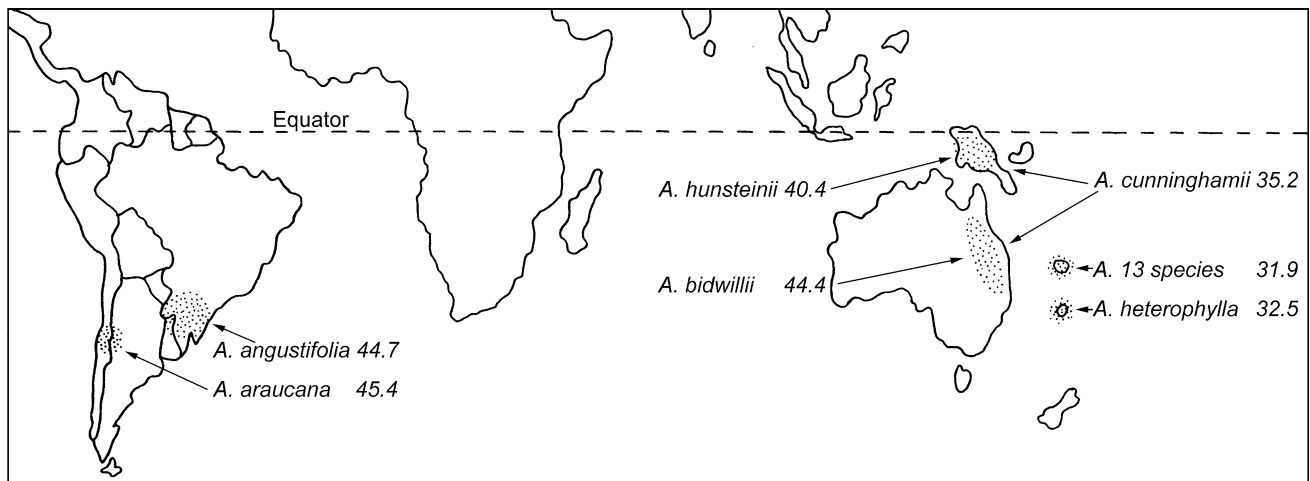


Fig. 1 Geographical distribution of all *Araucaria* species, followed by their nuclear DNA content in pg, and arranged according to their section. From left to right: Section *Araucaria*: *A. angustifolia* (Brazil, Argentina, Paraguay), *A. araucana* (Chile, Argentina), Section *Intermedia*: *A. hunsteinii* (New Guinea) Section *Bunya*: *A. bidwillii*

and var. *papuana* from New Guinea with 34.7 pg. It could be a coincidence, but the genome size of the Australian/New Guinean *A. cunninghamii* with $2C = 35.2$ pg is intermediate between the New Caledonian species with 31.9 pg and the New Guinean *A. hunsteinii* with 40.4 pg.

The 13 species occurring in New Caledonia, belonging to section *Eutacta*, are primarily restricted to ultrabasic soil, with exception of *A. columnaris*, which is found along the coast on coral remains. De Laubenfels (1972) remarks that the 13 species of New Caledonia differ in size and shape of the adult leaves and in the shape of the male cones, but hardly in any other character. Without the age of the tree and the height where it grows, it is even impossible to distinguish a branch with young leaves of species A from a branch with adult leaves of species B. Coupled with near identical *rbcL* genes and near identical genome sizes one wonders if the union of these 13 species into a single species (with different subspecies) would not better represent affinities within the genus *Araucaria*. However, from a conservation standpoint 13 species might be preferable.

Section *Bunea*

This section has only one species *A. bidwillii* Hook. from Australia. Its range partly overlaps those of *A. cunninghamii* of section *Eutacta*. According to DNA evidence (Setoguchi et al. 1998) it is placed in the same clade as section *Araucaria* (with *A. araucana* (Molina) K. Koch and *A. angustifolia* (Bertol.) Kuntze) and section *Intermedia* (with *A. hunsteinii* K. Schum.). These four species are sister to the other araucarias from section *Eutacta*. The

(E. Australia), Section *Eutacta*: *A. cunninghamii* (E. Australia, New Guinea), *A. bernieri*, *biramurata*, *columnaris*, *humboldtensis*, *laubenfelsii*, *luxurians*, *montana*, *muelleri*, *nemorosa*, *rulei*, *schmidii*, *scopulorum*, *subulata* (13 species, New Caledonia) *A. heterophylla* (Norfolk Island)

genome size ($2C = 44.4$ pg) does not support section *Bunya* as the oldest section, as is suggested by the fossil record (Stockey and Taylor 1978). Its genome size corroborates the earlier placement in this clade, based on *rbcL* (Setoguchi et al. 1998) and AFLP (Stefenon et al. 2006) analyses.

Section *Intermedia*

The single species of this section, *A. hunsteinii* from New Guinea is, with 89 m, not only the largest of all araucarias, but also the tallest tree of New Guinea. It is somewhat isolated, being placed in a section by itself, because it is intermediate in some characteristics between section *Eutacta* and the others. Its nuclear DNA content of 40.4 pg shows that it is closely related to the other three species of section *Araucaria* s.l., all with approximately 45 pg. The other *Araucaria* of New Guinea is *A. cunninghamii*, placed in section *Eutacta* and their territories partly overlap.

Section *Araucaria*

This section contains two species: *A. araucana* from Chile, the type plant of *Araucaria*, and *A. angustifolia* from S. Brazil, overflowing into adjacent countries. They are close in nuclear DNA content: 45.4 pg for *A. araucana* and 44.7 pg for *A. angustifolia* and can be crossed successfully. Both species are the only araucarias that are mostly dioecious and are (or were) important timber trees. The large seeds of both, produced abundantly, have been an important food source for local people and wildlife. The common name monkey puzzle tree is somewhat fanciful, because no monkeys live in its native range.

Agathis Salisb.

Agathis is distributed over Indonesia, Malaysia, the Philippines, Melanesia, Australia, New Zealand, and Fiji.

Resting buds, covered with imbricate scales, are well developed, the petioled leaves are flat, not needle-like, and seeds have a very large wing. Despite its economic importance both for timber and resins there is surprisingly little consensus on an appropriate classification. The characters of the species are all intergrading, so the number of species can vary from 12 to 21 in different classifications and the different groupings of species overlap only a little (Eckenwalder 2009). The only species here measured, *Agathis dammara* has $2C = 26.9$ pg.

Wollemia Jones, Hill and Allen

The discovery in 1994 of this conifer belonging to the Araucariaceae caused much excitement among botanists (Jones et al. 1995). *Wollemia* has several characteristics of both *Araucaria* and *Agathis*. The fossil record goes back at least a 100 million years making it one of the oldest living conifer genera. There are fewer than 40 unique trees in a single canyon in southeastern Australia. It can be grown from cuttings and via tissue culture and the latter has provided many young plants that can now be obtained worldwide. Setoguchi et al. (1998) place *Wollemia* basal to *Agathis* + *Araucaria* but this might be because of their choice of out-group's. According to other/late DNA studies (Gilmore and Hill 1997; Liu et al. 2009) *Wollemia* is more similar to *Agathis* than to *Araucaria*. This is supported by a genome size of $2C = 28.4$ pg for *Wollemia nobilis* that is close to *Agathis dammara* with 26.9 pg and less so to the araucarias varying from 31.3–45.4 pg. *Wollemia* was previously measured (Hanson 2001) with a similar genome size of $2C = 27.9$ pg.

Relation of genome size to geographical distribution

The species are arranged in Fig. 1 according to their taxonomic position and geographic distribution. Figure 1 and Table 1 reveal the relationship between genome sizes, sectional division, and geographic spreading. They clearly show that the sections have similar amounts of DNA. Moreover the species closest in genome size are also geographically closest. The relatively small genome sizes for the section Eutacta suggest that these are the most primitive. Their near identity in DNA analyses (Setoguchi et al. 1998), corroborated by a very similar genome size and a limited morphological diversity, suggest recent radiation. If 32 pg is the basal value for *Araucaria*, they must have evolved and survived elsewhere by having a strong, nearly 50 % increase in their genome sizes. Maybe

only the few that were able to acquire this extra DNA were the extant survivors. This extra DNA might have led to slow but enduring growth making them often the largest trees in the forest. This seems important, maybe essential, for wind pollinated trees. All others might have succumbed to the dryness of the climate and the domination of the angiosperm trees. Although the genome sizes suggest this, it seems unlikely they radiated from the very isolated New Caledonia to other places.

Ploidy

Ploidy seems not to have been involved in the speciation of Araucariaceae. The absence of polyploidy might be because araucarias already have a large amount of nuclear DNA, approximately 20 times more than the average angiosperm tree (Ahuja 2005). From *A. angustifolia* seeds also were available for measurement. The endosperm turns out to be haploid, as for all conifers measured so far (Zonneveld, unpublished results). More peculiar is the fact that the embryo had 50 % of cells with nuclear DNA content inferring tetraploidy.

Conclusion

Flow cytometry can be regarded as a quick and useful means of producing systematic data. Moreover, it can be used to investigate imported plants, precluding the need to grow them to maturity for identification purposes. The amount of nuclear DNA ($2C$ value) ranges from 31.3–45.4 pg. This almost 50 % difference in DNA content without any difference in the number of chromosomes may be because of genomic changes, for example insertions, but is more likely to be the result of a vast increase in the number of transposable elements. Depending on the size of the total genome, one picogram amounts to several thousand genes. Moreover, the largest genome contains approximately 1.4×10^{10} more base pairs than the smallest, and has chromosomes that are, on average, 45 % longer. Therefore, conclusions and suggestions based on genome size seem intrinsically more informative than a single morphological character. The results presented here for genome sizes agree with recent classifications of Araucariaceae and with their geographic distribution. Flow cytometry as a taxonomic and diagnostic tool is applicable even to seeds (Zonneveld 2011), or juvenile plants, and also has applications in conservation monitoring.

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