

Evidence for apomictic seed formation in *Coprosma waima* (Rubiaceae)

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Abstract Facultative autonomous apomictic seed formation has been identified in *Coprosma waima* by progeny tests, flow cytometry, and DNA Amplified Fragment Length Polymorphism profiles. An isolated, cultivated, female plant of *C. waima* produced seeds, from which were raised female *C. waima* plants. Flow cytometry of seeds collected from these female plants had an embryo to endosperm ploidy ratio of 2:4. This is consistent with parthenogenetic embryo development from a single unreduced egg nucleus, and autonomous endosperm development from two unreduced polar nuclei. DNA Amplified Fragment Length Polymorphism profiles of the maternal plant and three female progeny were identical, indicating they were genetically homologous. Seeds from wild plants of *C. waima* have an embryo to endosperm ploidy ratio of 2:3, consistent with sexual reproduction. We also present evidence of apomictic seed formation in *C. parviflora*, *C. robusta*, and *C. serrulata*.

Keywords Rubiaceae; *Coprosma*; *C. parviflora*; *C. robusta*; *C. serrulata*; *C. waima*; apomixis; flow cytometry; Amplified Fragment Length Polymorphisms; New Zealand flora

INTRODUCTION

Coprosma (Rubiaceae) is a widespread, conspicuous, and ecologically important genus in New Zealand, with species also occurring on a number of islands scattered across the western Pacific Ocean (Oliver 1935; Heads 1996). In New Zealand, *Coprosma* includes about 54 named taxa that can be found in most main habitat types, including sand dunes, river beds, coastal and lowland forest, bogs, montane scrub, tussock grasslands, and subalpine herbfield (Allan 1961; Moore & Mason 1974; Orchard 1987; Druce 1989; Heads 1998; Molloy et al. 1999; de Lange & Heenan 2001). Leaf size and shape vary greatly among the species, as does growth habit, with several having a distinctive divaricate branching. Almost all species are dioecious, with two hermaphrodite exceptions reported, *C. moorei* and *C. talbrockiei* (Moore & Mason 1974). The flowers are adapted to wind pollination (anemophily), and the calyx and corolla are usually small and often inconspicuous. Male flowers typically have four stamens with long filaments, anthers held well beyond the perianth, and production of abundant pollen. Female flowers usually have two prominent and well-exserted stigmas. Occasionally, atypical hermaphrodite or polygamous flowers are found (Cheeseman 1887; Wild & Zotov 1930). *Coprosma* fruit are fleshy and mostly red, orange, white, or yellow (Lee et al. 1988), and each usually contains 1–2 seeds (pyrenes). Precociously fruiting female plants often occur, on which nearly every flower appears to have formed fruit.

New Zealand seed plants have been the subject of numerous studies on floral biology (see Thomson (1881), Heine (1937), and Godley (1979)), and are particularly well known for the large number of dioecious species (Webb et al. 1999). In contrast, the production of seeds by apomixis, whereby meiosis is either partly or completely avoided and embryos form from unreduced maternal cells rather than from the fusion of male and female gametes, has not been widely examined. Indeed, for the indigenous flora, apomixis has been demonstrated in only two species

of *Pomaderris* (Rhamnaceae) (Harvey & Braggins 1985), although an aposporus initial has been observed in *Ripogonum scandens* (Smilacaceae) (Macmillan 1972). Interestingly, the possibility of apomixis in *Coprosma propinqua* was suggested but discounted by Wardle (1971), who concluded that the species "is efficient, but indiscriminating, in its anemophily". To date, apomixis has been studied more often in New Zealand populations of naturalised plants than indigenous species. Examples of such studies include *Cortaderia jubata* (Connor 1965; Philipson 1978), *Elymus rectisetus* (Hair 1956; Crane & Carman 1987), and *Hieracium pilosella* (Houliston & Chapman 2001). We suspect that the few documented occurrences of apomixis in the indigenous New Zealand flora may reflect the difficulties of identifying and studying apomixis, rather than as evidence of its true abundance. In the past apomixis could only be detected from detailed histological examinations, complex genetic and cytological studies, and often-prolonged progeny tests.

Since 1991 one of us (PBH) has made observations on seed formation and production in cultivated and wild plants of a number of species of *Coprosma*, as well as undertaking progeny tests. Here we present these observations for *C. waima*, along with data from flow cytometry of DNA and analyses of DNA Amplified Fragment Length Polymorphisms (AFLPs). These observations and experimental data support the hypothesis that apomixis is occurring in *Coprosma*.

MATERIALS AND METHODS

Plant material and progeny tests

The presence of cultivated female plants of *C. waima* at Lincoln, Canterbury, South Island, provided an opportunity to investigate apomixis. *Coprosma waima* was discovered as recently as 1986, formally named in 1989, and is restricted to Waima Forest, Northland, North Island (Druce 1989). No wild plants of *C. waima* occur in close geographic proximity to the cultivated female plants at Lincoln, and no male plants are known to be cultivated nearby. Other species cultivated near the study plants of *C. waima* include *C. acerosa*, *C. grandifolia*, *C. propinqua*, *C. repens*, *C. robusta*, and a range of horticultural hybrids and cultivars. Seeds were collected from cultivated female plants of *C. waima* and sown on 15 July 1997. Glasshouse-grown female plants of *C. waima* flowered between July

and September 2001 and these formed a small number of fruit. Controlled pollinations between *C. waima* (female) and *C. robusta* (male) were made in October 2001.

Flow cytometry

Flow cytometry provides an accurate estimate of DNA content by measuring fluorescence caused by fluorochrome-stained nuclei passing through a focused light beam. The amount of fluorescence is proportional to the quantity of DNA present in the cell (Heslop-Harrison & Schwarzacher 1996). The Flow Cytometry Seed Screen (Matzk et al. 2000) was used to determine relative DNA contents present within the embryo and endosperm tissue of single seeds of *C. waima*. Freshly matured fruits from female plants, which were isolated during flowering from potential pollen sources, were collected during August, September, and October 2001. Material analysed included 48 seeds collected from the cultivated plants and these gave two different embryo-to-endosperm ratios. Further analysis of 54 seeds from 27 fruit (2 seeds per fruit, and each fruit 4–5 mm long) was undertaken to systematically determine the distribution and frequency of the different embryo-to-endosperm ratios. In addition, 10 seeds from wild-collected plants were removed from herbarium material in Allan Herbarium (CHR). This included seeds from specimens collected in 1987 (e.g., CHR 394514).

We removed the two seeds from each fruit and excised the embryo and endosperm from the woody endocarp, which were then finely chopped in 0.5 ml of buffer for cell extraction and liberation. The cell suspension was then filtered through a 30 µm mesh into a sample tube and incubated for 2 min before adding 2 ml of DAPI stain. The buffer and stain are included in the proprietary Partec T kit (CyStain UV precise T (05-5003)). Fresh leaf material of *C. waima* was treated in the same way to provide a standard to identify the embryo (2C) peak, assuming the ploidy of the embryo and leaf tissue were similar.

The suspensions were passed through a flow-cytometer (Partec Ploidy Analyser PA-II) with the detector operating at 355 nm. A minimum of 5000 nuclei were counted for each sample (20–50 nuclei per second). Data analysis was performed using PA-II's Partec FloMax software. Ploidy levels of the nuclei of the endosperm and embryo tissue were determined by comparing the ratio between the different peaks. Normal distribution curves were fitted to the histograms, and coefficients of variation were calculated to provide a measure of variation.

Fig. 1 *Coprosma waima* fruit. **A**, apomictic fruit containing large and small seeds; **B**, fruit formed by crossing *C. waima* (♀) with *C. robusta* (♂) and containing similar-sized seeds.

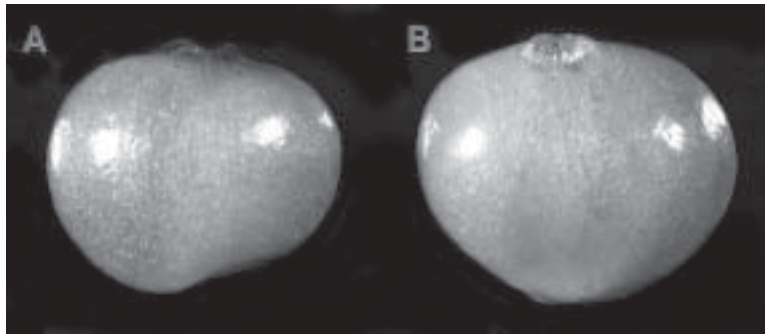
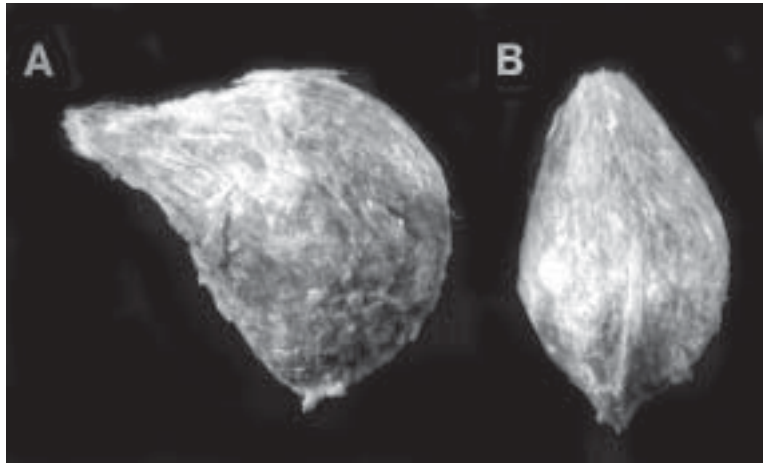


Fig. 2 *Coprosma waima* seeds from a single apomictic fruit. **A**, large seed; **B**, small seed.



AFLP profiles

Two female plants of *Coprosma* and three seedlings of each were selected for AFLP fingerprinting (experimental garden accession numbers in brackets):

- a, *C. waima* ♀ parent (G17485); b, *C. waima* seedling (G302/97-3); c, *C. waima* seedling (G302/97-4); d, *C. waima* seedling (G304/97); e, *C. serrulata* ♀ parent (G19433); f, *C. serrulata* seedling; g, *C. serrulata* seedling; h, *C. serrulata* seedling (G300/97).

At least 1 µg of DNA was extracted from the leaves of live plants using the protocols and contents of a Qiagen Plant Minikit. The extract concentrations were standardised to 25 ng µl⁻¹. Template DNA was prepared using *Mse*I and *Pst*I primers and adaptors. Six *Mse*+3 primers (CCA, CGA, CTG, GAG, GCC, GCT) were used in the selective amplification of template DNA. PCR amplification products were mixed with STR x-loading buffer, and loaded into

the wells of a polyacrylamide gel preceding electrophoresis. After electrophoresis, banding patterns were developed using a silver staining technique. It is assumed that the AFLP fragments are homologous. This method essentially follows that first described for the AFLP technique by Vos et al. (1995).

RESULTS

Progeny tests

The isolated female plants of *C. waima* grown at Lincoln developed fruit and formed seeds, and these were collected and sown in 1997. A number of plants from these seeds were raised to flowering. Vegetative and floral morphology of the progeny confirmed that four plants were typical of *C. waima* and, as would be expected if they resulted from apomictic seed formation, they were all female. No pollen-bearing male or hermaphrodite flowers were

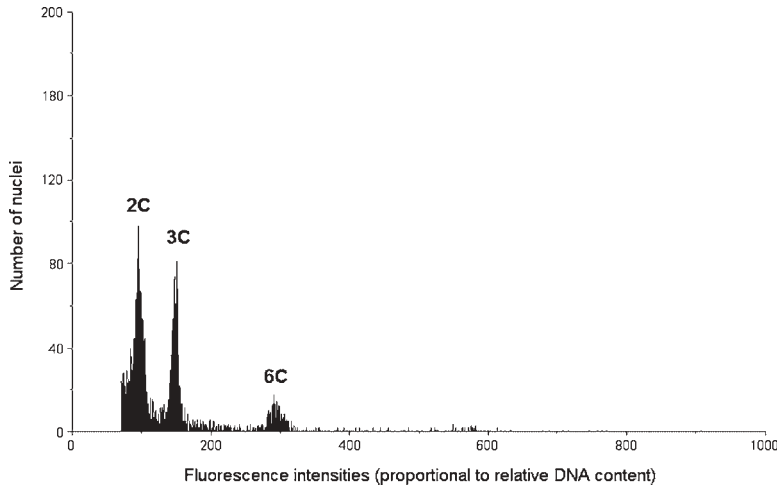


Fig. 3 Flow cytometry peaks with an embryo to endosperm ratio of 2:3, indicating sexually formed seeds. Coefficients of variation: 2C = 9.0%; 3C = 3.9%; 6C = 3.2%.

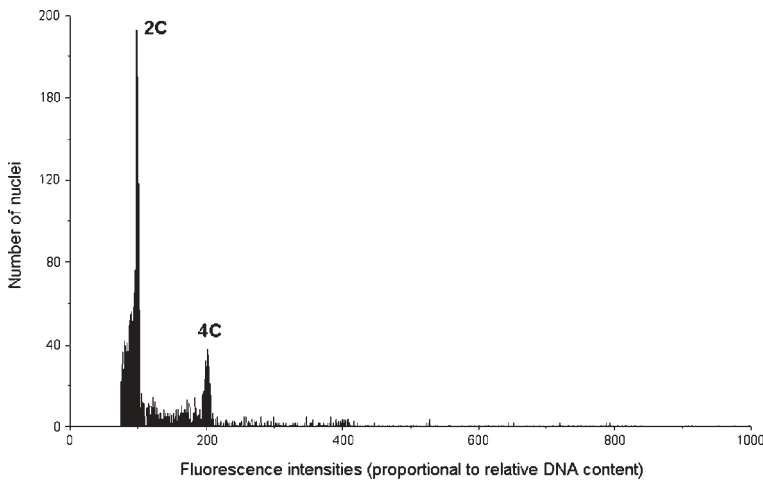


Fig. 4 Flow cytometry of small seeds with two peaks, corresponding to an embryo to endosperm ratio of 2:4, indicating apomictically formed seeds. Coefficients of variation: 2C = 3.5%; 4C = 2.6%.

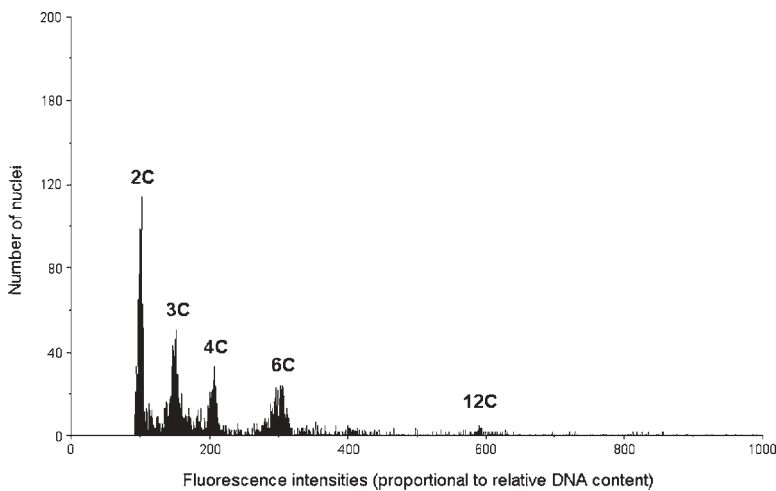


Fig. 5 Flow cytometry of large seeds with four main peaks and a much smaller fifth peak. Coefficients of variation: 2C = 3.8%; 3C = 7.4%; 4C = 3.2%; 6C = 3.4%; 12C = 3.3%.

observed on any of the *C. waima* cultivated female plants. Another 20 plants raised from these seeds included large-leaved putative hybrids between *C. waima* and *C. grandifolia*, and between *C. waima* and *C. robusta*, as well as a number of other small-leaved putative *C. waima* hybrid plants of unknown parentage.

During July to September 2001, the original female *C. waima* plant and three of the female progeny flowered and set fruit when grown in a glasshouse. No other species of *Coprosma* were observed in flower at Lincoln during this time; for example, flowering began for *C. robusta* in late September and for *C. repens* during mid October. Fruit formed on the four female plants of *C. waima* were all asymmetric, with one large seed and one small seed (Fig. 1A, 2). On the female clone of *C. waima* used for this study, we estimate that the number of fruit formed through apomixis is less than 1%.

Fruit formed on female *C. waima* from hand pollination with *C. robusta* were symmetrical and with two similar-sized seeds (Fig. 1B).

Flow cytometry

Flow cytometric analysis of nuclei from the 10 seeds from wild-collected herbarium material had an embryo to endosperm ploidy C-ratio of 2:3 (Fig. 3). This ratio characterises sexual reproduction in seed plants (Matzk et al. 2000). The 2C embryo peak is consistent with a single reduced egg nucleus having been fertilised by a reduced sperm nucleus, and the 3C endosperm peak is consistent with two reduced polar nuclei being fertilised by a single reduced sperm nucleus. In contrast, two different ploidy ratios were obtained for embryo and endosperm tissue of seeds gathered from mature fruit of isolated glasshouse-grown plants of *C. waima*. These mature fruit were asymmetric, with each containing one small seed and one large seed (Fig. 4, 5). The small seeds had an embryo to endosperm ratio of 2:4, and the large seeds had multiple ploidy peaks, with ratios of either 2:3:4 or 2:3:4:6, and occasionally 2:3:4:6:12 (Table 1). In immature fruit the large seeds (each 2–3 mm long) had embryo to endosperm ratios of 2:4, and the 3C and 6C peaks were either not detected or only incipient.

Differences in the percentage of embryo versus endosperm tissue were observed between the two sizes of seed (Table 1). Small seeds had more embryo tissue (72%), whereas large seeds had less embryo tissue (35%). In the small seeds the 4C endosperm peak comprised about 26% of the tissue.

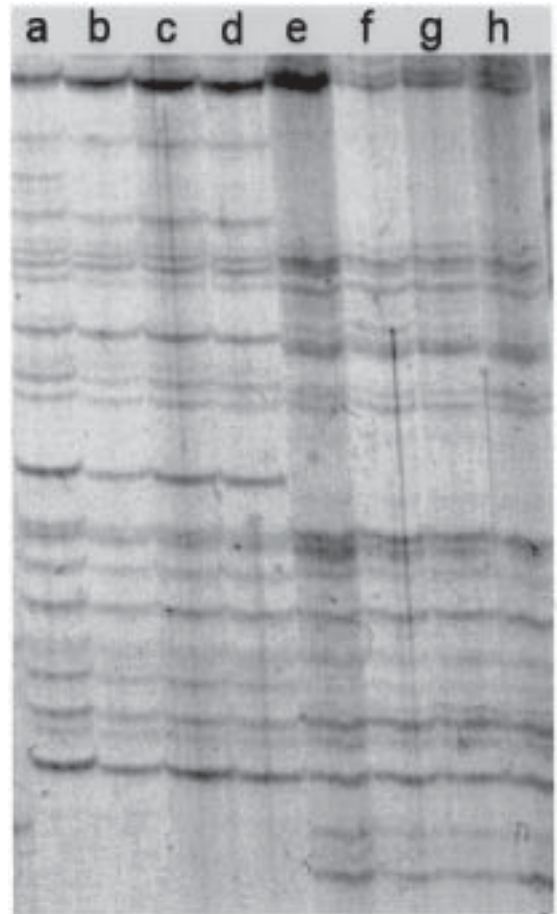


Fig. 6 Portion of AFLP plate (CGA primer) showing identical bands for the *C. waima* parent (a) and progeny (b, c, d), and for the *C. serrulata* parent (e) and progeny (f, g, h).

In the large seeds, it was difficult to establish the percentage of endosperm due to the presence of 3C, 4C, and 6C peaks, and the unknown origin of the tissue producing these peaks.

AFLP profiles

The AFLP techniques produced well-resolved, discrete banding patterns (Fig. 6). With each of the six primers, some bands were unique to *C. waima* or *C. serrulata*. However, other bands were common to both species. Significantly, the banding pattern of each parent was identical to all of its seedlings. As far as we could determine, through visual comparison, this was true for all primers used, providing strong evidence that the seedlings were genetically identical to their respective parents.

Table 1 Distribution (%) of inferred ploidy levels (C-values) in large and small seeds from the same fruit of glasshouse-grown *C. waima* (sample size = 27).

Seed type	C-values			
	2C	3C	4C	6C
Large seeds				
Mean	35.2 ± 9.7	31.5 ± 7.7	20.5 ± 7.7	10.7 ± 7.0
Range	14–65	14–47	4–36	0–26
Small seeds				
Mean		72.4 ± 6.0		26.4 ± 5.8
Range		60–82		17–39

Alternatively, if the seedlings were a result of hybridisation with one of the other species, horticultural hybrids, or cultivars grown in the area, then the AFLP profile of the resultant progeny would be expected to be distinct from its seed parent.

DISCUSSION

Evidence from fruit formation on isolated plants, progeny tests, flow cytometry of embryo and endosperm tissue, and AFLP profiles is consistent with the occurrence of low levels of apomixis in *C. waima*. In small seeds (Fig. 4), the 2C peak is consistent with apomixis in which a single, unreduced egg nucleus forms the embryo. The 4C peak is probably a composite of the two unreduced polar nuclei forming the endosperm combined with the 2C replicative peak of the embryo. Therefore, *C. waima* has parthenogenetic embryo formation and autonomous endosperm development, and should be considered an autonomous facultative apomict. This appears to be the first verified record of apomixis for the family Rubiaceae and superorder Gentiananae (Carman 1997). However, Osunkoya & Swanborough (2001) suggested that a dioecious species of *Gardenia* (Rubiaceae) from Queensland, Australia, might be apomictic, on the basis of bagged female flowers forming fruit in the absence of pollen.

The species-rich genus *Coprosma* is an important, but perhaps not surprising, addition to the list of genera whose species have an apomictic reproductive strategy. *Coprosma* has a number of attributes that are typical of other well-studied apomictic taxa (Asker & Jerling 1992; Mogie 1992). These include high levels of polyploidy (Beuzenberg 1983; Dawson 2000 and references therein), a

putative hybrid origin for some polyploid species (e.g., Molloy et al. 1999), dioecism, and probable radiation during the Pleistocene, a common feature of European and North American apomictic taxa (Gustafsson 1947; Bayer & Stebbins 1983).

Precocious fruiting has been widely observed in *Coprosma* and is generally considered to be the result of efficient wind pollination (e.g., for *C. propinqua* see Wardle 1971). However, other species of *Coprosma* may also be apomictic, and we have obtained evidence of this reproductive strategy in *C. parviflora*, *C. robusta*, and *C. serrulata*. An isolated female plant of *C. serrulata* in cultivation at Lincoln formed a number of fruit, the seeds of which were sown, and these produced a progeny of four female *C. serrulata* plants. The AFLP profiles of the female *C. serrulata* parent and its progeny are identical (Fig. 6). By using flow cytometry as a screen for reproductive pathways in other species of *Coprosma*, whereby 2C and 4C peaks indicate autonomous apomictic seed formation (Matzk et al. 2000), we have also identified possible apomixis in *C. parviflora* and *C. robusta*.

A relationship between apomixis and dioecious species has been recognised (Stebbins 1932; Gustafsson 1947; Connor & Dawson 1993), and the species-rich *Coprosma* provides a further important example. Dioecism is considered to have evolved as a reproductive strategy in response to selection pressures favouring the separation of male and female resource allocation in species with expensive reproductive structures (Bawa 1980), such as the fleshy fruit of *Coprosma*. In dioecious species, the evolution of an alternative breeding system, such as apomixis, to reduce dependency of female plants on fertilisation by pollen from conspecific male plants may be favoured (Gustafsson 1947). Apomixis has

advantages over other strategies, such as inconstant dioecism and gynodioecism, in that the “cost of meiosis” (Charlesworth 1980) and inbreeding are avoided. Autonomous embryo and endosperm formation, as occurs in *C. waima*, may also be advantageous for a dioecious species. This is because pseudogamy, where fertilisation is required for endosperm formation, will be disadvantaged by males becoming uncommon as the number of apomictically derived females increases in a population (Richards 1990).

The asymmetric fruit shape with two different seed sizes in apomictically produced fruit of *C. waima* provides a useful morphological marker correlated with apomictic processes. However, similar asymmetrical fruit morphology may be obtained through sexual reproduction if one of the ovules in a flower is fertilised and the other is not. Some of the fruit examined on herbarium specimens contained seeds of two different sizes, and when each was dissected the large seed contained embryo and endosperm tissue but the smaller seed was empty. These large seeds had embryo to endosperm C-value ratios of 2:3, consistent with sexual reproduction.

Approximately two-thirds of the New Zealand species of *Coprosma* have $2n = 44$, and this appears to be the functional diploid complement (see Dawson 2000 and references therein). Two chromosome counts of $2n = 22$ have been reported in this genus, but these are of uncertain validity (Dawson 1995). Fifteen taxa in *Coprosma* have been reported as high polyploids, including $2n = 88$ and $2n = 132$. For *C. waima* $2n = 44$ (Druce 1989), and based on this we assume that the 2C values of the leaf and embryo tissue are of this ploidy. For the large seeds of *C. waima*, the multiploid peaks were an unexpected result and their origin is unclear. The 3C and 6C peaks presumably represent $2n = 66$ and $2n = 132$, respectively. Their triploid base suggests the involvement of a fertilised endosperm, yet this seems unlikely as the plants appeared to set seed in the absence of concurrent male flowering. It is, however, not uncommon in seed plants for extensive endopolyploidy to occur during endosperm cell proliferation (Chamberlin et al. 1993; Grafi & Larkins 1995). We intend to investigate this issue further.

The presence of apomixis in *C. waima* is an exciting discovery that raises a number of new questions. Future research on *C. waima* should establish the fecundity of the large and small seeds, the origin and function of the 3C and 6C multiploid tissue in the large seeds, the range of intraspecific

variation, and the type of apomixis and embryology. In populations of *C. waima*, sex ratios (e.g., Gordon 1959) should be examined to discover whether skewed male-to-female ratios occur. Indeed, female-biased sex ratios may suggest apomixis is occurring in the population. A systematic screening of other species of *Coprosma* should also be undertaken to determine the extent of apomixis in the genus.

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