Stomatal guard cell length as an indicator of genome size in Orchidaceae

Victoria Butler

BSc Biological Sciences

Birkbeck, University of London

I declare that the practical work presented in this report is my own unless otherwise indicated, and that that the work of other authors (published or unpublished) has been acknowledged. I understand that I am required to submit a digital copy of the work and I agree to this being tested with software designed to detect plagiarism. Furthermore, I affirm that I have neither fabricated nor falsified any data reported herein.

Signature: Date: 5th May, 2008

ACKNOWLEDGEMENTS

I would like to thank the following people for their help with this project. At the Royal Botanic Gardens, Kew: Mike Fay, Ilia Leitch, Imalka Kahandawala, Lynda Hanson, Hazel Wilkinson, Christopher Ryan, Peter Brasson and John Sitch. I am also grateful to Martin Ingrouille at Birckbeck for supervising me and making the arrangements for me to do this project at Kew.

ABSTRACT

DNA C-values have been published for 213 orchid species to date (Bennett & Leitch, 2005) out of a total of approximately 25,000 known species. Sampling is currently low because techniques for estimating genome size are time-consuming and require expensive specialist equipment. In order for any analyses of genome size trends over the evolutionary history of Orchidaceae to be made, further data is needed. The objective of this study is to investigate whether the significant relationship between cell size and genome size that has been found for angiosperms is consistent throughout Orchidaceae. If a strong correlation is found, it should be possible to estimate genome size by measuring stomatal guard cell length. Measurements were taken for 71 orchid species with known 1C-values, covering as wide a range of clades and genome sizes as possible. A significant positive correlation ($r = 0.75$) between guard cell length and 1C-value was obtained. The technique was then used to measure guard cell lengths in dried herbarium specimens from the subfamily Apostasioideae, which is sister to all the remaining orchids (Cameron, 2004). The species in Apostasioideae were found to have smaller guard cells than the species from the other subfamilies, suggesting that primitive orchids have small genomes which have increased through evolution in many clades.

INTRODUCTION

Eukaryotic genome size varies 600,000-fold. Although these variations are well-documented, the reasons behind them are still not clear. A correlation between genome size and cell size has been observed in many previous studies (Mirsky and Ris, 1951, Commoner, 1964, Bennett, 1972) as well as a negative correlation with duration of cell cycle (Van't Hof and Sparrow, 1963). Many recent studies have used cell size as a proxy for genome size (Masterson, 1994, Almada et al., 2006, Gregory, 2001), although for plants, most studies are concerned with sizes within species at varying levels of polyploidy. Comparisons of genome size across species are sparse and results have so far been inconsistent. For example, Price (citation 1973) results showed a correlation of 1.00, whereas the figure was -0.48 for Grime's data (Grime et al., 1997).

At present, the most popular methods for estimating genome size are flow cytology and microdensitometry. These techniques are time-consuming and require expensive equipment. It has been postulated that if cell size is strongly correlated with genome size, it should be possible to obtain a C-value estimate by measuring cells. In angiosperms, epidermal cells are ideal for this as they can be easily be observed by making an epidermal impression using clear nail varnish. Stomatal guard cells have the added benefit in that they are of relatively consistent size within each species (Dunn et al., 1965), which appears not to be affected by stomatal aperture or different environmental conditions, such as temperature and humidity (Sharma and Dunn, 1968). Results showing a linear correlation between genome size and guard cell length have already been achieved for angiosperms in general (Beaulieu unpublished) and more comprehensively, for the orchid genus *Cypripedium* (Kahandawala, unpublished). There is also evidence that this technique can be used for dried and fossilised leaves (Masterson, 1994)(citation – Masterson & Kahandawala), which is not possible for flow cytology or microdensitometry.

Orchidaceae is of particular interest to evolutionary biologists, because this family is one of the most species-rich and diverse of the plant world. The Royal Botanic Gardens, Kew currently lists approximately 25,000 species, with new species regularly being discovered and described (Govaerts et al., 2006). Furthermore it is one of the best-studied families of the angiosperms in terms of infra-familial phylogenetic relationships (Chase et al., 2003)(citation – Chase). The family is split into five subfamilies: Apostasioideae, Vanilloideae, Cypripedioideae, Orchidoideae and Epidendroideae, with the bulk of species contained in the latter group (Figure 1). Apostasioideae is sister to the clade formed by the other subfamilies and thus is considered to contain the most primitive orchid species. There is a ~130-fold range in genome size over Orchidaceae, although the majority of orchid species have fairly small genomes. Larger genome sizes are found in *Cypripedium*, *Paphiopedilum* and a few other terrestrial species, based on data from the Plant C-values Database (Bennett and Leitch, 2005) and from more recent estimates carried out by Beaulieu *et al.* (2007) and Kahandawala (unpublished). There are currently no genome size estimates for any species in Apostasioideae.

Variation in DNA content between closely-related species is usually either a result of polyploidy (Mishra, 1997) or an accumulation of repetitive non-coding DNA. Polyploidy is extremely widespread in angiosperms in general, with multiple rounds of polyploidy events occurring throughout their evolution (Adams and Wendel, 2005). More relevant to this is the variation in orchid genome size due to the insertion of non-coding DNA, in particular, long terminal repeat- (LTR-) retrotransposons (SanMiguel et al., 1996), which are the most common type of transposable element in plants (Kumar et al., 1999). Retrotransposons replicate themselves to RNA then back to DNA via reverse transcriptase, thus rapidly increasing the copy numbers of genetic elements. LTR-retrotransposons make up a large part of plant nuclear genomes, for example, over 70% in maize (Sanmiguel and Bennetzen, 1998) (SanMiguel and Bennetzen, 1998).

In a recent investigation of genome size in a phylogenetic context, Leitch *et al* (Leitch et al., 2005) superimposed C-values for 2,802 angiosperm species including 72 orchids onto a robust phylogenetic tree. It showed that in 15 higher order groups, species had small C-values, and very large C-values occurred in only two distantly related groups. Within these two groups, small C-values were present at the lower taxonomic levels, with very large C-values restricted to species in the more derived families (Leitch et al., 1998). From this, they concluded that the ancestral angiosperm genomes were small. Paleobotanical evidence supports this conclusion. In a study using stomatal guard cell length as a proxy for genome size in fossilized plants, Masterson (Masterson, 1994) found that members of Magnoliales, Lauraceae and Platanaceae from the Cretaceous period when angiosperms first began to appear in the fossil record had C-values smaller or at the lower end of the range of those extant today. Bennetzen and Kellogg agree with this theory of a small ancestral genome which gradually increases through evolution via amplification of transposable elements (Bennetzen and Kellogg, 1997).

In this study, guard cell length was measured for a range of orchid species with known genome sizes in order to confirm the relationship between them. Once the correlation was proved to be significant, guard cell measurements were taken from species of *Apostasia* and *Neuwiedia*. The data should indicate whether these species have genomes smaller than those of higher orchids as hypothesized.

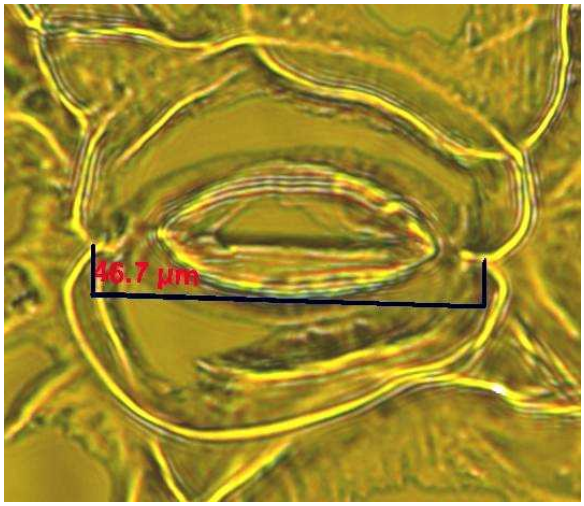
MATERIALS AND METHODS

Guard cell measurements

Epidermal impressions were made using clear nail varnish (Boots Natural Collection) applied to a section of the leaf approximately halfway between the base and apex and to either side of the midrib, away from the margins. All measurements were taken from the abaxial leaf surface. Epidermal peels were examined using a light microscope at 40x magnification and guard call measurements to the nearest micrometer were recorded. The length was measured as shown in Figure 1 rather than

the shorter axis, as the latter varies depending on whether the pore is open or closed. For each sample, 50 randomly selected stomata were measured. Although, the quality of the peels did not appear to deteriorate over time, each sample was measured within three days from the initial preparation of the slide.

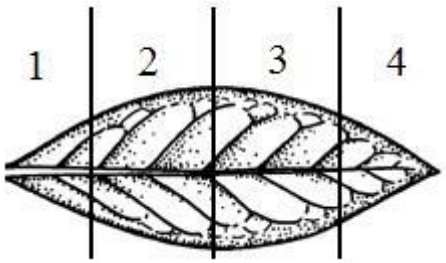
Figure 1. An orchid stoma showing how guard cell lengths measurements were taken.



Sampling

All fresh samples were from plants growing in nurseries at the Royal Botanic Gardens, Kew and the dried samples were obtained from Kew's herbarium collection. Although the choice of leaves was somewhat restricted in certain cases (for example, due to extensive damage to the plant), only fully mature leaves were selected. In order to find out if guard cell length varied in different parts of the leaf, samples were selected from five orchid species: *Bletilla striata, Bulbophyllum lepidum, Ceologyne massangeana, Ornithophora radicans* and *Dendrobium speciosum*. Species were selected on the basis of having diverse leaf shapes. Epidermal peels were made in each the sections of the leaf away from the margins and midrib (Figure 2).

Figure 2. Diagram showing how the leaves were divided into sections.



For the main set of measurements, 71 species with known genome sizes were selected, including species from as many different tribes as possible (at least one specimen for each of the subfamilies except Apostasioideae). Estimates of 1C-values were mainly taken from the Plant DNA C-values database maintained at RBG, Kew (Bennett and Leitch, 2005), but a small number were from recent unpublished studies not yet included on the database. A wide range of genome sizes was also desirable, therefore as the majority of orchids have small C-values, it was necessary to include a relatively large number of samples from Cypripedioideae. Of these, 40 of the samples were oven-dried and re-measured to see whether the drying process has any effect on guard cell length.

For the herbarium specimens, epidermal peels were taken from all available species in Apostasioideae (genera *Apostasia* and *Neuwiedia*). For each species three specimens were selected from different countries of origin, except for *A. latifolia* and *A. elliptica*, of which only one specimen of each currently exist in the herbarium collection at Kew.

Analytical methods

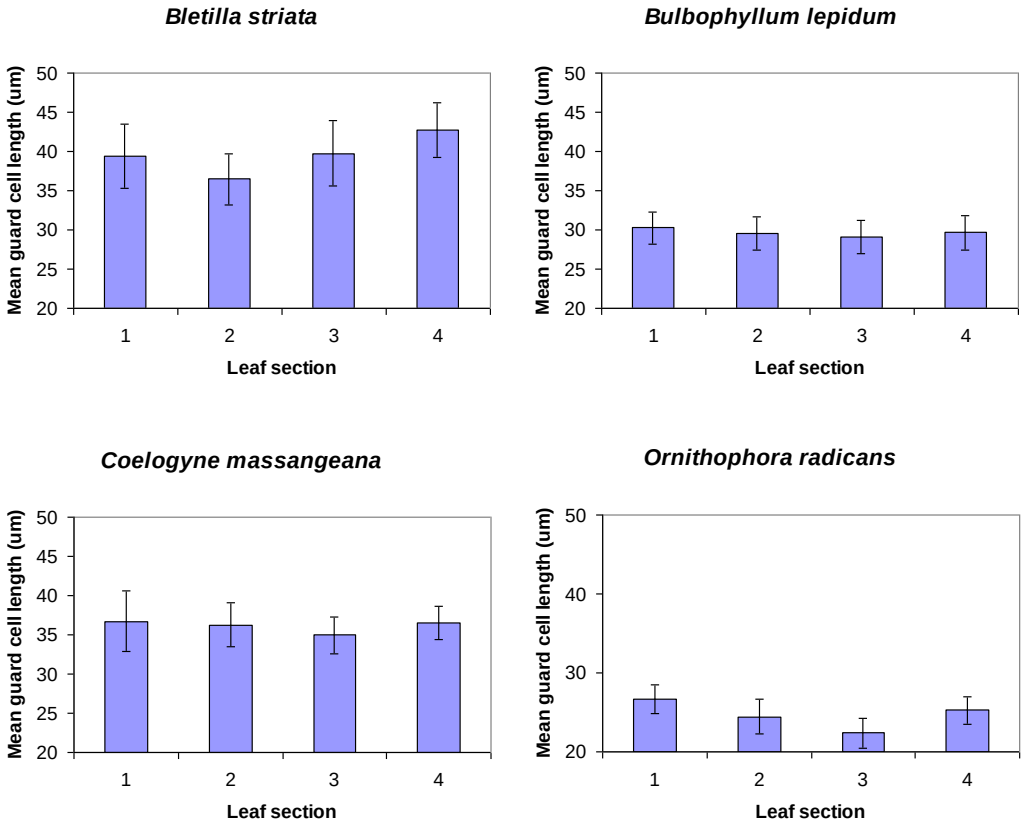
ANOVA were performed on the data from the different leaf sections using Minitab ® Release 14.1. Variances between guard cell length measurements and between the different were determined using this method.

Regression lines were produced for mean guard cell lengths of the species with known genome sizes and a correlation analysis carried out using Minitab ® Release

14.1. Likewise, a regression line was produced for the guard cell lengths before an after drying. A paired *t*-test was performed to determine whether there is a significant change in guard cell length after the leaf is dried.

Results

Figure 3. Mean guard cell lengths with standard errors from numbered leaf sections in different Orchidaceae species. There are differences of varying degrees between mean guard cell lengths across the different leaf sections in each species. The guard cells in the centre sections generally appear to be smaller than those at the base and apex, although this is not the case for *Dendobrium speciosum*. The differences in guard cell length between the species are evident, the smallest on average being in *Ornithophora radicans* and the largest in *Bletilla striata*. Untransformed data from which this figure is derived are tabulated in Appendix 4(a).



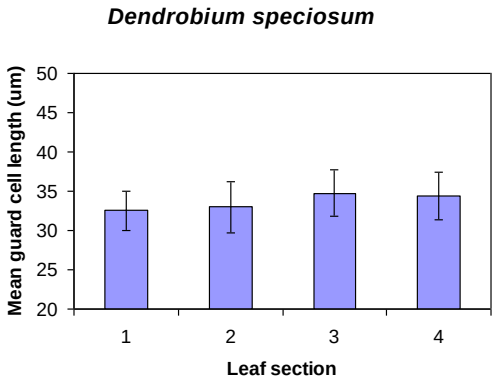


Table 1. Results for ANOVA of guard cell length between leaf sections. The results show that there is significant variance for all species at *P*=0.05 (full data are found in Appendix 4b). The species with the least variance between sections was *Bulbophyllum lepidum*.

Species	***F***	***P***
Bletilla striata	6.72	<0.001
Bulbophyllum lepidum	2.75	0.044
Coelogyne massangeana	3.81	0.011
Ornithophora radicans	43.38	<0.001
Dendrobium speciosum	6.72	<0.001

The variance in mean guard cell length between the species is also significant (*F*=11.06, *P*=<0.001). (Full data in Appendix 4c).

Figure 4. Regression of the relationship between 1C DNA amount and guard cell length with 95% confidence interval.

There is a positive linear correlation between the two variables, but with a number of outliers. The majority of points are clustered at the lower end of the range. Untransformed data from which this figure is derived are tabulated in Appendix 5.

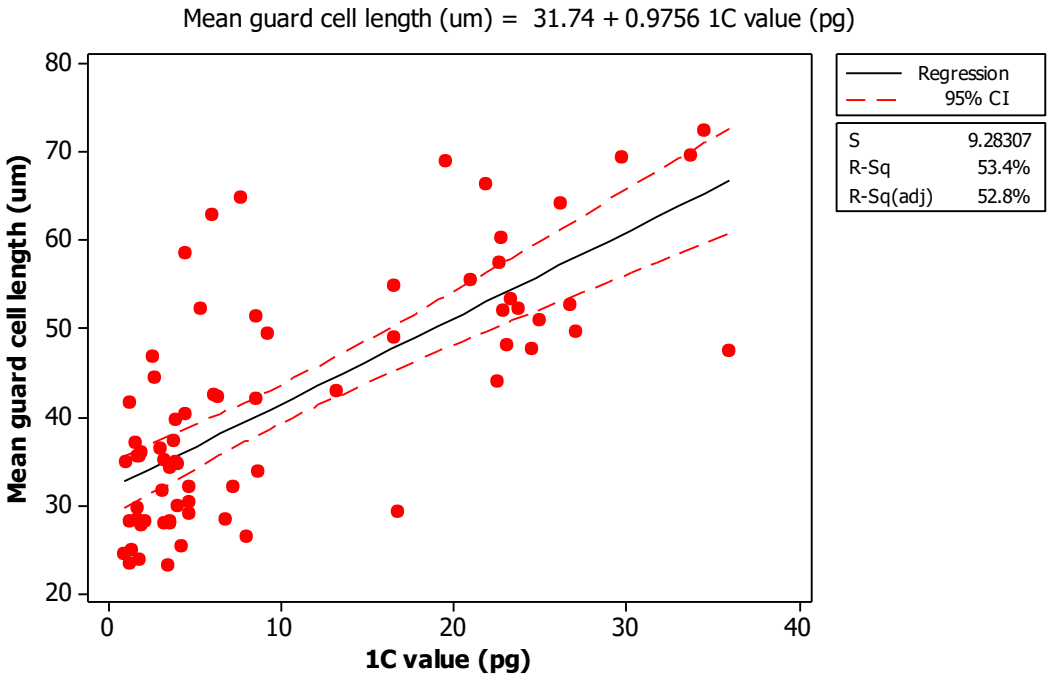
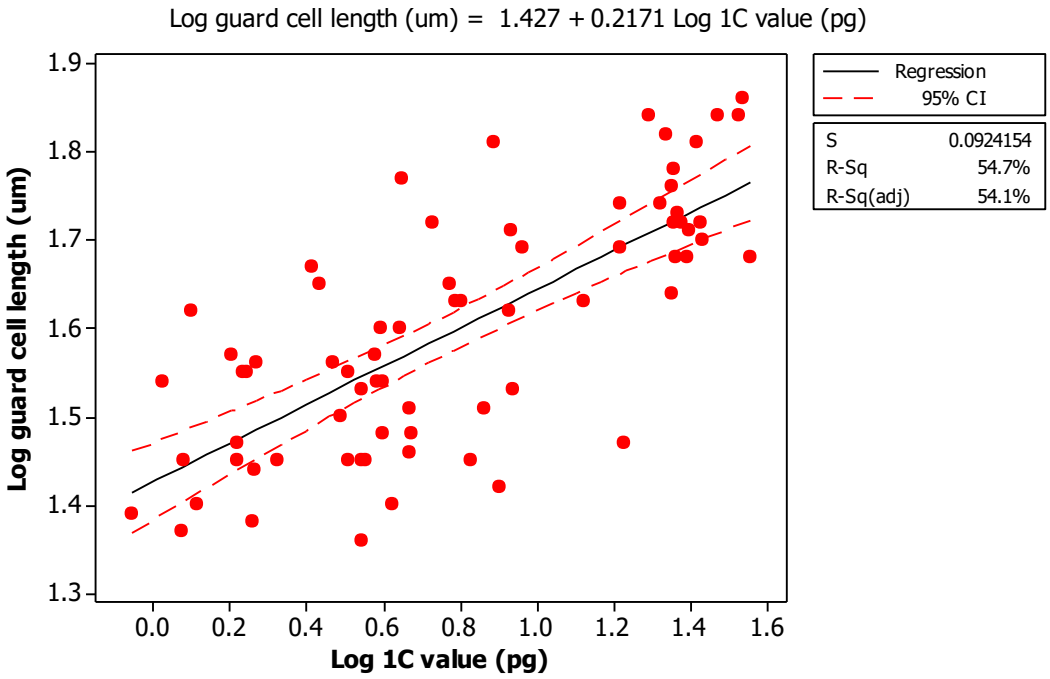


Figure 5. Regression of the relationship between log values of 1C DNA amount and guard cell length with 95% confidence interval.

The log shows a strong correlation and points are more evenly distributed throughout the range than in Figure 4. Untransformed data from which this figure is derived are tabulated in Appendix 5.



Regression analysis across all species showed that 1C DNA content was positively associated with guard cell length. Correlation analysis found a significant correlation at p=0.01 (*r*=0.75). Genome size accounted for 79% of the variation.

Figure 6. Scatter plot of correlation between mean guard cell length before and after drying. The line shows the point where there is no variation between results. There is a strong linear relationship between the two variables and most points lie on the line. Untransformed data from which this figure is derived are tabulated in Appendix 7.

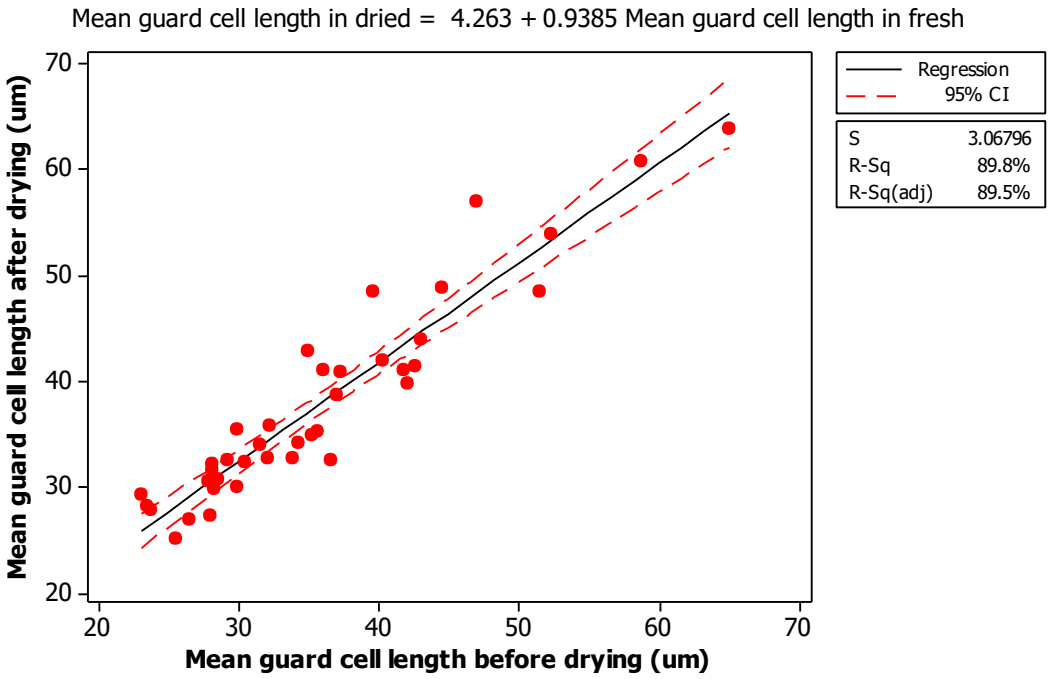


Figure 7. Floating bars showing the range of 1C-values of Orchidaceae classed by lifeform (classifications from World Monocot Checklist (Govaerts et al., 2006)). The black line shows the mean 1C-value.

The highest 1C-values are found in hemicryptophytes, namely *Paphiopedilum* and *Phragmipedium*. The smallest range and mean 1C-values are found in epiphytes. Although the range for epiphytes appears to extend to 1C-values higher than those for geophytes, this is only due to *Vanda coerulea* (16.8 pg). Without this figure, the range would extend to 8.5 pg. Untransformed data from which this figure is derived are tabulated in Appendix 5.

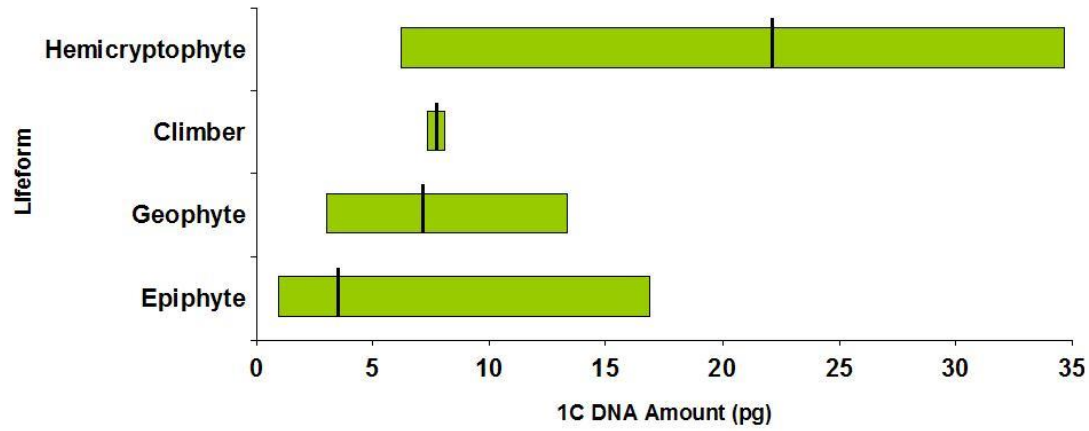


Figure 8. Floating bars showing the range of mean guard cell lengths of Orchidaceae by lifeform (classifications from World Monocot Checklist (Govaerts et al., 2006)). The black line shows the mean species guard cell length.

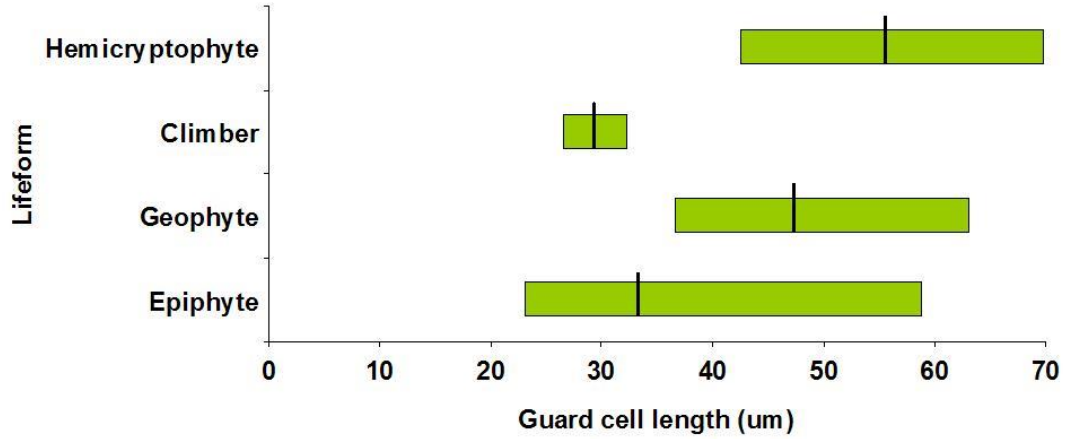
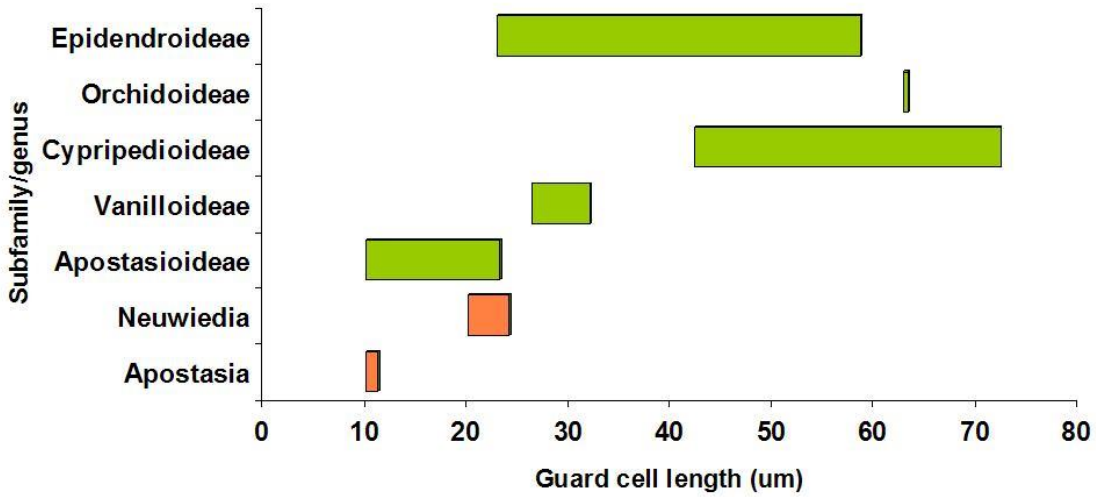


Figure 9. Graph showing ranges of guard cell measurements for *Apostasia*, *Neuwiedia* and the subfamilies of Orchidaceae.

Apostasia has the lowest range of measurements. *Neuwiedia* has a marginally higher range that overlaps slightly with the lower end range for Epidendroideae.

Untransformed data from which this figure is derived are tabulated in Appendix 7.



Discussion

The main purpose of this study was to examine the relationship between genome size and guard cell length within Orchidaceae, using a fairly large species set and a comparative approach. Across the four subfamilies, the linearity in the relationship between genome size and guard cell length is clear. However, the relationship is not sufficiently strong to enable an estimate of 1C-value based on the guard cell length.

There are numerous possible explanations for the variation, one of which concerns the diversity of stomatal shapes (see Appendix 8). For example, within the species investigated there are both elliptical and circular stomata. Some form an indentation where the two guard cells meet, whereas others extrude where they join, giving the stoma a “lemon” shape. It has been documented that within an individual plant there may be more than one stomatal shape, which can be related to factors such as the age of the leaf or the light quality under which it has developed (Stefano and Rosario, 2003, Brutti et al., 2002). It is possible that different types of stomatal shape have a varying relationship with genome size and are not suitable for a consistent comparison. To test this, a selection of samples with similarly shaped stomata were plotted, but a stronger correlation was not found. Neither was a relationship found between the volume of guard cells for 25 samples and their respective genome sizes.

Across the samples, stomatal aperture varied from closed to fully open. With time restrictions, it would not have been possible to measure only stomata in a particular stage of opening. In any case, this should not affect guard cell length, which has been shown to remain constant regardless of the stomatal aperture (Willmer and Fricker, 1995, Franks et al., 2001).

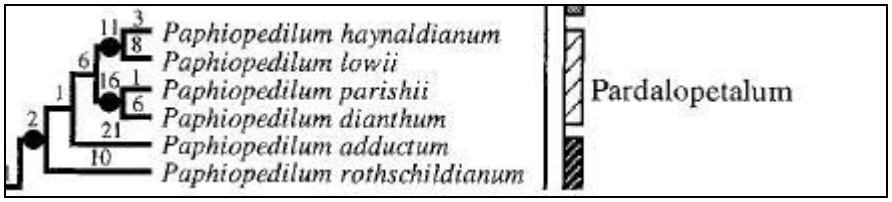
Guard cells close to the margins and midrib may significantly vary in size from the overall mean (Willmer and Fricker, 1995, Poole et al., 2000), (Sena Gomes and Kozlowski, 1987). The initial part of the study also showed that guard cell length varies depending on its proximity to the base and apex of the leaf (Fig. 3), although the sample used to investigate this was too small to give a reliable indication of which parts of the leaf contained larger or smaller stomata. The results suggest that guard cells measured in just one part of the leaf may not give a realistic representation of the mean for the entire leaf. If this is the case, a method which involves taking impressions in several parts of the leaf may obtain guard cell lengths that correlate more strongly with genome size.

The genome size estimates were taken from a number of different studies, dating back as far as 1989, which used varying methods. This may have led to inconsistencies in estimates and it would not surprising to find that a few estimates are inaccurate. In some cases ploidy level has not been recorded, which leads to uncertainties about whether all 1C-values are for specimens with identical ploidy levels. The 1C-values for some species were determined by dividing estimated 4C-values (Jodrell unpublished data). This may not give an accurate value because the cell size increases with levels of polyploidy, but is difficult to detect because cell size parameters do not precisely double as ploidy level doubles (Melaragno et al., 1993).

The two species with the highest residual values are *Paphiopedilum dianthum* and *Vanda coerulea*. In both cases, it may be possible that the C-values obtained for these are inaccurate. One of the most parsimonious phylogenetic trees showing subgeneric relationships for *Paphiopedilum*, places *P. dianthum* in a group with *P.*

haynaldianum, *P. lowii*, *P. parishii*, *P adductum* and *P. rothschildianum* (Figure 10). 1C-value estimates for these (excluding *P. parishii* for which there is no estimate) range from 22.60 pg to 27.03 pg. The known 1C-values for the other species in the group Pardalopetalum are 22.85 pg (*P. haynaldianum*) and 24.53 pg (*P. lowii*). The 1C-value 35.90 pg for *P. dianthum* seems unusually large, considering that in general the species that are closely-related, according to this phylogeny have very similar 1C-values.

Figure 10. Parsimonious ITS DNA tree showing subgeneric relationships in *Paphiopedilum*.



The 1C-value of 16.8 pg for *Vanda coerulea* seems unusually high when the estimated 1C-values for other *Vanda* species are 2.05 pg (*V. lamellata*) and 4.40 pg (*V. cristata*). A 1C-value closer to these would bring *V. coerulea* further towards the line of best fit.

The results of a recent study using the same technique as this study with the orchid genus *Cypripedium* showed a considerably stronger correlation between guard cell length and genome size than the broad range of orchid species in this one (Kahandawala unpublished). A reason for this may be that the stomata for this individual genus are all of a consistent shape, resulting in a meaningful comparison of guard cell lengths. Another explanation may be that Kahandawala also carried out her own genome size estimates and chromosome counts on the samples, which meant that the methods used were identical throughout. This may have resulted in more consistent data than when genome size estimates from a number of different studies are used. The method Kahandawala used to measure guard cell length differed from

this study in that epidermal impressions were photographed using a QICAM 12-bit Fast 1394 camera mounted to a Leitz Laborlux compound microscope and measurements were taken using Qcapture Pro 5 software. It is possible that this resulted in more accurate guard cell lengths than those obtained using a stage micrometer.

Even with several outliers, the correlation between guard cell length and genome size was found to be significant ($r = 0.75$). Moreover, genome size was found to account for nearly 80% of the total variation in guard cell length. The cause of the remaining variation is not clear and would require further sampling to rule out any of the possible explanations previously discussed. The strength of the relationship between DNA amount and guard cell length allows assumptions to made about the genome size of Apostasioideae species based on the data from dried herbarium specimens. The guard cell lengths of *Apostasia* in particular indicate that this genus contains genome sizes smaller than those of other orchids, although it is important to bear in mind that genome sizes for only a tiny proportion of species in Orchidaceae have so far been estimated. The data currently available supports the suggestion that the genus *Apostasia* is the most primitive of Orchidaceae, based on the hypothesis of a small ancestral genome (Leitch et al., 1998), although in terms of anatomy, *Neuwiedia* is closest to the hypothetical ancestral Ur-orchid (Dressler, 1993).

If the variation in genome size was simply a result of regular amplification of retrotransposons over evolution, it would be reasonable to expect the largest genome sizes to be found at the highest taxonomic levels, for example, higher Epidendroideae. This is not the case, however; the largest genome sizes are in fact contained in Cypripedioideae. There are two possible explanations for this: either a process of retrotransposon deletion has taken place in some lineages, a process that has been observed in other angiosperms, (Bennetzen and Kellogg, 1997, Vitte et al., 2007) or the increase in genome size has occurred more rapidly in others e.g. *Cypripedium*. From chromosome counts of species in Oncidiinae, Chase et al (Chase

et al., 2005) postulate that fusing chromosomes might be involved in the process leading to reduction in genome size.

The strength of the relationship suggests that there is a functional link between genome size and cell size. Because polyploid species have larger cells it may be assumed that genome size sets cell size and not the other way around. There is even a hypothesis that cells may actively enlarge cells by electing endomitotic cycling as a means to increase growth rate (Galbraith et al., 1991). Additional factors such as genes (Nadeau and Sack, 2002) and environmental conditions are also likely to play an important role in determining cell size, but only through the adjustment of the final cell size from the average laid down by DNA content. Variation in genome size can sometimes lead to phenotypic adjustments other than cell size, such as duration of mitosis and meiosis, minimum generation time and seed size. If certain phenotypes prove advantageous to the plant, the genome size with evolve with the corresponding traits as they are selected for (Petrov, 2001).

Figs. 7 and 8 show that epiphytic orchids have smaller genomes than their terrestrial counterparts. This agrees with findings by Fay et al (Fay et al., 2006). and Chase et al (Chase et al., 2005). One hypothesis for this could be that genomes have remained small in epiphytes due to selection pressures that are not experienced by terrestrial species, whereas the trend for retroelement amplification has continued with less restrictions in terrestrial species. One of the disadvantages of having a large genome is that it leads to an increase in minimum generation time (Bennett, 1972). As many epiphytic orchid species select precarious sites such as the twigs of shrubs, from which other epiphytic plants are absent (Chase et al., 2005), the aptitude for rapid cycling is likely to be advantageous. Terrestrial orchids tend to grow in sites less prone to disturbance or sudden change, therefore do not gain anything by possessing smaller genomes. Another constraint on cell size in epiphytic orchids is related to limited water availability. The presence of adaptations found typically in drought tolerant plants, such as thick cuticles and water storage organs, strengthens this hypothesis (Chase et al., 2005). The results of this study agree with other findings

that the largest genomes are generally found in plants that have a distinct brief period of growth followed by period of dormancy, for example, during a cold or dry season (Chase et al., 2005), which is case in most terrestrial orchids. In this case, having a large genome size may allow for rapid cell expansion while conditions are optimal.

In conclusion, Orchidaceae is an extremely diverse angiosperm family, but despite this there is a significant correlation between guard cell length and genome size across the subfamilies. As a result, it is possible to use guard cell length to obtain an approximate indication of genome size with both fresh and dried specimens. Using this method, it was found that species in Apostasioideae have very small genome sizes in relation to species in the higher subfamilies, which fits predictions of a small ancestral genome.

References

Adams, K. L. & Wendel, J. F. (2005) Polyploidy and genome evolution in plants. *Current Opinion in Plant Biology,* 8**,** 135-141.

Almada, R. D., Davina, J. R. & Seijo, J. G. (2006) Karyotype analysis and chromosome evolution in southernmost South American species of Crotalaria (Leguminosae). *Botanical Journal of the Linnean Society,* 150**,** 329-341.

Bennett, M. D. (1972) Nuclear DNA content and minimum generation time in herbaceous plants. *Proceedings of the Royal Society of London Series B-Biological Sciences,* 181**,** 109-135.

Bennett, M. D. & Leitch, I. J. (2005) Plant DNA C-values database (release 4.0, October 2005). < http://data.kew.org/cvalues/homepage.html> Accessed [18 April 2008].

Bennetzen, J. L. & Kellogg, E. A. (1997) Do plants have a one-way ticket to genomic obesity? *Plant Cell,* 9**,** 1509-1514.

Brutti, C. B., Rubio, E. J., Llorente, B. E. & Apóstolo, N. M. (2002) Artichoke leaf morphology and surface features in different micropropagation stages. *Biologia Plantarum,* 45**,** 197-204.

Cameron, K. M. (2004) Utility of plastid psaB gene sequences for investigating intrafamilial relationships within Orchidaceae. *Molecular Phylogenetics and Evolution,* 31**,** 1157-1180.

Chase, M. W., Cameron, K. M., Barrett, R. L. & Freudenstein, J. V. (2003) DNA data and Orchidaceae systematics: a new phylogenetic classification. In Dixon, K. W., Kell, S. P., Barrett, R. L. & Cribb, P. J. (Eds.) *Orchid Conservation.* Kota Kinabalu, Sabah: Natural History Publications.

Chase, M. W., Hanson, L., Albert, V. A., Whitten, W. M. & Williams, N. H. (2005) Life history evolution and genome size in subtribe Oncidiinae (Orchidaceae). *Annals of Botany,* 95**,** 191-199.

Commoner, B. (1964) Roles of deoxyribonucleic acid in inheritance. *Nature***,** 960-968.

Dressler, R. L. (1993) *Phylogeny and classification of the orchid family,* Cambridge: Cambridge University Press.

Dunn, D. B., Sharma, G. K. & Campbell, C. C. (1965) Stomatal Patterns of Dicotyledons and Monocotyledons. *American Midland Naturalist,* 74**,** 185-195.

Fay, M. F., Leitch, I. J., Kahandawala, I., Lysak, M. A. & Chase, M. W. (2006) Genome size in Orchidaceae – variation, evolution, and consequences. *Botany 2006* conference paper. California State University, Chico.

Franks, P. J., Buckley, T. N., Shope, J. C. & Mott, K. A. (2001) Guard cell volume and pressure measured concurrently by confocal microscopy and the cell pressure probe. *Plant Physiology,* 125**,** 1577-1584.

Galbraith, D. W., Harkins, K. R. & Knapp, S. (1991) Systemic endopolyploidy in Arabidopsis-Thaliana. *Plant Physiology,* 96**,** 985-989.

Govaerts, R., Dransfield, J., Zona, S. F., Hodel, D. R. & Henderson, A. (2006) World checklist of monocotyledons. The Board of Trustees of the Royal Botanic Gardens, Kew. < http://apps.kew.org/wcsp/home.do> Accessed [18 April 2008].

Gregory, T. R. (2001) The bigger the C-value, the larger the cell: Genome size and red blood cell size in vertebrates. *Blood Cells Molecules and Diseases,* 27**,** 830-843.

Grime, J. P., Thompson, K., Hunt, R., Hodgson, J. G., Cornelissen, J. H. C., Rorison, I. H., Hendry, G. A. F., Ashenden, T. W., Askew, A. P., Band, S. R., Booth, R. E., Bossard, C. C., Campbell, B. D., Cooper, J. E. L., Davison, A. W., Gupta, P. L., Hall, W., Hand, D. W., Hannah, M. A., Hillier, S. H., Hodkinson, D. J., Jalili, A., Liu, Z., Mackey, J. M. L., Matthews, N., Mowforth, M. A., Neal, A. M., Reader, R. J., Reiling, K., Rossfraser, W., Spencer, R. E., Sutton, F., Tasker, D. E., Thorpe, P. C. & Whitehouse, J. (1997) Integrated screening validates primary axes of specialisation in plants. *Oikos,* 79**,** 259-281.

Kumar, A. & Bennetzen, J. L. (1999) Plant retrotransposons. *Annual Review of Genetics,* 33**,** 479-532.

Leitch, I. J., Chase, M. W. & Bennett, M. D. (1998) Phylogenetic analysis of DNA C-values provides evidence for a small ancestral genome size in flowering plants. *Annals of Botany,* 82**,** 85-94.

Leitch, I. J., Soltis, D. E., Soltis, P. S. & Bennett, M. D. (2005) Evolution of DNA amounts across land plants (embryophyta). *Annals of Botany,* 95**,** 207-217.

Masterson, J. (1994) Stomatal size in fossil plants - evidence for polyploidy in majority of angiosperms. *Science,* 264**,** 421-424.

Melaragno, J. E., Mehrotra, B. & Coleman, A. W. (1993) Relationship between endopolyploidy and cell-size in epidermal tissue of *Arabidopsis*. *Plant Cell,* 5**,** 1661-1668.

Mirsky, A. E. & Ris, H. (1951) The desoxyribonucleic acid content of animal cells and its evolutionary significance. *Journal of General Physiology*, 451-462.
Mishra, M. K. (1997) Stomatal characteristics at different ploidy levels in *Coffea* L. *Annals of Botany,* 80**,** 689-692.
Nadeau, J. A. & Sack, F. D. (2002) Control of stomatal distribution on the *Arabidopsis* leaf surface. *Science,* 296**,** 1697-1700.
Petrov, D. A. (2001) Evolution of genome size: new approaches to an old problem. *Trends in Genetics,* 17**,** 23-28.
Poole, I., Lawson, T., Weyers, J. D. B. & Raven, J. A. (2000) Effect of elevated CO2 on the stomatal distribution and leaf physiology of *Alnus glutinosa*. *New Phytologist,* 145**,** 511-521.
Sanmiguel, P. & Bennetzen, J. L. (1998) Evidence that a recent increase in maize genome size was caused by the massive amplification of intergene retrotransposons. *Annals of Botany,* 82**,** 37-44.
Sanmiguel, P., Tikhonov, A., Jin, Y. K., Motchoulskaia, N., Zakharov, D., Melakeberhan, A., Springer, P. S., Edwards, K. J., Lee, M., Avramova, Z. & Bennetzen, J. L. (1996) Nested retrotransposons in the intergenic regions of the maize genome. *Science,* 274**,** 765-768.
Sena Gomes, A. R. & Kozlowski, T. T. (1987) Stomatal characteristics, leaf waxes, and transpiration rates of *Theobroma cacao* and *Hevea brasiliensis* seedlings. *Annals of Bontany,* 61**,** 425-432.
Sharma, G. K. & Dunn, D. B. (1968) Effect of environment on the cuticular features in *Kalanchoe fedschenkoi*. *Bulletin of the Torrey Botanical Club,* 95**,** 464-473.
Stefano, M. & Rosario, M. (2003) Effects of light quality on micropropagation of woody species. In Jain, S. M., & Ishii, K. (Ed.) *Micropropagation of Woody Trees and Fruits.* Dordrecht, Netherlands: Kluwer Academic Publishers.
Van't Hof, J. & Sparrow, A. H. (1963) A relationship between DNA content, nuclear volume, and minimum mitotic cycle time. *Proceedings of the National Academy of Sciences of the United States of America,* 49**,** 897-902.
Vitte, C., Panaud, O. & Quesneville, H. (2007) LTR retrotransposons in rice (*Oryza sativa*, L.): recent burst amplifications followed by rapid DNA loss. *Bmc Genomics,* 8**,** 15.
Willmer, C. M. & Fricker, M. D. (1995) *Stomata,* Kluwer Academic.

ADAMS, K. L. & WENDEL, J. F. (2005) Polyploidy and genome evolution in plants. *Current Opinion in Plant Biology,* 8**,** 135-141.
ALMADA, R. D., DAVINA, J. R. & SEIJO, J. G. (2006) Karyotype analysis and chromosome evolution in southernmost South American species of Crotalaria (Leguminosae). *Botanical Journal of the Linnean Society,* 150**,** 329-341.
BENNETT, M. D. (1972) NUCLEAR DNA CONTENT AND MINIMUM GENERATION TIME IN HERBACEOUS PLANTS. *Proceedings of the Royal Society of London Series B-Biological Sciences,* 181**,** 109-&.
BENNETT, M. D. & LEITCH, I. J. (2005) Plant DNA C-values database (release 4.0, October 2005).
BENNETZEN, J. L. & KELLOGG, E. A. (1997) Do plants have a one-way ticket to genomic obesity? *Plant Cell,* 9**,** 1509-1514.

BRUTTI, C. B., RUBIO, E. J., LLORENTE, B. E. & APÓSTOLO, N. M. (2002) Artichoke Leaf Morphology and Surface Features in Different Micropropagation Stages. *Biologia Plantarum,* 45**,** 197-204.

CAMERON, K. M. (2004) Utility of plastid psaB gene sequences for investigating intrafamilial relationships within Orchidaceae. *Molecular Phylogenetics and Evolution,* 31**,** 1157-1180.

CHASE, M. W., CAMERON, K. M., BARRETT, R. L. & FREUDENSTEIN, J. V. (2003) DNA data and Orchidaceae systematics: a new phylogenetic classification. IN DIXON, K. W., KELL, S. P., BARRETT, R. L. & CRIBB, P. J. (Eds.) *Orchid Conservation.* Kota Kinabalu, Sabah, Natural History Publications.

CHASE, M. W., HANSON, L., ALBERT, V. A., WHITTEN, W. M. & WILLIAMS, N. H. (2005) Life history evolution and genome size in subtribe Oncidiinae (Orchidaceae). *Annals of Botany,* 95**,** 191-199.

COMMONER, B. (1964) Roles of deoxyribonucleic acid in inheritance. *Nature,* 960-968.

DRESSLER, R. L. (1993) *Phylogeny and classification of the orchid family,* Cambridge, Cambridge University Press.

DUNN, D. B., SHARMA, G. K. & CAMPBELL, C. C. (1965) Stomatal Patterns of Dicotyledons and Monocotyledons. *American Midland Naturalist,* 74**,** 185-195.

FAY, M. F., LEITCH, I. J., KAHANDAWALA, I., LYSAK, M. A. & CHASE, M. W. (2006) Genome size in Orchidaceae – variation, evolution, and consequences. *Botany 2006.* California State University, Chico.

FRANKS, P. J., BUCKLEY, T. N., SHOPE, J. C. & MOTT, K. A. (2001) Guard cell volume and pressure measured concurrently by confocal microscopy and the cell pressure probe. *Plant Physiology,* 125**,** 1577-1584.

GALBRAITH, D. W., HARKINS, K. R. & KNAPP, S. (1991) SYSTEMIC ENDOPOLYPLOIDY IN ARABIDOPSIS-THALIANA. *Plant Physiology,* 96**,** 985-989.

GOVAERTS, R., DRANSFIELD, J., ZONA, S. F., HODEL, D. R. & HENDERSON, A. (2006) World checklist of monocotyledons. The Board of Trustees of the Royal Botanic Gardens, Kew.

GREGORY, T. R. (2001) The bigger the C-value, the larger the cell: Genome size and red blood cell size in vertebrates. *Blood Cells Molecules and Diseases,* 27**,** 830-843.

GRIME, J. P., THOMPSON, K., HUNT, R., HODGSON, J. G., CORNELISSEN, J. H. C., RORISON, I. H., HENDRY, G. A. F., ASHENDEN, T. W., ASKEW, A. P., BAND, S. R., BOOTH, R. E., BOSSARD, C. C., CAMPBELL, B. D., COOPER, J. E. L., DAVISON, A. W., GUPTA, P. L., HALL, W., HAND, D. W., HANNAH, M. A., HILLIER, S. H., HODKINSON, D. J., JALILI, A., LIU, Z., MACKEY, J. M. L., MATTHEWS, N., MOWFORTH, M. A., NEAL, A. M., READER, R. J., REILING, K., ROSSFRASER, W., SPENCER, R. E., SUTTON, F., TASKER, D. E., THORPE, P. C. & WHITEHOUSE, J. (1997) Integrated screening validates primary axes of specialisation in plants. *Oikos,* 79**,** 259-281.

KUMAR, A., BENNETZEN, J. L. & WM (1999) Plant retrotransposons. *Annual Review of Genetics,* 33**,** 479-532.

LEITCH, I. J., CHASE, M. W. & BENNETT, M. D. (1998) Phylogenetic analysis of DNA C-values provides evidence for a small ancestral genome size in flowering plants. *Annals of Botany,* 82**,** 85-94.

LEITCH, I. J., SOLTIS, D. E., SOLTIS, P. S. & BENNETT, M. D. (2005) Evolution of DNA amounts across land plants (embryophyta). *Annals of Botany,* 95**,** 207-217.

MASTERSON, J. (1994) Stomatal size in fossil plants - evidence for polyploidy in majority of angiosperms. *Science,* 264**,** 421-424.

MELARAGNO, J. E., MEHROTRA, B. & COLEMAN, A. W. (1993) RELATIONSHIP BETWEEN ENDOPOLYPLOIDY AND CELL-SIZE IN EPIDERMAL TISSUE OF ARABIDOPSIS. *Plant Cell,* 5**,** 1661-1668.

MIRSKY, A. E. & RIS, H. (1951) The desoxyribonucleic acid content of animal cells and its evolutionary significance. *Journal of General Physiology***,** 451-462.

MISHRA, M. K. (1997) Stomatal characteristics at different ploidy levels in Coffea L. *Annals of Botany,* 80**,** 689-692.

NADEAU, J. A. & SACK, F. D. (2002) Control of stomatal distribution on the Arabidopsis leaf surface. *Science,* 296**,** 1697-1700.

PETROV, D. A. (2001) Evolution of genome size: new approaches to an old problem. *Trends in Genetics,* 17**,** 23-28.

POOLE, I., LAWSON, T., WEYERS, J. D. B. & RAVEN, J. A. (2000) Effect of elevated CO2 on the stomatal distribution and leaf physiology of Alnus glutinosa. *New Phytologist,* 145**,** 511-521.

SANMIGUEL, P. & BENNETZEN, J. L. (1998) Evidence that a recent increase in maize genome size was caused by the massive amplification of intergene retrotransposons. *Annals of Botany,* 82**,** 37-44.

SANMIGUEL, P., TIKHONOV, A., JIN, Y. K., MOTCHOULSKAIA, N., ZAKHAROV, D., MELAKEBERHAN, A., SPRINGER, P. S., EDWARDS, K. J., LEE, M., AVRAMOVA, Z. & BENNETZEN, J. L. (1996) Nested retrotransposons in the intergenic regions of the maize genome. *Science,* 274**,** 765-768.

SENA GOMES, A. R. & KOZLOWSKI, T. T. (1987) Stomatal Characteristics, Leaf Waxes, and Transpiration Rates of Theobroma cacao and Hevea brasiliensis Seedlings. *Annals of Bontany,* 61**,** 425-432.

SHARMA, G. K. & DUNN, D. B. (1968) Effect of Environment on the Cuticular Features in Kalanchoe fedschenkoi. *Bulletin of the Torrey Botanical Club,* 95**,** 464-473.

STEFANO, M. & ROSARIO, M. (2003) Effects of light quality on micropropagation of woody species. IN JAIN, S. M., & ISHII, K. (Ed.) *Micropropagation of Woody Trees and Fruits.* Dordrecht, Netherlands, Kluwer Academic Publishers.

VAN'T HOF, J. & SPARROW, A. H. (1963) A Relationship between DNA Content, Nuclear Volume, and Minimum Mitotic Cycle Time. *Proceedings of the National Academy of Sciences of the United States of America,* 49**,** 897-902.

VITTE, C., PANAUD, O., QUESNEVILLE, H. & NF (2007) LTR retrotransposons in rice (Oryza sativa, L.): recent burst amplifications followed by rapid DNA loss. *Bmc Genomics,* 8**,** 15.
WILLMER, C. M. & FRICKER, M. D. (1995) *Stomata,* Kluwer Academic.

Appendix 1: Progress Form
Appendix 2: Project Plan & Safety Assessment
Appendix 3: Glossary

Hemicryptophyte A perennial plant with overwintering buds located at the soil surface.

Appendix 4(a): Table of raw data from sampling within leaf sections

	Mean guard cell length (um)			
Species	**Section 1**	**Section 2**	**Section 3**	**Section 4**
Bletilla striata	39.39 ± 4.15	36.46 ± 3.25	39.73 ± 4.17	42.74 ± 3.44
Bulbophyllum lepidum	30.27 ± 2.08	29.54 ± 2.07	29.07 ± 2.09	29.67 ± 2.21
Ceologyne massangeana	36.72 ± 3.86	36.25 ± 2.82	34.96 ± 2.37	36.51 ± 2.11
Ornithophora radicans	26.66 ± 1.84	24.47 ± 2.17	22.36 ± 1.89	25.24 ± 1.78
Dendrobium speciosum	32.51 ± 2.48	32.98 ± 3.24	34.74 ± 2.99	34.40 ± 3.04

Appendix 4(b): Results of ANOVA of guard cell length between leaf sections for each species.

Bletilla striata

Source	DF	SS	MS	F	P
Sections	3	175.539	58.513	6.72	0.000
Error	196	1706.719	8.708		
Total	199	1882.259			

Bulbophyllum lepidum

Source	DF	SS	MS	F	P
Section	3	36.864	12.288	2.75	0.044
Error	196	877.258	4.476		
Total	199	914.122			

Coelogyne massangeana

Source	DF	SS	MS	F	P
Section	3	93.814	31.271	3.81	0.011
Error	196	1610.017	8.214		
Total	199	1703.830			

Ornithophora radicans

Source	DF	SS	MS	F	P
Section	3	483.14	161.05	43.38	0.000
Error	196	727.58	3.71		
Total	199	1210.73			

Dendrobium speciosum

Source	DF	SS	MS	F	P
Section	3	175.539	58.513	6.72	0.000
Error	196	1706.719	8.708		
Total	199	1882.259			

Appendix 4(c): Results of ANOVA for guard cell length between each species.

Source	DF	SS	MS	F	P
Species	4	8.5032	2.1258	11.06	0.000
Error	15	2.8834	0.1922		
Total	19	11.3866			

Appendix 5: Table of raw data from sampling of fresh specimens of species with known genome sizes

Species	Guard cell length (um)		1C value (pg)	Genome size source	Lifeform	Accession No.
	Mean	+/-				
Phaius tankervilleae	42.05	3.08	8.5	Narayan et al., 1989	Geophyte	2002-2582
Calanthe tricarinata	42.96	2.69	13.25	Narayan et al., 1989	Geophyte	2003-475
Barkeria lindleyana	28.42	1.70	1.65	Jones et al., 1998	Epiphyte	2004-31
Cattleya forbesii	29.76	1.65	1.65	Jones et al., 1998	Epiphyte	-
Laelia rubescens	41.67	2.12	1.25	Jones et al., 1998	Epiphyte	1983-5544
Bletilla striata	36.46	3.25	2.95	Zonneveld et al., 2005	Geophyte	1969-32689
Coelogyne flaccida	58.65	3.07	4.45	Narayan et al., 1989	Epiphyte	1998-2369
Pholidota imbricata	31.52	2.93	3.08	Narayan et al., 1989	Epiphyte	1984-3301
Pleione bulbocodioides	52.31	4.17	5.35	Zonneveld et al., 2005	Geophyte	2003-674
Ada aurantiaca	34.19	2.09	3.5	Jodrell	Epiphyte	1975-464
Ansellia africana	35.91	1.91	1.85	Jones et al., 1998	Epiphyte	1981-1922
Aspasia lunata	28.04	1.84	3.56	Jodrell	Epiphyte	2002-2517
Disa tripetaloides	62.86	6.66	5.95	Suda	Geophyte	2004-1941
Brassia verrucosa	39.56	2.04	3.92	Jodrell	Epiphyte	1984-1741
Caucaea nubigena	34.77	2.63	3.94	Jodrell	Epiphyte	1999-2767
Cochlioda noezliana	23.05	1.68	3.48	Hanson et al., 1999	Epiphyte	1999-2954
Cuitlauzina pendula	27.99	1.60	3.5	Jodrell		1998-4207
Grammatophyllum scriptum	35.53	2.05	1.7	Jones et al., 1998	Epiphyte	2005-2709
Helcia sanguinolenta	34.83	2.57	3.83	Hanson et al., 1999	Epiphyte	2000-4462
Lockhartia oerstedii	23.74	1.79	1.8	Hanson et al., 1999	Epiphyte	1975-2611
Miltonia regnellii	30.36	1.77	4.71	Jodrell	Epiphyte	1979-1762
Odontoglossum wyattianum	29.86	1.90	3.95	Hanson et al., 1999	Epiphyte	2005-2617
Ornithophora radicans	24.47	2.17	0.88	Jodrell	Epiphyte	2003-2884
Peristeria elata	32.06	2.36	4.65	Jones et al., 1998	Epiphyte	2003-277
Rossioglossum grande	51.42	3.22	8.505	Knight et al	Epiphyte	2002-2100
Odontoglossum sanguineum	25.41	2.19	4.18	Jodrell	Epiphyte	2004-120
Trichoceros antennifer	40.26	3.85	4.38	Hanson et al., 1999	Epiphyte	2005-995
Trigonidium egertonianum	35.56	2.60	1.75	Jodrell	Epiphyte	2005-1025
Aerides odorata	37.24	2.15	3.78	Narayan et al., 1989	Epiphyte	2002-133
Cleisostoma subulatum	27.97	1.65	3.2	Jones et al., 1998	Epiphyte	1992-899
Doritis pulcherrima	28.25	2.61	6.75	Lin et al., 2001	Epiphyte	1979-311
Rhynchostylis retusa	46.91	2.77	2.58	Narayan et al., 1989	Epiphyte	1984-4643
Schoenorchis gemmata	35.20	2.46	3.2	Narayan et al., 1989	Epiphyte	2004-27
Smitinandia micrantha	28.08	2.40	2.1	Jones et al., 1998	Epiphyte	2004-108
Vanda coerulea	29.19	2.54	16.8	Zonneveld et al., 2005	Epiphyte	2002-2731

Vanilla pompona	32.12	2.28	7.25	Jones et al., 1998	Climber	2003-309
Vanilla planifolia	26.40	2.89	7.95	Arumuganathan and Earle, 1991	Climber	1990-1994
Phragmipedium longifolium	42.48	2.36	6.1	Cox et al., 1996	Hemicr	1988-2127
Oncidium marshallianum	27.76	1.41	1.83	Hanson et al., 1999	Epiphyte	2004-68
Phalaenopsis amabilis	23.44	1.75	1.18	Nagl and Capesius, 1977	Epiphyte	1998-2397
Paphiopedilum appletonianum	64.90	3.31	7.7	Hanson et al., 1999	Hemicr	1981-1588
Dendrobium pulchellum	36.97	1.46	1.6	Jones et al., 1998	Epiphyte	1993-1957
Bulbophyllum cocoinum	44.42	2.11	2.7	Jones et al., 1998	Epiphyte	1933-18505
Liparis condylobulbon	33.82	2.38	8.695	Suda	Epiphyte	1984-3212
Dendrobium crumenatum	24.89	1.61	1.3	Jones et al., 1998	Epiphyte	2002-2116
Dendrobium lindleyi	28.09	2.20	1.2	Jones et al., 1998	Epiphyte	2004-3527
Phragmipedium pearcei	42.36	2.62	6.33	Cox et al., 1996	Hemicr	1987-2715
Phragmipedium caudatum	49.38	2.26	9.2	Cox et al., 1996	Hemicr	1986-2247
Paphiopedilum wardii	72.41	3.84	34.5	Cox et al., 1996	Hemicr	1990-266
Paphiopedilum exul	49.11	2.26	16.5	Hanson et al., 1999	Hemicr	1981-3284
Paphiopedilum gratrixianum	51.00	3.07	25	Cox et al., 1996	Hemicr	2002-1341
Paphiopedilum philippinense	53.31	2.74	23.25	Cox et al., 1996	Hemicr	1989-3410
Paphiopedilum lowii	47.79	2.55	24.53	Hanson et al., 1999	Hemicr	1998-2146
Paphiopedilum rothschildianum	57.52	2.65	22.6	Cox et al., 1996	Hemicr	1983-2788
Paphiopedilum insigne	48.16	3.38	23.05	Cox et al., 1996	Hemicr	1983-5460
Paphiopedilum druryi	52.77	1.72	26.75	Cox et al., 1996	Hemicr	1976-952
Paphiopedilum adductum	49.68	2.05	27.03	Hanson et al., 1999	Hemicr	1992-3661
Paphiopedilum haynaldianum	52.06	2.43	22.85	Hanson et al., 1999	Hemicr	1990-211
Paphiopedilum lawrenceanum	64.20	2.71	26.13	Hanson et al., 1999	Hemicr	1990-256
Paphiopedilum micranthum	60.39	3.30	22.78	Cox et al., 1996	Hemicr	1990-195
Paphiopedilum primulinum	55.54	2.47	20.93	Cox et al., 1996	Hemicr	2002-3414
Paphiopedilum villosum	44.07	2.18	22.48	Narayan et al., 1989	Hemicr	1981-1548
Paphiopedilum sukhakulii	69.51	3.45	29.75	Cox et al., 1996	Hemicr	1981-3290
Paphiopedilum delenatii	66.34	3.49	21.83	Cox et al., 1996	Hemicr	1998-2187
Paphiopedilum concolor	68.96	2.81	19.48	Cox et al., 1996	Hemicr	1987-4004
Paphiopedilum glanduliferum	52.25	2.44	23.75	Cox et al., 1996	Hemicr	1953-38501
Paphiopedilum dianthum	47.55	2.92	35.9	Hanson et al., 1999	Hemicr	1990-215
Paphiopedilum barbatum	69.63	3.04	33.75	Cox et al., 1996	Hemicr	2002-3417
Dendrobium moschatum	28.98	1.54	4.65	Narayan et al., 1989	Epiphyte	1984-4674
Dendrobium gouldii	34.80	1.87	1.05	Jones et al., 1998	Epiphyte	1982-2351
Paphiopedilum exul	54.90	2.39	16.5	Hanson et al., 1999	Hemicr	1981-3284

Appendix 6: Results of ANOVA for 71 orchid species with known genome size.

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	6977.4	6977.4	89.90	0.000
Residual Error	69	5355.6	77.6		
Total	70	12333.0			

Appendix 7: Table of raw data comparing specimens before and after drying

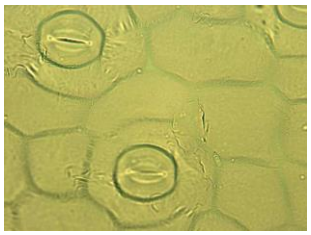
Species	**Guard cell length in fresh specimen (µm)**		**Guard cell length in dried specimen (µm)**	
	Mean	**+/-**	**Mean**	**+/-**
Phaius tankervilleae	42.05	3.08	39.65	2.81
Calanthe tricarinata	42.96	2.69	43.92	2.76
Barkeria lindleyana	28.42	1.70	30.6525	1.93
Cattleya forbesii	29.76	1.65	35.441	2.44
Laelia rubescens	41.67	2.12	40.992	2.21
Bletilla striata	36.46	3.25	32.551	3.89
Coelogyne flaccida	58.65	3.07	60.634	2.73
Pholidota imbricata	31.52	2.93	33.977	2.69
Pleione bulbocodioides	52.31	4.17	53.924	4.04
Ada aurantiaca	34.19	2.09	34.221	2.07
Ansellia africana	35.91	1.91	40.931	1.91
Aspasia lunata	28.04	1.84	30.744	1.55
Brassia verrucosa	39.56	2.04	48.434	2.44
Cochlioda noezliana	23.05	1.68	29.158	2.80
Cuitlauzina pendula	27.99	1.60	31.5675	1.64
Helcia sanguinolenta	34.83	2.57	42.8525	2.71
Lockhartia oerstedii	23.74	1.79	27.8465	2.82
Miltonia regnelli	30.36	1.77	32.3605	1.80
Odontoglossum wyattianum	29.86	1.90	29.9815	2.01
Peristeria elata	32.06	2.36	32.757	3.18
Rossioglossum grande	51.42	3.22	48.495	2.96
Symphyglossum sanguineum	25.41	2.19	25.1625	2.25
Trichoceros antennifer	40.26	3.85	41.968	3.85
Trigonidium egertonianum	35.56	2.60	35.2275	2.37
Aerides odorata	37.24	2.15	40.748	2.33
Cleisostoma subulatum	27.97	1.65	27.267	1.76
Doritis pulcherrima	28.25	2.61	29.7985	2.23
Rhynchostylis retusa	46.91	2.77	57.0045	3.70
Schoenorchis gemmata	35.20	2.46	34.892	2.08
Smitinandia micrantha	28.08	2.40	32.0555	2.93
Vanda coerulea	29.19	2.54	32.5435	3.47
Vanilla pompona	32.12	2.28	35.807	3.21
Vanilla planifolia	26.40	2.89	26.8705	2.26
Phragmipedium longifolium	42.48	2.36	41.3275	2.79
Oncidium marshallianum	27.76	1.41	30.4695	1.96
Phalaenopsis amabilis	23.44	1.75	28.243	2.84
Paphiopedilum appletonianum	64.90	3.31	63.867	3.63
Dendrobium pulchellum	36.97	1.46	38.5825	3.67
Bulbophyllum cocoinum	44.42	2.11	48.7085	2.43
Liparis condylobulbon	33.82	2.38	32.7265	2.20

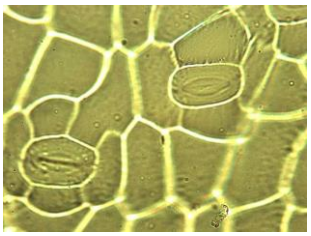
Appendix 8: Table of raw data from sampling of dried *Apostasia* and *Neuwiedia* specimens

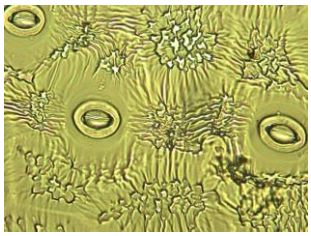
Species	Mean guard cell length (µm)	Standard deviation guard cell length	Origin	Collector	Collector's no.	Collection date
A. elliptica	10.16	1.00	Borneo	Poulsen, A.D.	89B	25/05/1991
A. latifolia	10.68	1.45	Malay Peninsula	Scortechini	868	1888
A. nuda	9.61	1.12	Burma	Griffith, W.	100	
A. nuda	12.38	1.12	Malay Peninsula	Robinson, H.C.	1913	
A. nuda	11.77	1.22	Borneo	Perumal, B. & Dewol, S.	134994	09/07/1994
A. odorata	8.78	1.55	China	Henry, A.	13738	
A. odorata	12.87	1.04	India	n/k	n/k	
A. odorata	9.79	1.14	Malay Peninsula	Corner, E.J.H.		15/11/1941
A. wallichi	9.61	1.41	India	Clarke, C.B.		
A. wallichi	10.31	0.93	India	Cowan, J.M.		04/12/1923
A. wallichi	9.91	1.89	Burma	Kerr, A.F.G.	11	
A. wallichi	11.96	1.23	Sri Lanka	Waas, S.	1949	23/02/1977
N. borneensis	23.61	1.15	Sabah	Lamb, A. & Lohok, H.	355/85	
N. borneensis	22.11	2.30	Brunei	Forman, L.L.	1113	27/10/1989
N. borneensis	22.30	2.01	Indonesia	Rachman, I.	15456	27/03/1998
N. elongata	24.13	2.05	Borneo	Kostermans, A.	12988	13/09/1956
N. griffithii	20.25	2.10	Malay Peninsula	Maingay, A.C.	10268	14/01/1928
N. griffithii	18.73	2.03	Sumatra	Yates, H.S.	2258	
N. griffithii	23.85	1.75	Sumatra	Bartlett, H.H.	6421	
N. veratrifolia	17.14	2.15	Malay Peninsula	Henderson, M.R.	24054	16/10/1930
N. veratrifolia	21.69	1.62	New Guinea	Streimann, N.G.F.	24478	28/04/1972
N. veratrifolia	21.84	1.74	Solomon Islands	Dennis, G.F.C.	12	10/05/1984
N. zollingeri var. singapureana	24.13	1.59	Malay Peninsula	n/k	12154	Dec 1905
N. zollingeri var. singapureana	21.01	1.55	Indonesia	Henderson, M.R.	20407	15/04/1928
N. zollingeri var. singapureana	23.06	1.92	Thailand	Kerr, A.F.G.	15940	

Appendix 9. Photographs of selected orchid stomata

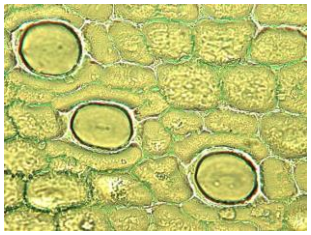
Phaius tankervilliae *Calanthe tricarinata* *Barkeria lindleyana* *Cattleya forbesii*

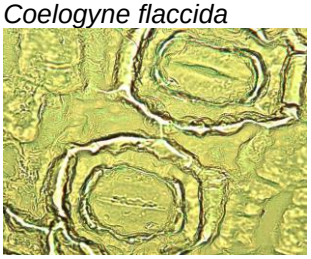

Laelia rubescens

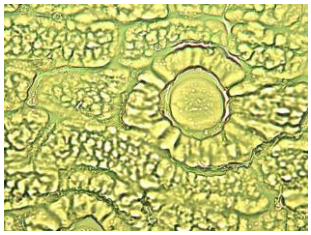

Coelogyne flaccida

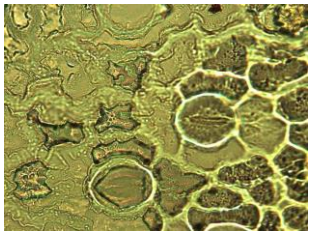

Pholidota imbricata


Ada aurantiaca


Ansellia Africana


Aspasia lunata

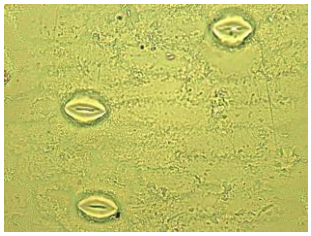

Brassia verrucosa


Cochlioda noezliana

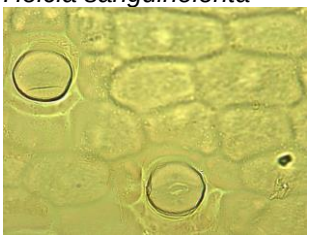

Cuitlauzina pendula

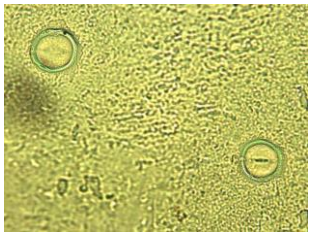

Helcia sanguinolenta


Lockhartia oerstedii


Miltonia regnellii

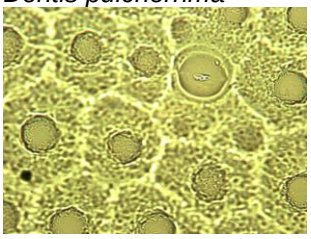

Aerides odorata

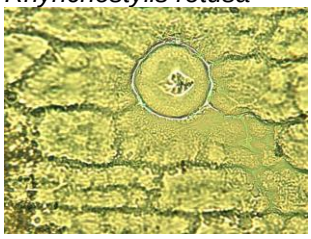

Doritis pulcherrima


Rhynchostylis retusa

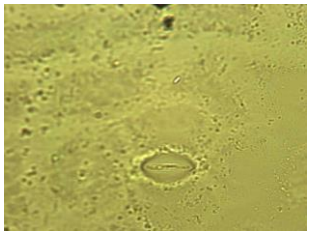

Smitinandia micrantha

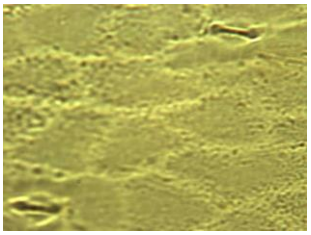

Vanilla pompona

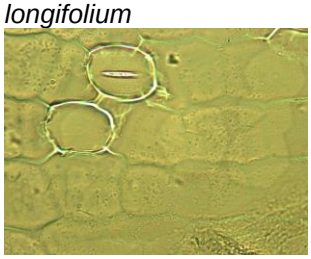

Vanilla planifolia

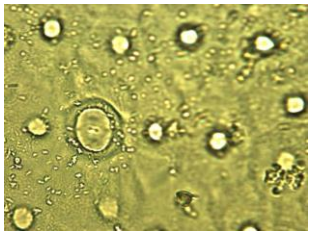

Phragmipedium longifolium

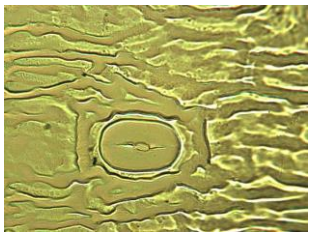
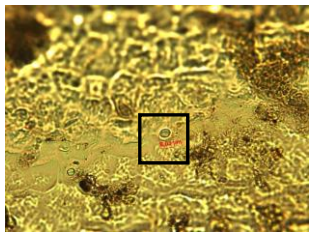
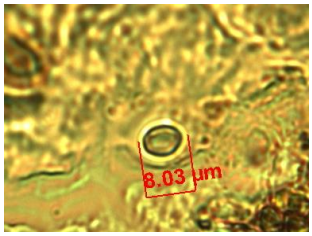

Phalaenopsis amabilis


Bulbophyllum cocoinum


Apostasia odorata


Apostasia odorata (enlarged)


Neuwiedia veratrifolia




8.03 µm