

## **Investigation of *Passiflora* hybrids using flow cytometry**

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### **Abstract**

Using flow cytometry to measure the mass of nuclear DNA in leaf cells, it has been shown that cross-fertilization of a tetraploid with a diploid pollen parent can give rise to a fertile triploid *Passiflora* hybrid. This technique also confirmed the status of several presumed tetraploids, and allowed the DNA ratio of certain parent and hybrid species to be calculated indirectly. The nuclear DNA ratio of the taxa examined here is consistent with other data on *Passiflora*. Flow cytometry not only offers a means of investigating the ploidy of *Passiflora* hybrids, but is potentially capable of throwing light on the parentage of hybrids with an uncertain ancestry.

### **Introduction**

The term, "ploidy," refers to the number of sets of chromosomes in cells. Most living organisms are diploid: because an individual has two parents, the chromosomes in each somatic (body) cell occur in pairs, although their gametes (sex cells) are haploid and contain unpaired chromosomes. The actual number of chromosomes in a set varies among different species, and is denoted ' $n$ '. Thus diploid organisms are denoted as ' $2n$ '. In the genus *Passiflora*, most species in subgenus *Passiflora* have  $2n = 18$ , whereas most species in subgenus *Decaloba* have  $2n = 12$ .

Polyploidy, or having more than two sets of chromosomes, occurs naturally through various mechanisms, but is rare in wild passifloras. Polyploid species (or individuals) are morphologically almost identical to their diploid relatives, but often have larger flowers. About a dozen records of wild natural polyploids have been confirmed, mostly from subgenus *Decaloba* [1, 2]. The only known naturally-occurring polyploidy in subgenus *Passiflora* is *P. incarnata*, a tetraploid at  $4n = 36$  [3,4], and an octaploid in an unidentified species [2].

Polyploidy can be induced by chemical agents such as colchicine. In this way Knight [5] created the first artificial *Passiflora* tetraploid ( $4n = 36$ ), based on a complex cross of *P. incarnata* and *P. edulis*. The resulting hybrid, known as *P. 'Byron Beauty'*, has four copies of each chromosome in somatic cells. Fischer then made a number of other tetraploid passifloras, both species and hybrids, using the colchicine technique. These tetraploids included *P. caerulea* 'Emil Kugler' [6], *P. 'Jara'* and others. In theory, colchicine treatment can produce further polyploids such as hexaploids ( $6n = 54$ ) and octaploids ( $8n = 72$ ), but there are no confirmed examples in cultivated *Passiflora*.

The ready availability of tetraploid *Passiflora* has allowed them to be used for hybridisation. Two such hybrids are *P. 'Betty Myles Young'* (*P. 'Clear Sky'* ♀ × *P. loefgrenii* 'Iporanga' ♂) and *P. 'Lambiekins'* (*P. caerulea* 'Emil Kugler' ♀ × *P. loefgrenii* 'Iporanga' ♂) as shown in Figure 1.

**Figure 1. Left to right: *Passiflora* 'Betty Myles Young', *P. 'Lambiekins'* and an unregistered sister of *P. 'Betty Myles Young'*, with diploid *P. caerulea* below.**



These hybrids were presumed to be either triploids or tetraploids. Triploid plants can arise when a tetraploid (hybrid or species) is crossed with a diploid plant. The only confirmed triploid ( $3n = 27$ ) in *Passiflora* is *P. ×caponii*. It appears that this arose unexpectedly when the two diploid parents were hybridised. However, some uncertainty exists about its parentage [7]. Both *P. ‘Betty Myles Young’* and *P. ‘Lambiekins’* are partially fertile; *P. ‘Betty Myles Young’* in particular sets fruit readily with open pollination from other passifloras. Many triploid plants are infertile because their chromosomes are unable to segregate correctly during meiosis (pollen and egg production). It was therefore believed that the most likely explanation was that these two hybrids were tetraploids and that they had arisen either because the male parent (*P. loefgrenii* ‘Iporanga’) had produced unreduced (i.e. diploid) gametes or that *P. loefgrenii* ‘Iporanga’ is itself a tetraploid [8]. Some evidence for the latter could be found in the observation that the flowers of *P. loefgrenii* ‘Iporanga’ are much larger than those of *P. loefgrenii* ‘Corupa’, which, from its morphology, is almost certainly diploid.

The possibility that viable triploid forms of *Passiflora* might exist has been discussed for some time. In 2001, Kugler [9] described the experience of a colleague, Hans Schwartz, who had produced fruit on (tetraploid) *P.* ‘Byron Beauty’ after pollination with (diploid) *P. caerulea* ‘Constance Elliott’. The resulting seeds were viable and grew to mature plants. This cross was repeated by others with the same result, but the matter was complicated by the knowledge that some plants labeled as *P.* ‘Byron Beauty’ are in fact diploid hybrids of *P. incarnata*. In the same year, Fischer reported that *P.* ‘Clear Sky’ and *P.* ‘Jara’ (both tetraploids) produced fruits when open-pollinated with *P. caerulea* ‘Constance Elliott’ [10]. The fate of the seeds produced by these crosses is unknown, but Fischer took this as evidence that triploids might exist. Yet again, other explanations were possible, such as the pollinator was not *P. caerulea* ‘Constance Elliott’, but some other tetraploid taxon growing nearby, or that the fruit represented parthenogenesis, or that the offspring were tetraploids that arose from unreduced gametes produced by *P. caerulea* ‘Constance Elliott’.

Although there have been many reports of DNA sequences and chromosome counts of *Passiflora* species, little is known about the properties of their hybrids. It was decided that the above questions could be answered by an analysis of cellular DNA levels of a number of species and hybrids using flow cytometry. It is important to note that, unlike conventional microscopic chromosomal analysis, flow cytometry is a comparative technique which does not measure ploidy directly. It relies therefore on comparing samples with known existing standards.

## Methods

*Passiflora* samples were taken from our living collections. In each case, several leaves were stored in plastic bags before dispatch and analysis by [Plant Cytometry Services](#), P.O. Box 299, 5480 AG Schijdel, Netherlands. Leaf material (1–2 cm<sup>2</sup>, equivalent to 50–100 mg) was chopped with a sharp razor blade into about 2ml of pH 7.5 buffer at 4°C, in a plastic petri dish. A similar quantity of internal standard (*Buxus sempervirens*) was added. The buffer, slightly modified from that described by Arumuganathan and Earle [11], contained 2mg/L of 4,6-diamidino-2-phenylindole (DAPI). This fluorescent dye selectively complexes with double-stranded DNA to give a product that fluoresces at 465 nm. DAPI has specific DNA-binding properties with preference for adenine-thymine (AT)-rich sequences. The buffer containing cell constituents and large tissue remnants was passed through a 50µm mesh nylon filter. The filtrate normally contains thousands of cell nuclei. The solution with stained nuclei is passed through a flow cytometer (CyFlow ML, Partec GmbH, Otto Hahnstrasse 32, D-4400 Münster, Germany) fitted with a high pressure mercury lamp (OSRAM HBO 100 long life) and appropriate filter-settings for excitation (UG-1[for UV] and BG-38) and emission (GG 435). The fluorescence of the stained nuclei passing through the focus of the beam from the lamp was measured by a photomultiplier and converted into voltage pulses. These voltage pulses were electronically processed using Flomax version 2.4d (Partec GmbH) to yield integral and peak signals allowing DNA histograms to be produced. A single analysis was made for each *Passiflora* sample; the coefficient of variation of the method is known to be approximately 5%.

Apart from the immediate interest in the two hybrids of uncertain ploidy (i.e., *P.* ‘Betty Myles Young’ and *P.* ‘Lambiekins’), an opportunity was taken to confirm the status of their parents and examine a limited range of other taxa. Most of the

hybrids had *P. caerulea* in their ancestry, and this was chosen as the primary diploid reference standard. *Passiflora caerulea* has been reported as diploid ( $2n = 18$ ) in the literature [1,2]. As a check on the reproducibility of the analytical method, two different samples of *P. caerulea* as well as the white variant *P. caerulea* ‘Constance Elliott’ were included. In addition, *P. actinia* (reported as diploid by De Melo [2]) and its unnamed hybrid with *P. ‘Mini Lamb’* were also tested, partly because the latter is a parent of *P. ‘Poppet’*, a complex four-species hybrid of uncertain ploidy (Figure 2).

**Figure 2. *Passiflora* ‘Poppet’.**



## Results and Discussion

The typical output histogram of the flow cytometry method is shown in Figure 3. The first peak is the internal standard (*Buxus sempervirens*), the second peak is *P. 'Poppet'*. The relative DNA mass ("Index" in Figures 3 & 5) of this hybrid is 2.72 times greater than the internal standard (= 1.00). Since the relative DNA mass, also called the DNA ratio, of the diploid ( $2n = 18$ ) *P. caerulea* is 1.62, then it may be concluded that *P. 'Poppet'* has approximately 50% more nuclear DNA, and is therefore a triploid.

Figure 3. Flow cytometry of *Passiflora* 'Poppet'.

File: Sample\_09 + int st.fcs Date: 09-12-2009 Time: 13:52:13 Particles: 6050 Acq.-Time: 82 s

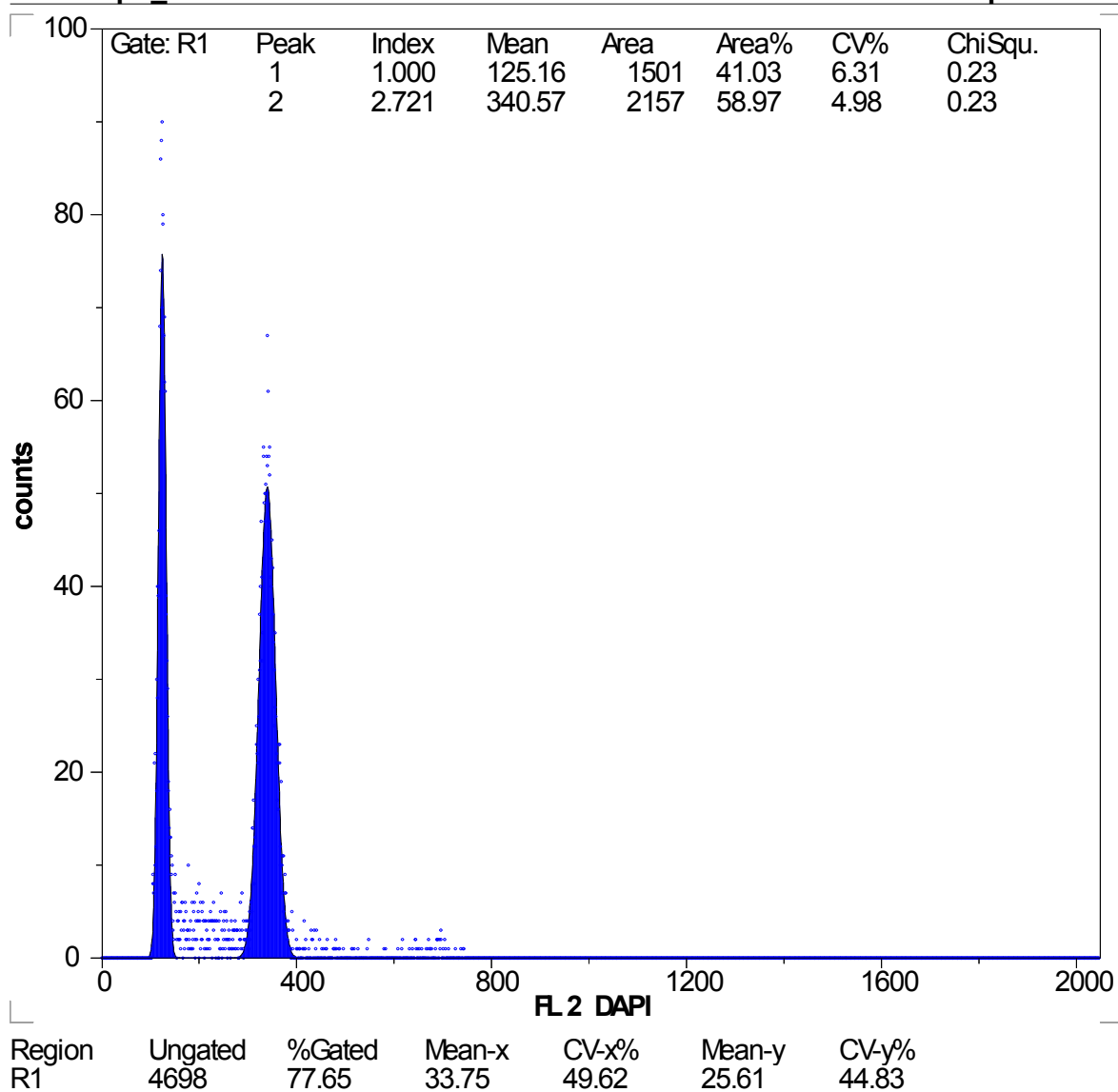


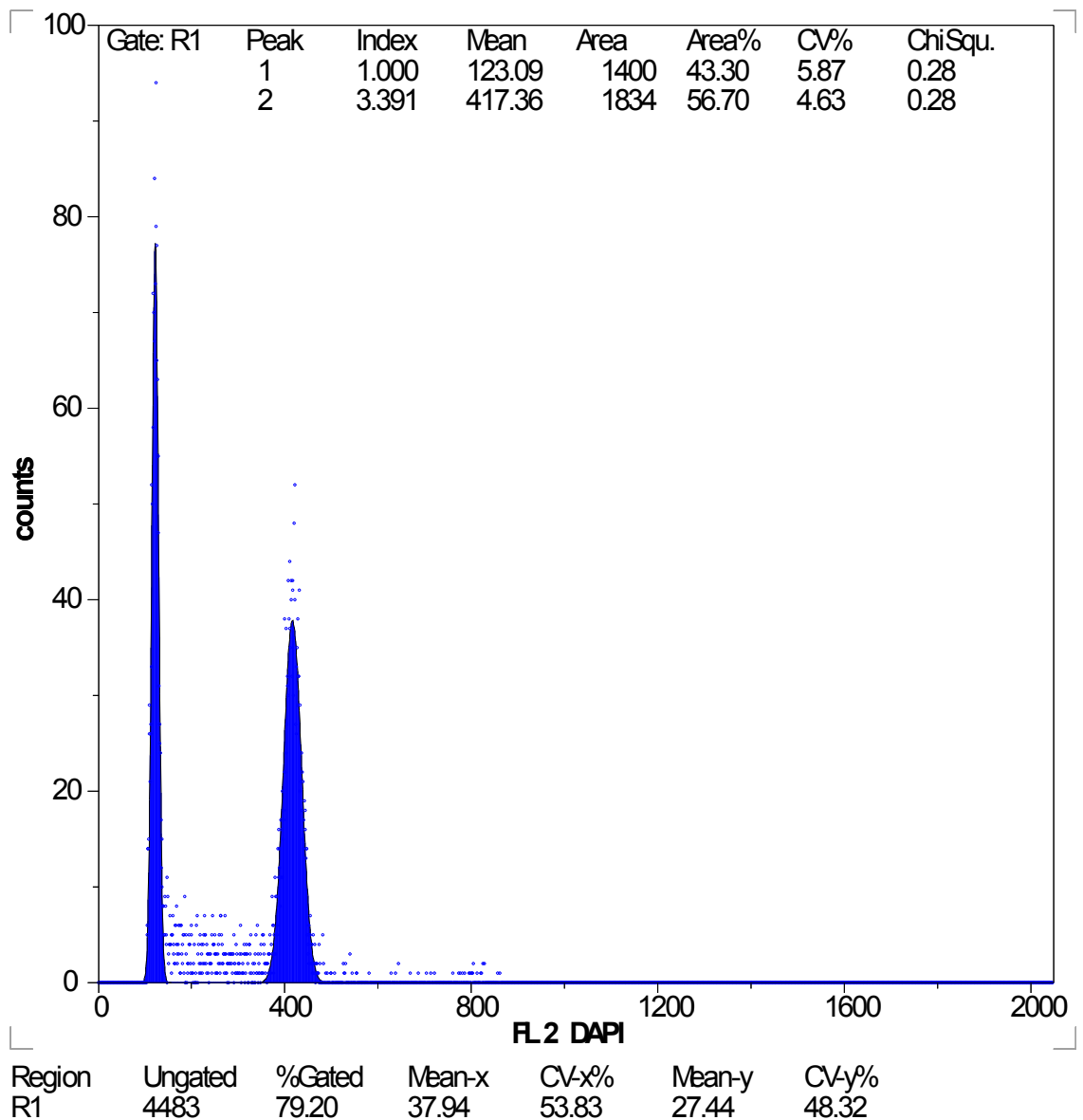
Figure 4 shows *P.* ‘White Wedding’, and its flow cytometry histogram is shown in Figure 5. The DNA mass was 3.39 times greater than the internal standard and almost exactly twice the mass of *P. caerulea*. It follows that *P.* ‘White Wedding’ is a tetraploid.

**Figure 4. *Passiflora* ‘White Wedding’.**



Figure 5. Flow cytometry of *Passiflora* 'White Wedding'.

File: Sample\_08 + int st.fcs Date: 09-12-2009 Time: 13:50:16 Particles: 5660 Acq-Time: 60 s



The DNA ratio (relative DNA mass, or "Index" in Figures 3 & 5) of all samples tested is shown in Table 1. There was good agreement between the two *P. caerulea* controls (Samples 1 and 2), each having 1.62 units DNA/cell. The DNA ratio of *P. caerulea* 'Constance Eliott' (Sample 3) at 1.60 was not statistically different from the normal forms of *P. caerulea*. The DNA ratio of *P. actinia* (Sample 4) was significantly lower at 1.10.

DAPI only binds to the bases adenine (A) and thymine (T). Since the AT frequency varies among different plant families [12], and rarely comprises 50% of the genome, the method used here does not allow absolute quantities of nuclear DNA to be calculated. However, the absolute nuclear DNA content of *P. caerulea* is known to be 2.90pg/cell [13]. Thus an approximate DAPI conversion factor of  $(2.90/1.62) = 1.80$  could be used to convert the DNA mass index into pg/cell. However, since it is not certain that the AT frequency is constant for all species in the *Passiflora* genus, the present results are left as DNA ratios representing the amount of DNA relative to the internal standard (*Buxus sempervirens*).

Samples 9 and 17 were laboratory-created by Fischer using colchicine, and samples 8, 13, and 14 were created by him using hand pollination and selection of the best offspring. *Passiflora* ‘Clear Sky’, for example, was selected for its hardiness from fifty seedlings. All these samples were presumed tetraploids, although this had not been confirmed experimentally in all cases. From Table 1 it will be seen that their DNA ratios ranged from 3.11 to 3.39 (mean = 3.29; range = +/- 5.5% of mean) units/cell, i.e. close to the amounts expected in a tetraploid if it is assumed that the other species involved in these hybrids have similar DNA ratios to *P. caerulea*. In the four hybrids (Samples 6, 10, 15, and 19), which had been created by crossing a tetraploid female parent with a diploid male parent, the DNA ratio ranged from 2.43 to 2.72 (mean = 2.54; range = +/- 4.3% of mean) units/cell. This is close to the amounts expected in a triploid, again assuming that the other parental species were homologous to *P. caerulea*.

**Table 1. The nuclear DNA mass of various *Passiflora* species and hybrids (relative to *Buxus sempervirens* = 1.0) and the deduced ploidy**

No.	Sample (Parentage is female × male)	DNA ratio	Deduced ploidy
1	<b><i>P. caerulea</i> #1</b> [Species]	1.62	Diploid control
2	<b><i>P. caerulea</i> #2</b> [Species]	1.62	Diploid control
3	<b><i>P. caerulea</i> ‘Constance Elliott’</b> [Selection of species]	1.60	Diploid control
4	<b><i>P. actinia</i></b> [Species]	1.10	Diploid
5	<b><i>P. ‘Berkeley’</i></b> (USA 2006?) [Hybrid of <i>P. subpeltata</i> ?]	2.27	Triploid ?
6	<b><i>P. ‘Betty Myles Young</i></b> (Irvine, UK 2005) [ <i>P. ‘Clear Sky’</i> × <i>P. loefgrenii</i> ‘Iporanga’]	2.50	Triploid
7	<b><i>P. ‘Blue Bouquet’</i></b> (Worley, USA 1990) [( <i>P. amethystina</i> × <i>P. caerulea</i> ) × ( <i>P. amethystina</i> × <i>P. caerulea</i> ) × <i>P. caerulea</i> ]	1.81	Diploid
8	<b><i>P. ‘Clear Sky’</i>(**)</b> (Fischer, Germany 2001) [( <i>P. amethystina</i> × <i>P. caerulea</i> ) × <i>P. caerulea</i> ] × <i>P. caerulea</i> ]	3.33	Tetraploid
9	<b><i>P. ‘Emil Kugler’</i>(*)</b> (Fischer, Germany 2000) [ <i>P. caerulea</i> × <i>P. caerulea</i> ]	3.25	Tetraploid
10	<b><i>P. ‘Lambiekins’</i></b> (Irvine, UK 2006) [ <i>P. ‘Emil Kugler’</i> × <i>P. loefgrenii</i> ‘Iporanga’]	2.43	Triploid
11	<b><i>P. ‘Lunametista’</i></b> (Vecchia, Italy 2005) [ <i>P. caerulea</i> ‘Constance Elliott’ × <i>P. loefgrenii</i> ‘Iporanga’]	1.67	Diploid
12	<b><i>P. ‘Mini Lamb’</i></b> (Irvine, UK 2005) [ <i>P. ‘Purple Haze’</i> × <i>P. loefgrenii</i> ‘Corupa’]	1.80	Diploid
13	<b><i>P. ‘Monika Fischer’</i>(**)</b> (Fischer, Germany 2003) [( <i>P. incarnata</i> × <i>P. amethystina</i> ) × ( <i>P. kermesina</i> × <i>P. caerulea</i> )]	3.11	Tetraploid
14	<b><i>P. ‘Panda’</i>(**)</b> (Fischer, Germany 2006) [ <i>P. ‘White Wedding’</i> × <i>P. ‘Monika Fischer’</i> ]	3.32	Tetraploid
15	<b><i>P. ‘Poppet’</i></b> (Irvine, UK 2008) [ <i>P. ‘White Wedding’</i> × <i>P. ‘Mini Lamb’</i> ]	2.72	Triploid
16	<b><i>P. ‘White Mirror’</i>(**)</b> (Wouters, Netherlands 2007) [ <i>P. ‘White Wedding’</i> × <i>P. caerulea</i> ‘Emil Kugler’]	3.39	Tetraploid
17	<b><i>P. ‘White Wedding’</i>(*)</b> (Fischer, Germany & Wouters, Netherlands 2004) [ <i>P. caerulea</i> ‘Constance Elliott’ × <i>P. eichleriana</i> ]	3.39	Tetraploid
18	<b>Unnamed hybrid #1</b> (Irvine, UK 2007) [ <i>P. ‘Betty Myles Young’</i> × <i>P. ‘Purple Haze’</i> ?]	2.49	Triploid
19	<b>Unnamed hybrid #2</b> (King, UK 2009) [ <i>P. ‘White Wedding’</i> × <i>P. caerulea</i> ]	2.49	Triploid
20	<b>Unnamed hybrid #3</b> (Irvine & King, UK 2009) [ <i>P. actinia</i> × <i>P. ‘Mini Lamb’</i> ]	1.43	Diploid

(\*) colchicine-created tetraploids (\*\*) hybrids from two tetraploid parents

The DNA ratio of some species and hybrids can be derived indirectly from these results. For example, *P. 'Lunametista'* (Sample 11; 1.67 units/cell) arose from the cross *P. caerulea* 'Constance Elliott'  $\times$  *P. loefgrenii* 'Iporanga'. It follows that the calculated DNA ratio of *P. loefgrenii* 'Iporanga' =  $2[1.67 - (1.62/2)] = 1.72$ . This is close to the *P. caerulea* diploid control and strongly suggests that *P. loefgrenii* 'Iporanga' is also diploid. Similarly, if it is assumed that both forms of *P. loefgrenii* (i.e. 'Corupa' and 'Iporanga') have the same DNA ratio then, from the unnamed hybrid #3 (Sample 20; 1.43 units/cell), it follows that *P. 'Mini Lamb'* =  $2[1.43 - (1.10/2)] = 1.76$  units/cell. Since *P. 'Mini Lamb'* (Sample 12) is the hybrid *P. 'Purple Haze'*  $\times$  *P. loefgrenii* 'Corupa', then *P. 'Purple Haze'* =  $2(1.76 - (1.72/2)) = 1.80$  units/cell. Finally, since *P. 'Purple Haze'* is a hybrid of *P. amethystina* and *P. caerulea*, then *P. amethystina* =  $2(1.80 - (1.62/2)) = 1.98$  units/cell. Using a DAPI conversion factor of 1.8 (see above), then the absolute amount of DNA in *P. amethystina* is approximately  $(1.98 \times 1.8)$ , i.e.  $\sim 3.56$  pg/cell. This is close to the published value for *P. amethystina* (3.36pg/cell) as presented by Souza et al. [14], and is concordant with the report that this species is diploid [15]. A collection of these derived results is shown in Table 2.

**Table 2. Estimated nuclear DNA mass of certain diploid hybrids and species (relative to *Buxus sempervirens* = 1.0; *P. caerulea* = 1.60)**

Hybrid/species	Source of estimate	DNA ratio
<i>P. amethystina</i>	<i>P.</i> ‘Purple Haze’ (see below)	1.98
<i>P. eichleriana</i>	#17 Tetraploid <i>P.</i> ‘White Wedding’ [ <i>P. caerulea</i> ‘Constance Elliott’ × <i>P. eichleriana</i> ]	1.58
<i>P. loefgrenii</i> ‘Corupa’	Assumed equal to <i>P. loefgrenii</i> ‘Iporanga’	1.72
<i>P. loefgrenii</i> ‘Iporanga’	#11 <i>P.</i> ‘Lunametista’ [ <i>P. caerulea</i> ‘Constance Elliott’ × <i>P. loefgrenii</i> ]	1.72
<i>P.</i> ‘Mini Lamb’	#20 Unnamed hybrid #3 [ <i>P. actinia</i> × <i>P.</i> ‘Mini Lamb’]	1.76
<i>P.</i> ‘Purple Haze’	#12 <i>P.</i> ‘Mini Lamb’ [ <i>P.</i> ‘Purple Haze’ × <i>P. loefgrenii</i> ‘Corupa’]	1.80

In the absence of data for certain species and hybrids, these indirect analyses serve some value. However, they should be treated with caution since experimental errors could accumulate during the calculations. Furthermore, although it is theoretically possible to derive similar results using data on triploid crosses, these may be prone to further uncertainties. For example, amongst the triploid hybrids examined here, the presence of aneuploidy cannot be ruled out. In other words, it would be quite conceivable for a triploid organism to lose one or several of the ‘redundant’ chromosomes yet retain viability. This would reduce the DNA ratio by an unpredictable amount.

Data on the DNA content of a number of other *Passiflora* species has been published by Souza et al. [16]. They found variable amounts of nuclear DNA/cell, for example: *P. edmundoi* (3.43pg); *P. nitida* (4.82pg); *P. giberti* (3.92pg) *P. edulis* f. *edulis* (3.16pg); *P. edulis* f. *flavicarpa* (3.19 – 3.21pg); *P. quadrangularis* (5.36pg); *P. galbana* (3.52pg); *P. mucronata* (3.40pg); *P. maliformis* (3.78pg) and *P. suberosa*

(1.85pg). For comparison, Ingle et al. [17] reported 3.00pg/cell for *P. antioquiensis*, while Arumuganathan and Earle [18] found 4.55pg/cell in *P. menispermifolia*.

The parentage of *P. 'Berkeley'* (Sample 5) is unknown, but from observation of its morphology it appears to be a hybrid of *P. subpeltata* and *P. caerulea* [19]. Somewhat surprisingly, the DNA ratio (2.27 units/cell) suggests that it could be a triploid.

Although it is conceivable that it could have a tetraploid species in its parentage (e.g. *P. 'Clear Sky'*), an alternative explanation is that it is diploid, but that *P. subpeltata*, reported as diploid by De Melo et al. [2], has a high DNA ratio relative to *P. caerulea*.

A further presumed triploid is shown in Table 1. Unnamed hybrid #1 (Sample 18) is the cross *P. 'Betty Myles Young' × P. 'Purple Haze?'*, i.e. [triploid × diploid]. It is vigorous and free flowering with well formed flowers. Depending on how chromosomes segregate at meiosis, this type of cross might have a number of theoretical outcomes (i.e. diploid, triploid, tetraploid, aneuploid). The fact that unnamed hybrid #1 is a triploid may be purely fortuitous. The pollen donor may indeed even have induced selfing. Both of the 'primary' triploid hybrids (i.e. *P. 'Betty Myles Young'* and *P. 'Lambiekins'*) have given rise to a number of unreleased daughter hybrids, but always when acting as female parents. Some of these subsequent hybrids, like *P. 'Lambiekins' × P. caerulea*, (see Figure 6), have appeared morphologically normal, and have grown to maturity and flowered. This confirms that some *Passiflora* triploids are fertile.

**Figure 6. Above: the hybrid *P. 'Lambiekins' × P. caerulea*; a morphologically normal progeny of a triploid female parent and a diploid male parent. Below: for comparison, two flowers of diploid *P. caerulea***



## Conclusions

The status of Fischer's tetraploids was confirmed. However, the most important finding of this research was that some *Passiflora* hybrids are partially fertile triploids. This is relatively unusual in that most plant triploids are sterile [20]. This partial fertility refers to the fact that the egg cells are capable of being pollinated, although we have no evidence that these triploids produce fertile pollen. Indeed, SEM imagery by Holthuysen [21], Philips Research, Eindhoven, shows evidence that the triploids *P.* 'Betty Myles Young' and *P.* 'Lambiekins' both have abnormalities of the pollen exine. Contrary to earlier expectations, *P. loefgrenii* 'Iporanga' was shown to be a diploid and not a naturally-occurring tetraploid. The nuclear DNA ratios of the taxa examined here are consistent with other data on *Passiflora*. Flow cytometry not only

offers a means of investigating the ploidy of *Passiflora* hybrids, but is potentially capable of throwing light on the parentage of hybrids with an uncertain ancestry.

## Acknowledgements

We thank Ir Gerard Geenen, Flow Cytometry Services, for carrying out the DNA analysis and for many helpful discussions on the technique. We also had useful discussions with David Costen, and thank him for alerting us to the potential of flow cytometry.

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