Cymbidium Chatter

A first flowering diploid seedling of Cym. Seamew.

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Contents

Latest News

Welcome to the final issue of *Cymbidium Chatter* for 2022. This issue took a little longer than anticipated to prepare, particularly as life has been very busy of late! I am expecting the first half of next year to likewise be quite demanding, so for 2023 I will be reducing the number of issues from six to four and releasing them quarterly.

I am still researching Dean Roesler's hybridising work and influence on the Australian Cymbidium scene for a future article and need photos – particularly some of him and his hybrids. If you have any, or a short story about Dean to share, please contact me at jwhite88@gmail.com. Thanks!

Hopefully you have all had a safe and happy Christmas and I wish you good growing in 2023!

Edited by Joshua White.
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Plant Profile: Orange Coral (with contributions by Allan Rae)

Allan Rae, of Tipperty Orchids in New Zealand, kindly sent me some photos and comments on this cross recently. I have added some background information on the parents to compliment them.

Allan: *I purchased a few Cymbidium flasks from Valley Orchids in 2009 in the hope of getting some oranges. Their hybrid Red Coral 'Mystery Orange' x Sundaani's Treasure 'Orange Crush' was the most obliging on that score. I registered this (with the owner's permission of course!) as Orange Coral.*

Left: Red Coral 'Mystery Orange'; Right: Sundaani's Treasure 'Orange Crush'. Photos courtesy of Graham Morris.

Cym. Red Coral was registered in 1994 and was a Norm Porter cross (Red Panther X Coratea). It has only six registered offspring – two from Valley Orchids under Graham Morris, three from Allan's Tipperty Orchids and one from Kevin Black.

Cym. Sundaani's Treasure was also from the Valley Orchids stable (eventually registered by Pauline Hockey of Sundaani Orchids in 2012) and is the cross of Pure Treasure 'Pirate' (a bright canary yellow alba) with Red Beauty 'Bronze Delight' 4n (a chance mutant from a batch of 2n clones). It is still in circulation in Australia (particularly in South Australia) and seen at shows on occasion.

Pauline Hockey has taken Sundaani's Treasure forward, with eight of the nine registered offspring being from her breeding (and five of these being registered in 2018). However, only Orange Coral has any further progeny, again from Pauline's breeding and all registered in 2022.

Allan: *[Orange Coral] has so far been awarded twice by the OCNZ. The first was 'Orange Treasure' which received an HCC in 2019, but even more orange was 'Sunrise' which received an AM this year. One of the photos shows the 6-spiked awarded plant. I have bred several oranges myself, but nothing as dark as this.*

Nado Lenkic's 2022 Seedlings (Part Two)

Nado Lenkic (of Springfield Orchids, WA) has kindly provided some photos and commentary from ten of his crosses. The first five were featured in the previous issue.

Cym. (Joan's Charisma X Brenda)

Cym. Joan's Charisma 'Vanity' 4n and Brenda 'Loveable' 4n.

Nado: *Joan's Charisma 'Vanity' seedlings vary enormously in quality, with the best (from my experience) being those from parents not possessing early flowering genes. [It] is a real Jekyll & Hyde breeder – producing either really good or really bad results, reminiscent of Wallara 'Gold Nugget'.*

Two seedlings of the cross.

Cym. Federer (De-Lovely X Yowie Rose)

Cym. De-Lovely 'BTC' 4n and Cym. Yowie Rose 'Cabernet' 4n, the parents of Cym. Federer.

***Nado:** I have a lot of time for Yowie Rose 'Cabernet'. This is the first of its progeny to bloom for me, with seedlings ranging from mid-pinks through to red shades. Classy.*

Two seedlings of Cym. Federer demonstrating the colour range of the grex.

Cym. Fremantle (West Coast X Hazel Fay)

Cym. West Coast 'Molten Gold' 4n and Hazel Fay 'Orange Squash' 4n, the parents of Cym. Fremantle.

Nado: *Aesthetically pleasing with mostly excellent presentation, in peach, orange and yellow shades.*

Two seedlings of Cym. Fremantle. The lip of West Coast seems to have dominated.

Cym. Kind (Tracey Reddaway X Gwen Thomas)

Cym. Tracey Reddaway 'Geyserland' and Gwen Thomas 'One & Only', the parents of Cym. Kind.

Nado: *Clean crisp greens and yellows with a touch of quality have resulted.*

Two seedlings of Cym. Kind.

Cym. Tytan (Titian X Royalty)


Cym. Titian 'Springfield' and Royalty 'Exquisite', the parents of Cym. Tytan.

Nado: *Vibrant miniature pinks with instant eye appeal. Have yet to see an ordinary one from this cross. [It is] rewarding to have bred both parents.*


Two seedlings of Cym. Tytan, demonstrating the compact and floriferousness nature of the grex.

A Detailed Look at Ploidy

Back in Issue 31 (June 2021), I briefly touched on the subject of ploidy in my article "Making Your Own Crosses". Recently I received a request to go into more detail, which conveniently lined up with my plans to document how to estimate ploidy using stomatal guard cell measurements. So, in this article, we will be taking a deeper dive into ploidy – its definition, history, and significance.

Chromosomes and Ploidy

Chromosomes are the individual DNA molecules that make up the genetic code for living things. Normally these are grouped into homologous sets, where homologous means that each chromosome in one set matches up neatly with its corresponding partner(s) in the other sets. The number of these homologous chromosome sets is called ploidy, whilst the number of chromosomes in each set is represented by the letter *n* (this allows for the same notation to be used across biology and botany, as the number of chromosomes in varies widely in the natural world).

There is no requirement for *n* to be an even number, nor even uniform across a genus. Whilst Cymbidiums are fortunately consistent with *n* = 20, Paphiopedilums have at least seven different values for *n* (although usually these are more stable within a section). The popular bearded Irises also have several different chromosome counts within the genus. These differences often lead to fertility challenges between groups with different values for *n*.

In nature, Cymbidiums (and most other orchids) have just two sets of chromosomes, otherwise known as diploids or 2n. However, over the last century, the combination of chance and deliberate breeding has given rise to polyploidy (higher levels of ploidy above 2n), and each of these multiples of *n* has its own name:

Ploidy	Name
1n	Haploid or Monoploid
2n	Diploid
3n	Triploid
4n	Tetraploid
5n	Pentaploid
6n	Hexaploid
7n	Heptaploid or Septaploid
8n	Octoploid

Cymbidiums have been recorded with every ploidy from 2n to 6n (with 8n reported not to survive the flasking process). Plants that are missing or possess a few extra chromosomes also exist, often from combinations of mismatched ploidy levels. These abnormalities are called *aneuploids* (from *euploid* meaning an exact multiple of the haploid chromosome count). Aneuploids often suffer from vigour and fertility issues and so generally ought to be avoided in any breeding program. Plants with odd ploidy (3n and 5n) also suffer fertility issues, as they are almost never fertile as pollen parents and only have limited fertility as pod parents. In most cases, diploids and tetraploids are the best for breeding.

Ideally, one would keep the ploidy of both parents consistent when making a cross. Except for the rare exception, the offspring will all be the same ploidy as the parents. Mixing ploidies in Cymbidium crosses can produce a range of results, as shown in the table below.

Pod Parent	Pollen Parent	Result
2n	3n	Exceedingly rare, but can produce 2n, 3n, 4n and aneuploids
2n	4n	3n
2n	5n	Exceedingly rare; outcome unknown
2n	6n	4n
3n	2n, 4n	Possible, but usually little to no seed produced and highly dependent on compatibility of parents. The same cross may work one year but not another. Produces aneuploids, 2n, 3n and 4n.
3n	3n, 5n	Extremely unlikely to produce viable seed
4n	2n	3n
4n	3n	Exceedingly rare, but can produce 3n, 4n and aneuploids
4n	5n	Exceedingly rare; aneuploids
4n	6n	5n
5n	2n	?
5n	3n, 5n	Extremely unlikely to produce viable seed
5n	4n	Little to no seed; will produce aneuploids
6n	2n	4n
6n	4n	5n

The majority of Cyms these days are tetraploids, as the higher ploidy increases flower size and substance and thus flower longevity. The presence of four sets of chromosomes instead of two also increases the possible number of gene combinations and introduces the concept of partial dominance, where the degree to which a trait is expressed depends on how many copies of the gene (1 to 4) are present.

However, since most species are still only found as diploids (some have been converted to tetraploid status; see below) and many older hybrids were diploid, the hybridiser only has two choices when wishing to introduce genes from a diploid line into a tetraploid one:

1. Self or clone the diploid (selfing may not be an option if it is a hybrid) and chemically treat the seedlings to induce tetraploidy in a percentage of them. The best tetraploid can then be used for breeding, but this means 4 to 6 years before the desired cross can be made.
2. Make the cross between the diploid and tetraploid parents and chemically treat the seedlings. This will yield a few hexaploids (6n) which can be crossed with a diploid to get back to tetraploids in the second generation. It is worth noting that the tetraploid parent will dominate over the diploid in the F1 and the hexaploid even more so in the F2.

The approach taken really depends on what the hybridiser is trying to achieve and which traits they are seeking from the diploid parent.

Table: Cym. species known to be converted to tetraploid status.

Species	Named 4n Clones	Comments
Cym. dayanum var. angustifolium		Extant in Australia
Cym. devonianum		Extant in Australia
Cym. eburneum	'New Horizon', 'TR2'	Extant in Australia

Cym. ensifolium		
Cym. erythrostylum	'Claude', 'Dale', 'Springfield', 'Tikitere'	Extant in Australia
Cym. floribundum		Both colour forms are extant in Australia
Cym. hookerianum		Held in a private collection
Cym. insigne	'Best Pink'	Extant in Australia
Cym. iridioides		Made by NHO and probably no longer in existence
Cym. lowianum	'Comte d' Hemptinne'	'CdH' exists as both 2n and 4n forms and both are in Australia
Cym. madidum	'New Horizon'	Extant in Australia
Cym. mastersii	'Supremo'	Extant in Australia
Cym. parishii var. sanderae	'Emma Menninger'	Selfings of 'EM' were made available in Australia
Cym. suave		Held in a private collection
Cym. suavissimum		Made by NHO and probably no longer in existence
Cym. tigrinum		
Cym. tracyanum	'New Horizon Alba', 'New Start', 'Tetra Royale'	Both colour forms are extant in Australia; Royale Orchids has offered 4n seedlings in the past
Cym. wenshanense		Extant in Australia

A Brief History of Polyploidy

Polyploidy arose early on in Cymbidium breeding, with several chance tetraploids being notable for their qualities at the time. The first was Cym. Alexanderi 'Westonbirt' FCC/RHS in 1922, which originated from a diploid cross and quickly became famous. Since it appeared on the scene, it has been one of the most used selections in breeding and produced a long line of progeny.

The tetraploid Cym. Alexanderi 'Westonbirt' (left) and a triploid Alexanderi (right) for comparison.

Cym. Pauwelsii 'Comte d' Hemptinne' FCC/RHS followed in 1931, this time the result of a triploid X diploid cross (*insigne* 'Bieri' X *lowianum* 'St. Denis'). It, too, lead to a significant number of polyploid offspring and the cross with Alexanderi 'Westonbirt' (Cym. Swallow) became famous in its tetraploid iteration. Greig Russell extensively researched Pauwelsii 'CdH' and its impact on breeding, which you can read at http://www.geocities.ws/pennypoint9/pauwelsii.html.

The chance tetraploid Cym. Rosanna 'Pinkie' was also awarded FCC/RHS in 1931 (Alexanderi X Kittiwake). Of its many offspring, Cym. Balkis (the backcross to Alexanderi) was the most famous and garnered the most awards. 'Pinkie' survives to the present day, although in only perhaps a handful of collections.

Cym. Rosanna 'Pinkie' (photo courtesy of Andy Easton).

Babylon 'Castle Hill' (reg. 1942) was another famous counted 4n that originated from Olympus 'Monarch' X Pauwelsii 'Comte 'd Hemptinne', probably a triploid X tetraploid cross. The grex was extensively used in breeding, although few of its progeny remain today. Cym. Vieux Rose and Burgundian are two well-known offspring still extant in Australia, whilst Babylon 'Castle Hill' still exists overseas.

The next breakthrough chance tetraploid Early Bird 'Pacific' first appeared in 1946, when it was awarded AM/RHS. Although registered as (Edward Marshall X *erythrostylum*), it is very likely that it instead originated from the crossing of Atlantes (3n) X *erythrostylum* (2n). Again, it has produced a long line of progeny, predominantly through Cyms. Stanley Fouraker, Fred Stewart and Earlyana.

A partially open bloom of Cym. Girrahween 'Enid' FCC/NSW

With a limited number of tetraploids available early on and initially no straightforward method of counting chromosomes, many crosses between plants of different ploidies were made. Aneuploids, triploids, tetraploids and even pentaploids were produced, occasionally giving rise to awarded plants. One such famous aneuploid was Cym. Girrahween 'Enid', the first Australian FCC in 1944. Its sibling, the yellow 'Gloria', was awarded an AM in the following year. 'Enid' had 76 chromosomes whilst 'Gloria' only had 69; the grex overall suffered poor fertility and only produced one registered hybrid. This chromosomal mess came about from the unlikely cross of Alexanderi 4n X Flamingo 5n yielding the aneuploid Flamenco pod parent, which was partnered with a diploid *lowianum* to make Girrahween. It serves as a cautionary tale for why breeders should avoid aneuploids.

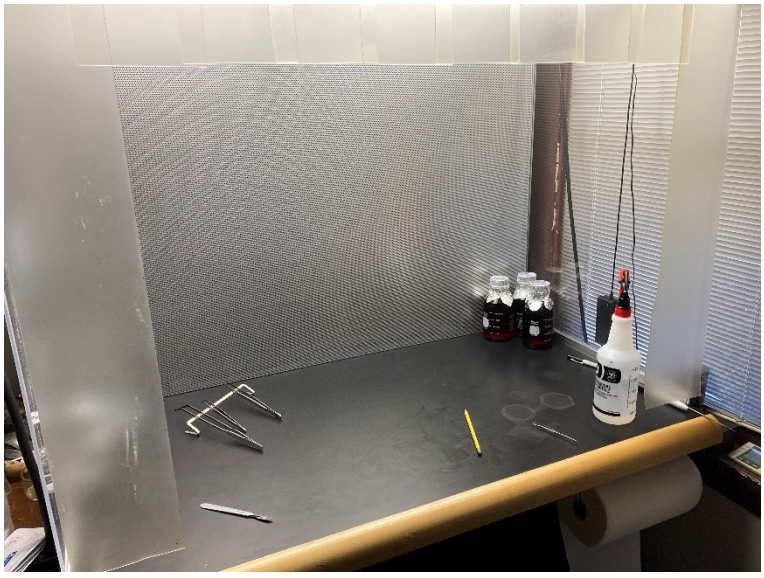
By the late 1940s, there were an abundance of polyploid and aneuploid Cyms in existence and the technology to carry out chromosome counts was becoming available. A flurry of activity result around the 1950s, with selections from species and hybrids available at the time were counted by Wimber himself, Gus Mehlquist, Emma Menninger, E. Wells and others. Kenneth Leonhardt collated much of this information as part of his PhD dissertation in 1977, which is still available online[1].

[1] Leonhardt, K.W. 1977. *Chromosome Numbers and Cross Compatibility in the Genus Cymbidium and some related Tropical Genera.* Available at: https://scholarspace.manoa.hawaii.edu/items/764e1acc-125c-4870-96fa-6dc46425b9be

The rise of chance tetraploids gave polyploid breeding programs an excellent start, but it wasn't until Donald E. Wimber and Ann Van Cott published their paper *Artificially induced polyploidy in Cymbidiums* at the Fifth World Orchid Conference in 1966 that it took off and the focus began to shift to working predominantly with tetraploid plants. Don had already published a method for mericloning Cymbidiums in 1963[2], building upon the earlier work of G. Morel who first published a technique in 1960[3]. Combining this knowledge with the colchicine treatment he and Ann had researched, it was now possible to readily convert existing diploid plants to tetraploids.

The increasing numbers of tetraploids prompted much discussion and comparisons with their diploid counterparts in the 1970s and 1980s, including articles in AOR by Merv Dunn[4,5] and Alvin Bryant[6]. Flower substance, longevity and colour were often improved in the converted tetraploids, although flower count normally dropped.

Interactions with Alan Moon at the Eric Young Foundation and Prof. Don Wimber, as well as a 1984 paper by Prof. Henry Wallbrunn[7], prompted Robert "Bob" Hamilton[8] of the University of California, Berkeley, to begin his own ploidy conversions using colchicine in 1985. In discussions with Bob, he explained that based his approach on that of Don Wimber, et al, and treated a range of genera. Several of the plants arising from his work went on to receive awards for the EYOF.



Bob Hamilton's laminar flow cabinet in his lab. Photo courtesy of Bob Hamilton.

By the early 1990s, research into the use of oryzalin as an alternative to colchicine for ploidy doubling was underway. Jaap van Tuyl, Bertus Meijer and Maria van Diën presented their work on its application in Liliums and Nerines at the 1992 International Symposium on Flowering Bulbs[9]. This prompted Bob to start his own experiments with oryzalin around 1998, using Surflan (an antimitotic herbicide with oryzalin in glycerol as the active ingredient). He had also read other papers

[2] Wimber, D.E. 1963. *Clonal multiplication of Cymbidiums through tissue culture of the shoot meristem*. AOS Bulletin, Vol. 32. Available online at: https://www.osti.gov/biblio/12491054

[3] Kerr, R. 1968. *Wonderful World of Meristems: A Symposium of Significant Excepts.* AOR Vol. 33 No. 2 (June 1968), available online at: https://archive.org/details/australianorchi3234orch

[4] Dunn, M. 1978. *Revelations*. AOR Vol. 43 No. 1 (March 1978), pg. 24-26. Available online at: https://archive.org/details/australianorchi4143orch

[5] Dunn, M. 1979. *The Case for Colchicine*. AOR Vol. 44 No. 3 (September 1979), pg. 145-149. Available online at: https://archive.org/details/australianorchi44orch

[6] Bryant, A. 1980. *Tetraploids Unlimited*. AOR Vol. 45 No. 2 (June 1980), pg. 109-111. Available online at: https://archive.org/details/australianorchi44orch

[7] Wallbrunn, H. 1985. *The art and science of orchid hybridising*. In Proceedings of the Eleventh World Orchid Conference, March 1984.

[8] Bob notes that there is another Robert M. Hamilton involved with orchids who goes by "Bert" and lived in Vancouver.

[9] van Tuyl, J.M., Meijer, B. and van Diën, M.P., 1992, May. *The use of oryzalin as an alternative for colchicine in in-vitro chromosome doubling of Lilium and Nerine.* In VI International Symposium on Flower Bulbs 325 (pp. 625-630). Available on at: http://www.liliumbreeding.nl/oryzaline.pdf

which reported on the use of amiprophos-methyl (APM), but this was not readily available whilst Surflan could be purchased at local hardware stores.

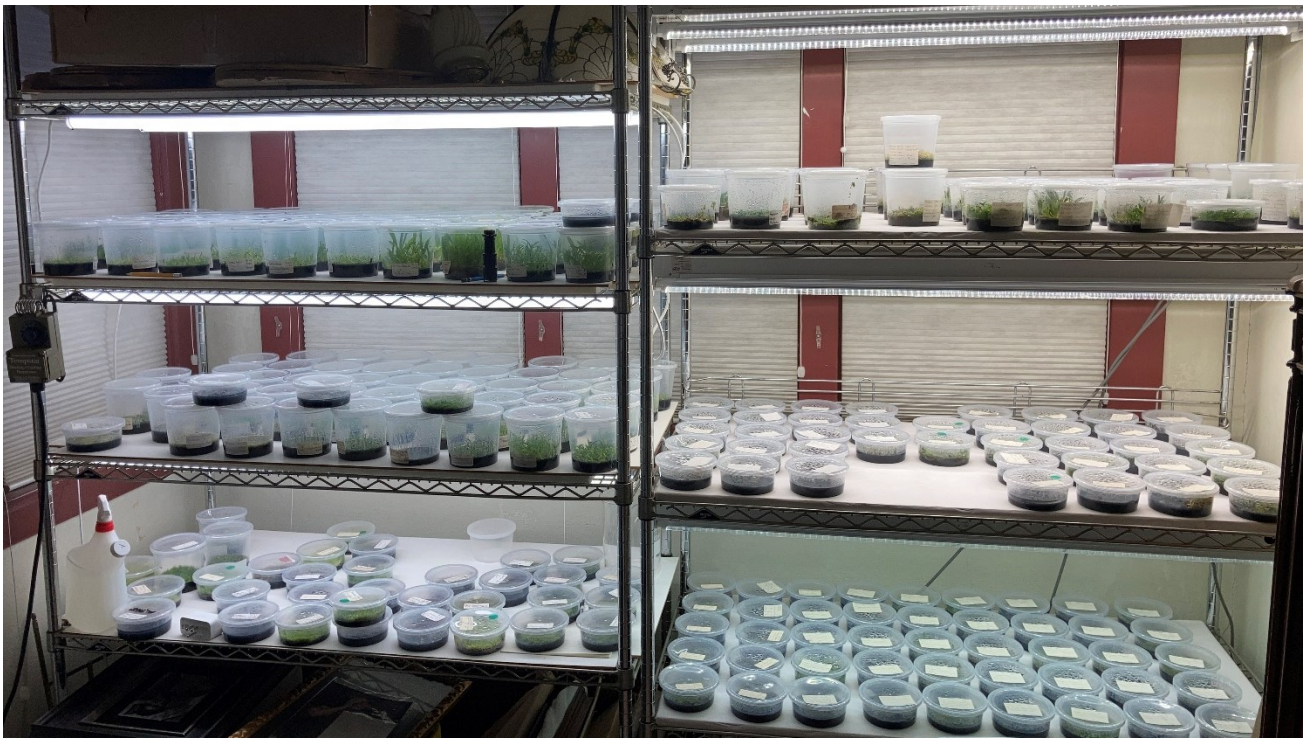
Bob's initial efforts with oryzalin replicated the Dutch protocol published by van Tuyl, et al, which he found to be phytotoxic to orchid protocorms. He soon reached the following conclusions:

1. Oryzalin is not heat labile and is stable in solution as long as it is protected from light (Bob uses amber bottles).
2. Given a low mitotic index (the rate at which cells divide), Orchids needed a much longer exposure time than Liliums and a much lower concentration of oryzalin than the Dutch used (the use of DMSO by van Tuyl was to increase the amount of oryzalin dissolved in the solution beyond its room temperature solubility of ~85 µM).
3. For 12-16 of days of exposure, there was no need for the presence of basal salts (i.e., orchid culture media).

Bob also kindly shared his protocol for oryzalin treatment, which the reader can find later in this issue.

Saturated oryzalin solution in a clear bottle to demonstrate precipitation. Photo courtesy of Bob Hamilton.

Oryzalin has now become the go-to for ploidy conversion, displacing the previously popular colchicine (although colchicine was still in use in Australia as recently as 2021, when Pauline's lab ceased operating). Today's hybridisers work predominantly with tetraploid lines, many of which have been at a tetraploid level for generations. Still, when working with species or earlier hybrids, diploid crosses are common. It is a good idea to have at least some seedlings from a diploid or triploid cross treated to provide the breeder with more options when making crosses for the next generation.



Bob and John's light racks populated with recently sown flasks. The racks are fitted with 5500-6500K LEDs and a thermostat switches off the lights when temperatures exceed ~28°C. Photo courtesy of Bob Hamilton.

Methods of Determining Ploidy

The most accurate method is counting chromosomes from root-tip samples, a tedious process that requires the appropriate equipment and chemicals (these are used for staining the chromosomes to make them readily visible). However, there are other approaches that can be used.

Cym. erythrostylum *2n (left) and* Cym. erythrostylum *4n (right).*

The most accessible method is examining the morphology – converted plants will have more substance, as the cell volume necessarily increases to accommodate the additional sets of chromosomes. One of the easiest parts of the plant to compare are the roots and the thickness can be used as an indicator of ploidy. It is important to keep in mind that root thickness varies across the genus, though, so comparisons between different species and primary hybrids is often not a reliable indicator. This method is best used when comparing seedlings from the same cross or clones.

A flask of treated plantlets from Bob Hamilton's work. Note the variation in root thickness indicative of 2n vs 4n. Photos courtesy of Bob Hamilton.

Another approach lies roughly half-way between the previous two – that of measuring stomatal guard cell length, which Don Wimber published in an article in the May 1967 Cymbidium Society News. Whilst not able to give an exact chromosome count, it provides a quicker and easier method of estimating ploidy. As with morphology, this relies on the fact that the cell volume is proportional to the amount of genetic material and hence can be used as a proxy measure for estimating the ploidy of a plant. Unfortunately, the range of possible cell sizes for each ploidy level overlap, so again this method is best used to compare clones or seedlings from the same cross. The hobbyist or enthusiast who is so inclined can do this themselves at home with the right setup.

A fourth method extends the stomatal guard cell approach to counting chloroplasts within the guard cells. This was in use in the 1970s for determining the ploidy of cotton[10] and is still used today, although I was not able to locate any published research where it has been applied to orchids. This approach has been reported to be more accurate than stomatal guard cell length, as the published results from measuring genera such as *Gossypium* (Cotton) and *Acacia* (Wattle)[11] indicate little to no overlap in the counts between diploid and tetraploid specimens.

[10] Chaudhari, H.K. and Barrow, J.R. 1975. *Identification of Cotton Haploids by Stomatal Chloroplast-count Technique*. Crop Science, 15: 760-763. https://doi.org/10.2135/cropsci1975.0011183X001500060006x

[11] Beck, S.L., Fossey, A. and Mathura, S. 2003. *Ploidy determination of black wattle (Acacia mearnsii) using stomatal chloroplast counts.* South African Forestry Journal, 2003(198). Publish online at https://hdl.handle.net/10520/EJC33929

Measuring Stomatal Guard Cell Length to Estimate Ploidy

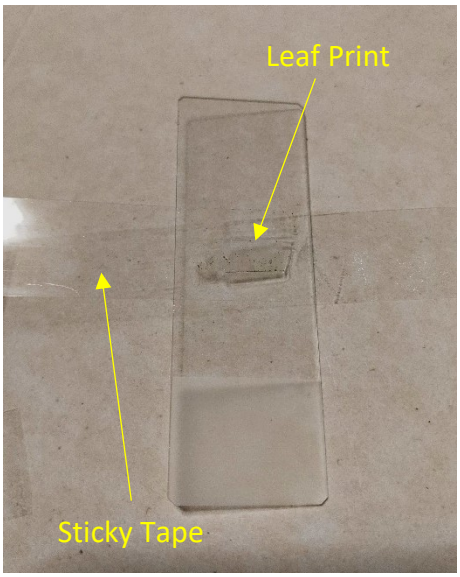
My venture into stomatal guard cell measurements started when I read Grieg Russell's article on the process[12]. Today's technology allows for easier measurements than what Grieg had to do back in 2003-2004, as it is now possible for the average person to get microscopes with digital camera eyepieces for viewing and capturing images on a computer. Some low-end models do not have adequate magnification with their eyepieces, however, so it is important to review the specifications carefully when seeking a microscope for this purpose.

The minimum requirements are:

- A microscope capable of 400x magnification (40x objective + 10x eyepiece)
- A micrometre/graticule/calibration slide
- Slides for samples
- Clear nail polish (Grieg mentions some alternatives in his article)
- High-quality clear sticky tape

Sample Preparation

There are two ways to prepare a sample for inspection under the microscope. One is to take an epidermal peel, where you physically remove the outermost layer of the leaf and place it on the slide. This is not ideal when dealing with orchids, as it requires either cutting the leaf to obtain a sample or causing a significant surface wound.



A sample being attached to a slide.

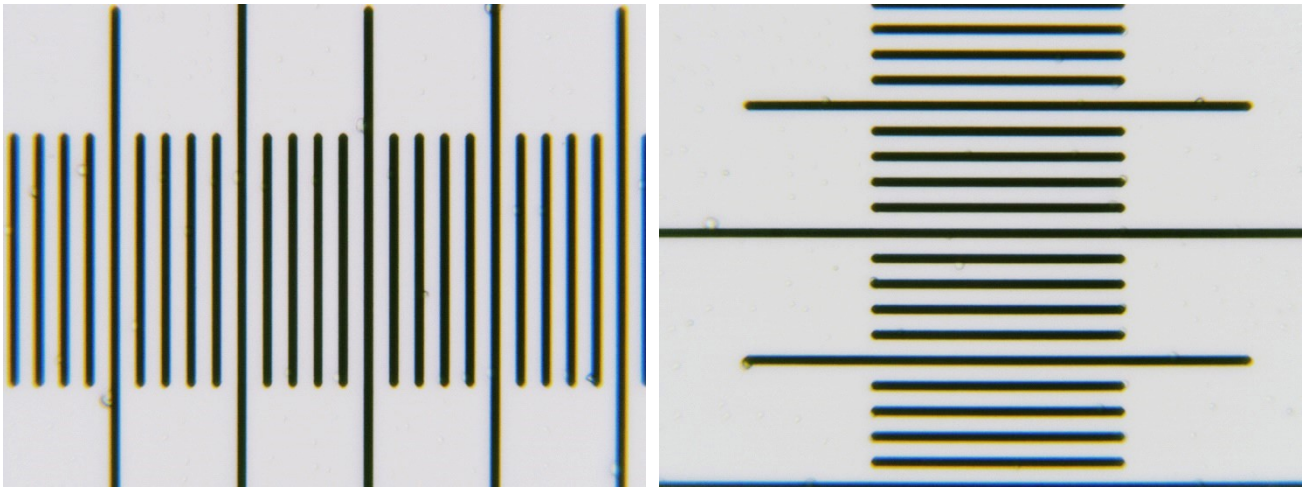
The second approach – favoured by Grieg and myself – is to create an impression or print of the leaf surface. Simply apply a thin coat of clear nail polish to a small area (no larger than 1x1cm) of the underside of the leaf. Once dry, place a section of clear tape over the top, allowing enough excess tape so that you can handle it without leaving fingerprints around the sample. You should then be able to peel the nail polish off the leaf and stick it down to the upper surface of your sample slide. It is important to get the leaf print centred and flat on the slide, as there needs to be sufficient tape to secure all the edges. Trim any excess tape off.

Capturing Images

Depending on your microscope, you may have a digital camera eyepiece or an attachment to allow a smartphone to be used in conjunction with the microscope. In my case, the digital camera eyepiece was partnered with ImageView, a program for capturing images and video. Before imaging any slides, it is a good idea to calibrate your microscope so that you know how many pixels in the image correspond to a real-world distance.

[12] Russell, G. 2003. *Stomatal Guard Cell Measurements Using Leaf Prints*. Available at: https://www.geocities.ws/pennypoint9/stomata.html

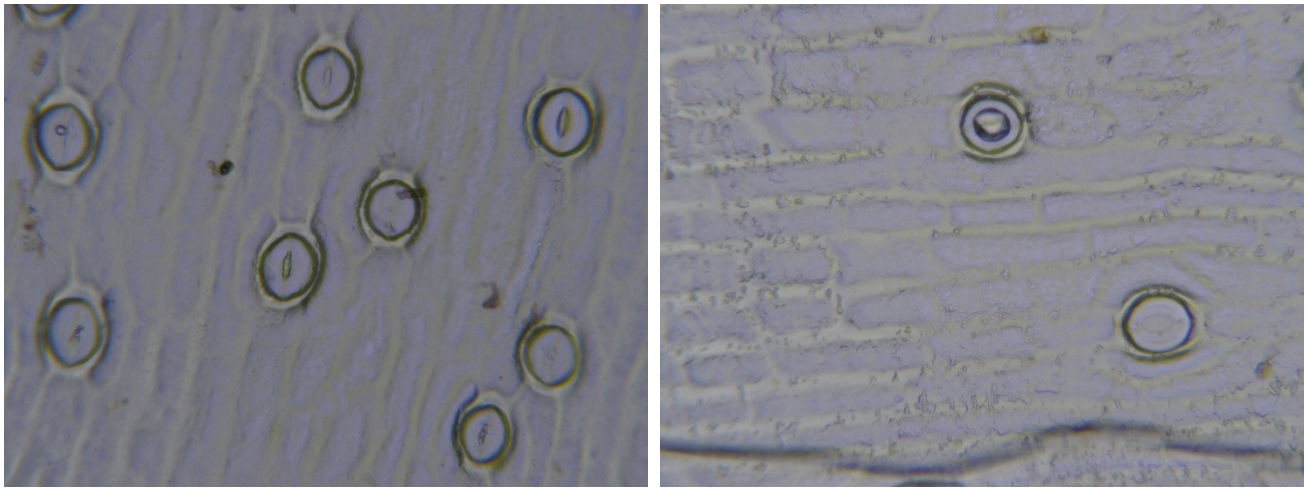
Place your calibration slide on the stage of the microscope and adjust the focus to get a clear, sharp image, then take a photo with the software or phone. Make sure you take photos of both the vertical and horizontal lines on the slide, as not all cameras will be symmetric in both axes. For example:



10-micron divisions on the calibration slide when viewed through the 40x objective and digital camera eyepiece.

Depending on your camera, you may find the edges slightly blurry or show chromatic aberration, where the colours start to separate (you can see an example of this in the photos above). Make note of the worst areas, as you don't want to be taking measurements there (in my case, you can see that the last two divisions towards the bottom and right of the frame suffer the worst chromatic aberration and so is not a good region of the image to use).

You can now place your prepared sample slide on the microscope stage and start capturing images. Move the slide around using the stage controls, as it is important to photograph a significant number of cells to get a statistically valid measurement. Some areas of the leaf print will be sparsely populated with stomata, whilst others will have many in the field of view.

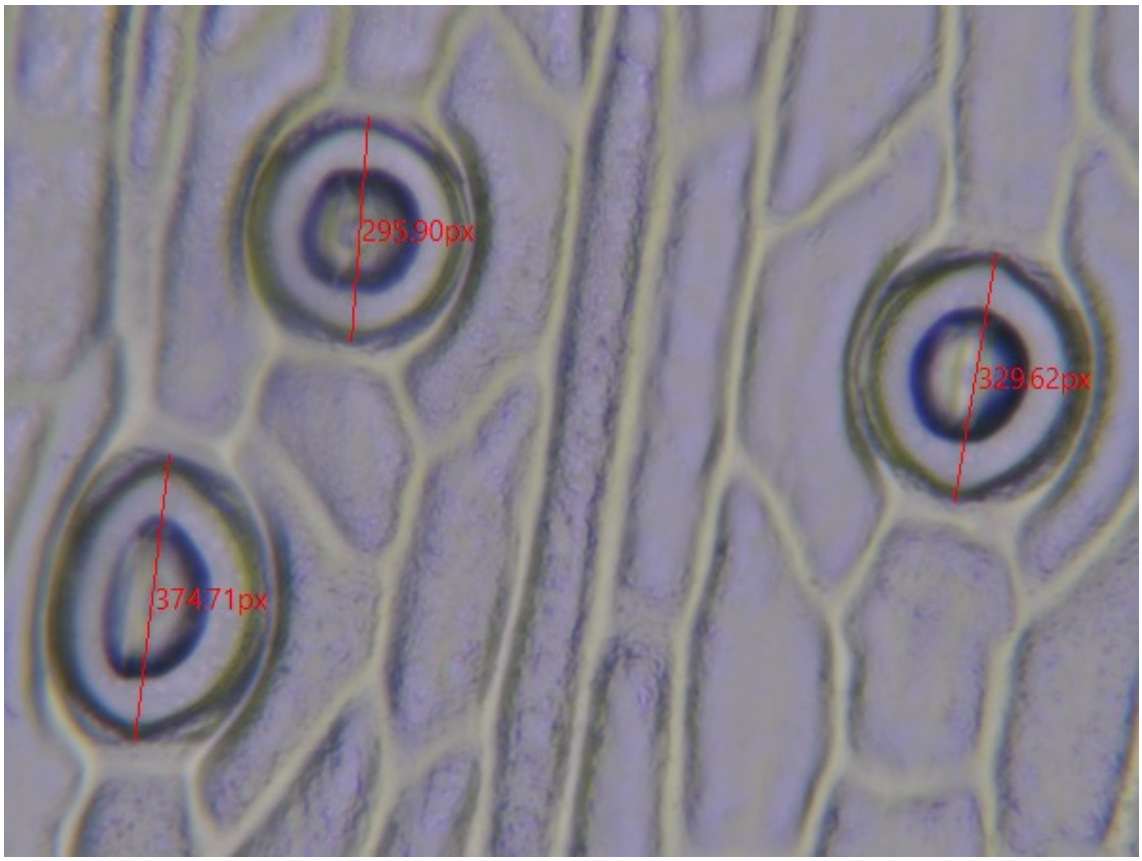


Examples of stomata from diploid (2n) plants. Only the top right stoma is open.

Depending on the plant and the conditions, the number of open stomata will vary. Across all the plants I have measured, I have found no great difference in the cell length between the open and closed stomata – the closed stomata tend to be about half a micron shorter than the open stomata, as more of the cell volume is used in closing the opening.

Capture at least 50 stomata in order to get a representative sample of the stomatal guard cell population. Measure the guard cell length using whichever software is most convenient (in my case

ImageView), taking the measurement across the stomata as shown. Sometimes the ends of the stomatal guard cells are obvious, other times not (in the image below, you can see that the axis of the stomata runs almost vertically and there is a bulge at this point where the two stomatal guard cells are in contact with each other).



Example screenshot showing the raw measurement (in pixels) of stomatal guard cells.

Once a sufficient number of stomata have been measured, calculate the average cell size to get the final result. I have calculated both the median and mean (average) for all the plants I have examined thus far and the two values rarely differ by more than three tenths of a micron.

Estimating Ploidy

I noted that the diploid cell size was not as narrowly constrained as what Grieg Russell had found – the average cell size for untreated plants (so either expected or known diploids) ranged in size between 22 and 27 microns, although the majority favoured 25 to 26 microns. Even within the same species, there was variation of a few microns. This means that one can expect higher ploidies to vary in size as well. We know that cell volume is approximately proportional to quantity of genetic material, such that:

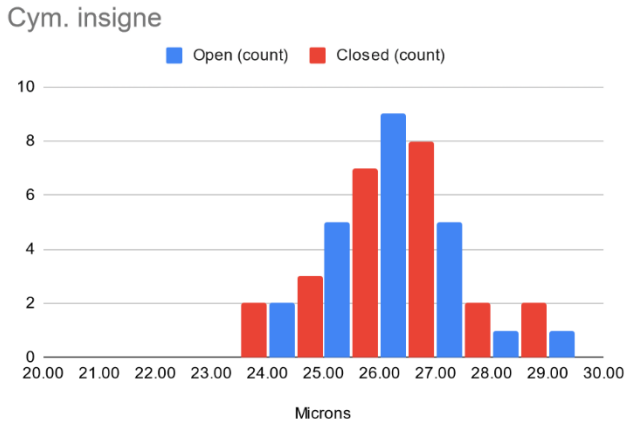
$$r = \sqrt[3]{\frac{n}{2}}$$

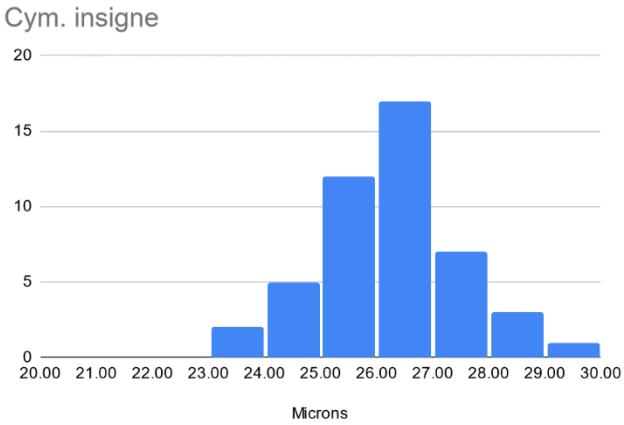
where *n* is the ploidy (e.g., 2 for diploid, 3 for triploid, etc.) and *r* is ratio of the cell size to the diploid cell size. This yields the following estimates for the range of cell sizes in higher ploidies:

Ploidy	Ratio	Diploid Cell = 24µ	Diploid Cell = 25µ	Diploid Cell = 26µ	Diploid Cell = 27µ
2n	1	24µ	25µ	26µ	27µ
3n	1.145	27.47µ	28.62µ	29.76µ	30.91µ

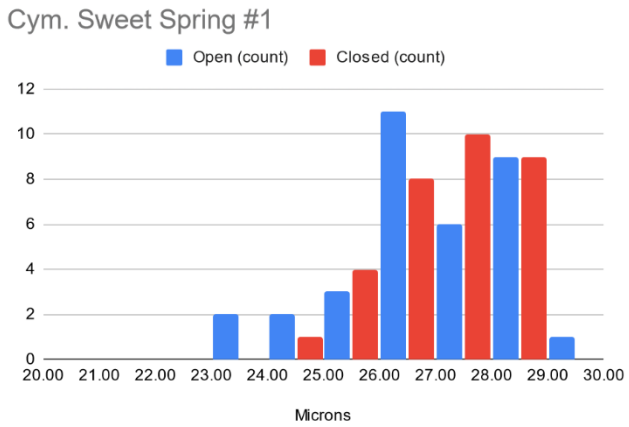
4n	1.26	30.24µ	31.50µ	32.76µ	34.02µ
5n	1.357	32.57µ	33.93µ	35.29µ	36.64µ
6n	1.442	34.61µ	36.06µ	37.50µ	38.94µ
7n	1.518	36.44µ	37.96µ	39.48µ	40.99µ
8n	1.587	38.10µ	39.69µ	41.27µ	42.86µ

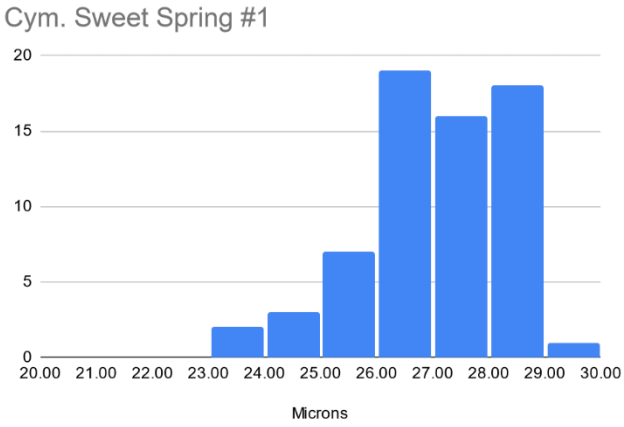
With these values, we now have a reference to use with any measurements taken. It is important to collect enough measurements to get a statistically meaningful average, as cells vary in size within a plant and, at least in untreated plants, approximate a normal distribution curve (which is why the mean and median values are very close). If you don't take enough measurements, you may find your results skew high or low and give a misleading result. Below is a pair of histograms that show a particularly clean distribution of cell sizes from a diploid plant (in this case, *Cym. insigne*).





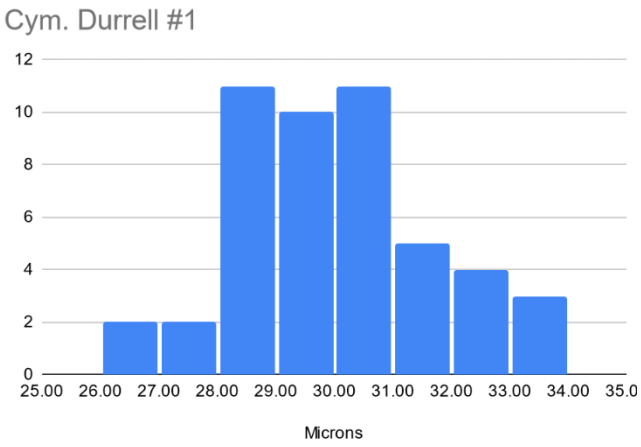
Treated plants sometimes have a recognisable normal distribution, but often not:



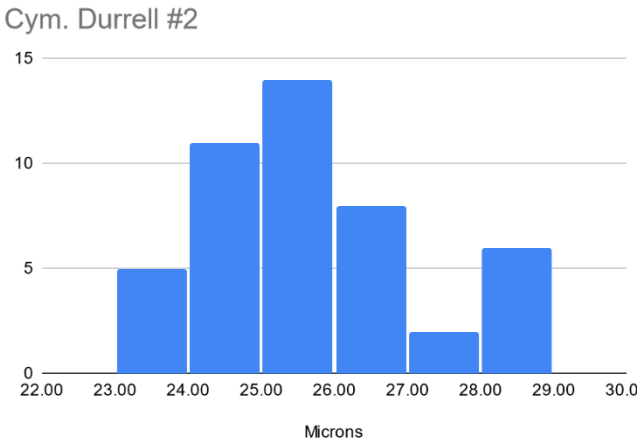


It is my hypothesis that this is because not all cells are converted when exposed to colchicine or oryzalin. As a result, the first generation of treated plants will have cells of varying ploidies and tend to favour one ploidy in particular (which will likely determine what it breeds like).

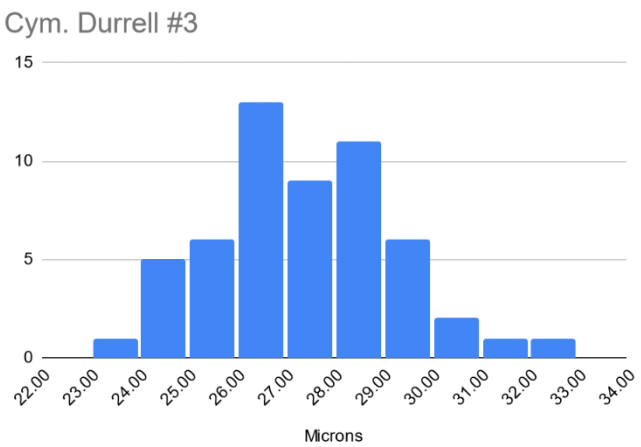
With this variation, it can be challenging to identify some treated plants. Here are the results from six seedlings of a diploid cross (Cym. Durrell) that was treated with oryzalin:



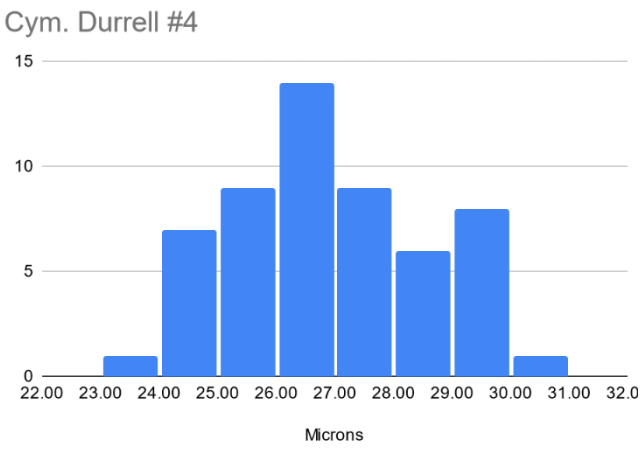
Durrell #1. Mean cell size 30.01µ, median cell size 29.81µ. Appears to be pollen and pod fertile. Likely 4n.



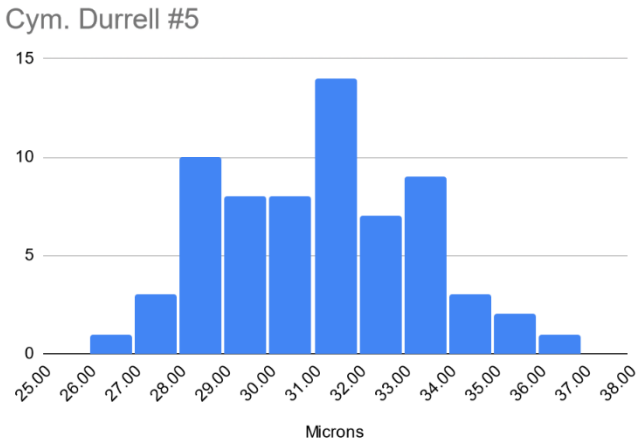
Durrell #2. Mean cell size 25.72µ, median cell size 25.49µ. Fertility untested. Clearly 2n.



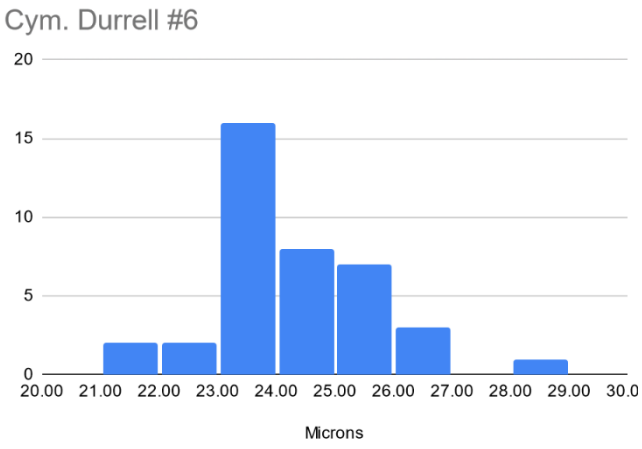
Durrell #3. Mean cell size 27.39µ, median cell size 27.28µ. Fertility untested.
Not sure which ploidy it will favour if used as a parent, although I suspect that it favours 2n based on the histogram.



Durrell #4. Mean cell size 26.92µ, median cell size 26.70µ. Fertility untested. Likely 2n.



Durrell #5. Mean cell size 31.18µ, median cell size 31.41µ. Appears to be pod and pollen fertile. Clearly 4n.



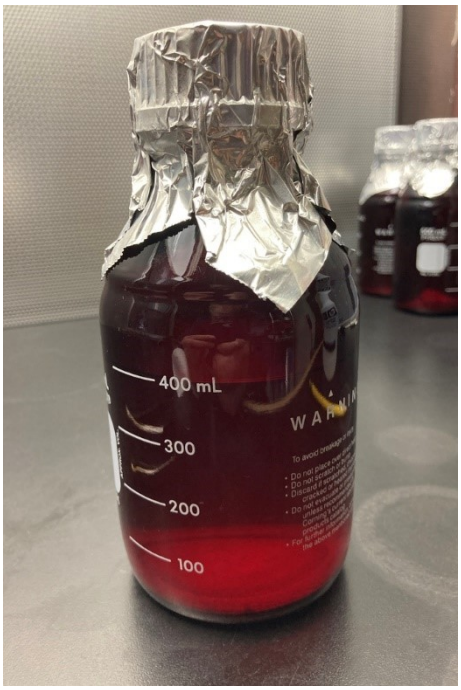
Durrell #6. Mean cell size 24.37µ, median cell size 23.98µ. Appears to be pod and pollen fertile. Clearly 2n.

Seedlings #3 and #4 are interesting cases, as from the histogram it is possible to identify how two separate normal distributions (one for 2n cells centred around 26µ, the other for 4n cells centred around 29-30µ) overlap to create the profile observed. Test crosses would be required to ascertain whether they breed as 2ns (which I suspect) or 4ns.

A Protocol for the use of Oryzalin to Double Orchid Chromosomes – Bob Hamilton

The following steps outline the use of the herbicide Surflan (40.4% oryzalin, the balance glycerides) to double the chromosome numbers of orchid plants. The room temperature solubility of oryzalin in water is ~2.5 mg/litre, which results in a sufficient concentration for effective orchid doubling. Oryzalin's low solubility simplifies using this chemical as a saturated solution is ideal for the effort. The preemergent herbicide Surflan is considered one of the more environmentally safe herbicides.

1) Add ~5-6 ml of Surflan to one litre of distilled water. This amount will exceed the room temperature solubility of its active ingredient, oryzalin. Oryzalin is a vivid orange chemical which gives Surflan its colour. Surflan is also a viscous liquid.
2) Autoclave the solution at 15 psi (103.5 kPa) for 30 minutes. I preheat the solution to ~90°C before placing its container in a pressure cooker to assure the solution reaches the 121°C of an autoclave at 15 psi in a reasonable amount of time and remains at that temperature for the duration of the sterilization cycle.
3) Allow the solution to cool to room temperature, being careful not to agitate the container. Within a few days a precipitate will form at the bottom of the container. This is because the solution's content of oryzalin exceeds its room temperature solubility. Be careful not to agitate the solution – it is important not to disturb the precipitate. This becomes the stock liquid. When the bottle is about ½ used, add more water and re-autoclave. I find I can do this 2-3 times before all the Surflan is dissolved.



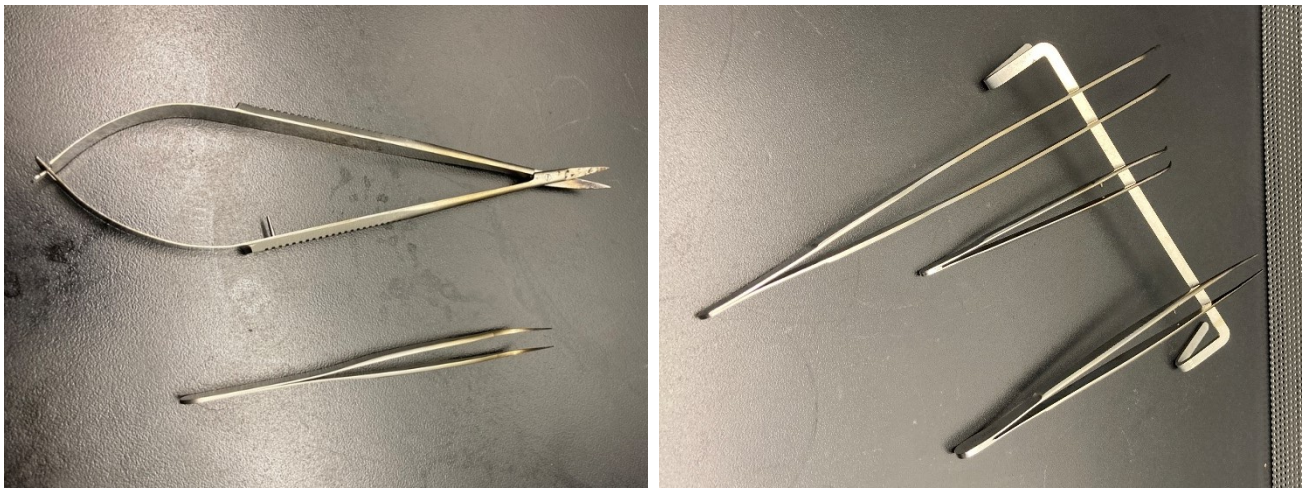
Oryzalin solution with precipitate visible at the bottom of the bottle. Photo courtesy of Bob Hamilton.

4) Store the sterile solution in an amber coloured bottle to prevent disassociation from ultraviolet (UV) light, as Oryzalin is UV-sensitive. I wrap the area of the bottle cap with aluminium foil to keep the area sterile.
5) Select a "mother" bottle of germinated orchid embryos at the proper stage of development, i.e., protocorms which have developed into small spheres, typically 2-4 mm in diameter for Oncidiinae, and which have begun to show the emergence of a leaf primordia, i.e., a small "tit". The leaf primordium is the most active growing part of a protocorm. The primordia have a high mitotic index.
6) Carefully pour the precipitate-free oryzalin solution into the mother bottle adding enough volume to approximately equal the volume of germination medium, e.g., if the germination medium is ~1 cm in height, add enough liquid for a total of ~2 cm height. Note: within 24 hours the salts and sugars of the germination media will equilibrate with the Surflan solution.
7) Expose the protocorms from 8-16 days. The effectiveness of exposure seems to vary with the cross and the protocorm stage of development. It is difficult to state an optimum exposure time of conversion versus mortality. Longer exposure increases conversion rates; however, it also increases mortality. I have treated different crosses for the same durations with virtually

no kill of one (*Odm. naevium* x *Cda. noezliana* v. *xanthina*) and complete kill for the other (*Odm. wyattianum*) after 16 days exposure. I recommend 10-12 days.

8) Carefully sterilize the outer area of the mother bottle and its lip before tilting and decanting the exposed protocorms into a sieve. I use a tea strainer perched on top of a beaker to capture the protocorms. Be careful not to tip so far as to spill out the mother medium. The reason for sterilizing the lip of the container is the invariable "roll back" of liquid from the outside of the container can introduce contamination.
9) Rinse the protocorms with ~100 ml of sterile water.
10) Transfer them to a container with replate medium.

The recovery time from exposure can vary from weeks to months. After a subsequent growing period from this "spread", select plants for the final relate. I empty plants ready for finals replate into a sterile stainless-steel pie container and search for plants that have obvious tetraploid (4n) morphology, i.e., thicker roots and leaves, leaves that end in an obtuse rather than acute point. I discard a lot of material as I do this.



Tools used by Bob in his lab work. Iris scissors are used for excising large root balls and a pair of forceps for small ones. Fine-pointed forceps are used to punch holes in the shrink band for gas exchange. Photos courtesy of Bob Hamilton.

When I am asked about my conversion rate, I point out that my final replates are done from plants visually selected as probably 4ns. Because of this selective replating, it is difficult to estimate a conversion rate. I can say the final yield rate is 25-50% are converted plants. I add the caveat that one never really knows the ploidy of a plant unless they count its chromosome numbers. However, an experienced grower can do a fair job of identifying the likely 4ns.

Over the decades I have treated many orchid families, first using colchicine and now the above protocol. These include *Ada, Cattleya, Cochlioda, Coelogyne, Cymbidium, Dendrobium, Dracula, Laelia, Lycaste, Masdevallia, Maxillaria, Neocogniauxia hexaptera, Odontoglossum* and *Oncidium*. I plan to continue using the above methods. My email is roberthamilton@berkeley.edu and I am available by email for those who need further information or have questions.

Robert Hamilton

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Plants Wanted

Cym. Mary Pinchess 'Sunbeam'.
Photo courtesy of John Harris.

John Harris is still looking for **Cym. Mary Pinchess 'Sunbeam'**, a hybrid from the late 1960s which was used in hybridising by Syd Monkhouse and then Adelaide Orchids to produce Scott's Sunrise 'Aurora'. He describes it as "a nice golden yellow with red veining in the petals and sepals and a nice barred and spotted lip."

Please contact John on 576djh@gmail.com or 0410477524.

The editor, Joshua White, is also looking for some older hybrids – in particular, Early Bird 'Pacific' and Sleeping Beauty 'Golden Queen'. Please email me at jwhite88@gmail.com if you can help!

Errata

In Vol. 3 No. 1 (Issue 35, February 2022) it was erroneously stated that *Cym. canaliculatum* was part of the main alba group. This species should have had an asterisk next to it to indicate that it is *presumed* to be part of the main alba group, as this has not yet been demonstrated to be the case. Furthermore, new evidence (a remake of Helen Bannerman using alba parents) seems to suggest *canaliculatum* may not be in the main alba group!

Acknowledgements and Contributions

I hope you have enjoyed this issue. If you have any feedback or would like to contribute (whether it be just one or two photos, an idea for an article, or to volunteer for an interview), please get in touch! I can be reached at jwhite88@gmail.com.

Previous issues are available at https://www.cosv.com.au/publications-and-resources. All material is copyright © the original owners and used with permission. Thanks to all those who have contributed to this issue, including Andy Easton, Robert "Bob" Hamilton, Nado Lenkic, Graham Morris and Allan Rae.

The next issue is planned for March 2023.