CLIVIA

Yearbook of the Clivia Society

13

2011

20th Anniversary Issue

CLIVIA **The Clivia Society** | www.cliviasociety.org

The Clivia Society caters for *Clivia* enthusiasts throughout the world. It is the umbrella body for a number of constituent Clivia Clubs and interest Groups which meet regularly in South Africa and elsewhere around the world. In addition, the Society has individual members in many countries, some of which also have their own Clivia Clubs. An annual Yearbook and quarterly Newsletters are published by the Society. For information on becoming a member and / or for details of Clivia Clubs and Interest Groups contact the Clivia Society secretary or where appropriate, the International contacts, at the addresses listed in the inside back cover.

THE OBJECTIVES OF THE CLIVIA SOCIETY

1. To coordinate the interests, activities and objectives of constituent Clivia Clubs and associate members;
2. To participate in activities for the protection and conservation of the genus *Clivia* in its natural habitat, thereby advance the protection of the natural habitats and naturally occurring populations of the genus *Clivia* in accordance with the laws and practices of conservation;
3. To promote the cultivation, conservation and improvement of the genus *Clivia* by:
   - 3.1 The exchange and mutual dissemination of information amongst Constituent Clivia Clubs and associate members;
   - 3.2 Where possible, the mutual exchange of plants, seed and pollen amongst Constituent Clivia Clubs and associate members; and
   - 3.3 The mutual distribution of specialised knowledge and expertise amongst Constituent Clivia Clubs and associate members;
4. To promote the progress of and increase in knowledge of the genus *Clivia* and to advance it by enabling research to be done and by the accumulation of data and dissemination thereof amongst constituent Clivia Clubs and associate members;
5. To promote interest in and knowledge of the genus *Clivia* amongst the general public; and
6. To do all such things as may be necessary and appropriate for the promotion of the above mentioned objectives.

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COVER PHOTOGRAPHS

FRONT COVER: *Clivia miniata* with a "Mandarin Duck" variegated umbel.
Grower: Peet van der Walt. Photo: Roger Dixon.

BACK COVER: *Clivia* 'Carmen Miranda'. Photo: Gordon Fraser.

Contents

Hirao polytepal Photo: Kina van der Merwe

A Tribute to Mick Dower

Mick Dower, surely one of the giants of the world-wide clivia fraternity, died in his sleep on 12 October 2011. He had been in increasingly poor health for some time. His contributions to the development of interest in the growing and breeding of clivias will be remembered long after his passing.

Mick attended the inaugural International Conference and Show in Pretoria in 1994 and, with John Winter and a few others in 1996, started the Cape Province Branch of the Clivia Club. He served on the committee of the Club and also of the Clivia Society for several years. With a small group he organized the second Clivia Conference in Cape Town in 1998. He was a founder of the Clivia Yearbook, and the lead editor

Figure 1. A photomontage of Mick Dower and plants he had bred or grown. Composed by Claude Felbert.

from number 1 up to number 5.

Mick was a lawyer with an incisive mind. He drafted the Constitutions of the Cape Province Branch of the Clivia Club and its successors, the Cape Clivia Club

Photo: Claude Felbert

Figure 2. Grown and bred by Mick Dower: #229 De Villiers Variegated Peach × #81 Nakamura pinstripe Yellow.

Photo: Claude Felton

Figure 3. *C. miniata* 'Floradale Apricot'. A favourite breeding plant of Mick Dower.

and the Eastern Province Clivia Club. He also assisted in the drafting of the initial Constitution of the Clivia Society. He was often called on, sometimes behind the scenes, for advice on legal matters and gave that advice most willingly. He also served on the Society's Research Committee and the Standards and Judging sub-committee.

John Winter, Curator of Kirstenbosch and close friend of Mick, played a pivotal role in the search for *Clivia* populations. From 1996, together with taxonomist John Rourke, they made many sorties into the hidden corners of *Clivia* habitat in southern Africa. They collected the well-known 'Apple Blossom' series, the 'Komgha Red', the Mbashe and Umtamvuma pastels and many other variants, all of which were added to the SANBI collection. On behalf of SANBI Mick helped, for many years, to distribute seeds from that collection to enthusiasts in South Africa and throughout the world.

After he retired from professional life Mick had more time to devote to his clivias and to clivia-related interests, particularly the genetics of *Clivia*. He contributed at conferences, in the Clivia Yearbook, the Society's Clivia News, and in the Cape Clivia News, which he established. He was also an active member of the Clivia Enthusiasts internet discussion group. Through that membership, his direct personal contacts, and his contacts with people to whom he distributed seeds and plants, Mick's name became known internationally.

Mick was active in Cape Clivia Club shows, exhibitions, workshops, plant sales and Kirstenbosch Garden Fairs. He utilised every opportunity to advance *Clivia*. His affection, enthusiasm and expertise were usually so contagious that many visitors became members of the Club. He impressed all at shows and exhibitions with the outstanding prize-winning plants he bred and grew. At Club meetings he

Photo: Mick Dower

Figure 4. *Clivia* 'JKD'. One of the six plants named by Mick Dower after his grandchildren.

Photo: Mick Dower

Figure 5. *C. miniata* 'Mopi Hirt' sent to Mick by a South African friend from America. He named it after her.

Photo: Mick Dower

Figure 6. *C. miniata* 'Julia D' bred out of *C. miniata* 'Floradale Apricot' x *C. miniata* 'Oribi Gorge Yellow'. One of the six plants named by Mick Dower after his grandchildren. A registered plant.

donated many of the raffle plants, usually exceptional and valuable specimens. As a result, his plant material has been spread across the membership of the Club. Mick was also generous in giving away top-class plants and suckers, particularly to younger people and to newcomers to the Club wanting to establish collections.

Mick contributed to a greater understanding of genetics through his experiences and knowledge of breeding which he freely shared with all. He was particularly keen to encourage Club members to exhibit plants that they had bred themselves, as opposed to those acquired from others. He persuaded the Club committee to introduce the "Own Breeding" class at Club shows, with various categories within the class. He donated a Trophy for the plant judged best out of all the entrants. His vision has paid off as the number of entries has grown over the years. The "Own breeding" class was in fact the largest class at the 2011 Cape Clivia Club Show.

In his retirement Mick became a very proficient photographer. Two of his plants featured on Yearbook front or back covers. One was "Emma Leslie". The other one was on the front cover of Yearbook 8 and was a winning entrant in the Clivia Society photographic competition that year. But that is not all by any means. He and Claude Felbert together developed and produced the two Cape Clivia Club colour charts which many of us regularly use.

A devoted family man, Mick leaves his wife Jill, children and their spouses, and grandchildren, all of whom supported him as he indulged in his passion for all things clivia.

The memorial to Sir Christopher Wren, architect of St Paul's Cathedral, London, includes the words in Latin, "Reader, if you seek his monument – look around you." In

Figure 7. *C. mirnata* 'Rosemary D'. One of the six plants named by Mick Dower after his grandchildren.

Figure 8. Mick Dower bred *C. mirnata* hybrid from (Warren Glover Yellow x Bill Morris Yellow) x (Fragrant Yellow x 'Floradale Apricot').

Figure 9. *C. mirnata* of Mick Dower's own breeding, a section winner at the 2018 Cape Cliva Club Show.

a clivia sense, the same thing can be said about Mick Dower. His monument is to be seen in the legacy of plants and plant material that he has left behind for so many of us throughout the world to enjoy and by which we can remember him.

This tribute to Mick was compiled by three of his friends who share his love of clivias and who remember him with affection.

October 2011

Photo: Claude Felten

Figure 10. A Mick Dower bred 'Ghost'-style *C. miniata*.

Photo: Mick Dower

Figure 11. *C. miniata* 'Emma Leslie'. One of the six plants named by Mick Dower after his grandchildren.

Photo: Claude Felten

Figure 12. A sibling of 'Emma Leslie' bred by Mick Dower.

Photo: Mick Dower

Figure 13. *C. miniata* 'Katie D'. One of the six plants named by Mick Dower after his grandchildren.

Photo: Mick Dower

Figure 14. *C. miniata* 'Margot D'. One of the six plants named by Mick Dower after his grandchildren.

Systematics and Phylogeography of *Clivia*

Ferozah Conrad & Dee Snijman

South African National Biodiversity Institute, Cape Town

The genus *Clivia* comprises six species: *C. miniata* (Lindl.) Bosse, *C. nobilis* Lindl., *C. gardenii* Hook, *C. caulescens* R.A.Dyer, *C. robusta* B.G.Murray, Ran. de Lange, Hammett, Truter et Swanev. and *C. mirabilis* Rourke. Five species (*C. miniata, C. nobilis, C. gardenii, C. caulescens* and *C. robusta*) are found in coastal and inland Afromontane forest along the east coast of southern Africa, from the Eastern Cape northwards to Limpopo Province and Swaziland (Rourke, 2002). One species, *C. mirabilis* which was discovered in 2002, occurs in the Oorlogskloof Nature Reserve, in a semi-arid valley in the Northern Cape.

The discovery of *C. mirabilis*, in a climate and locality so different from that previously known for *Clivia*, has prompted phylogeographic questions regarding this genus. *Clivia* has always been seen as belonging to densely forested, subtropical environments experiencing a summer rainfall, dry winter climatic regime. *Clivia mirabilis*, in direct contrast, occurs remotely in the arid Northern Cape with its strictly winter rainfall regime, isolated from the other *Clivia* species (Rourke, 2002). The distribution ranges of the tubular-flowered *Clivia* species (*C. nobilis, C. gardenii, C. caulescens* and *C. robusta*) are parapatric in relation to one another [they do not occupy the same geographical ranges but the ranges are contiguous (Wiley, 1981)], whereas the distribution range of the open-flowered *C. miniata* is partly sympatric [populations of two or more species are found together with all the tubular-flowered species, except for *C. mirabilis* in one part of their distribution range, but apart from them in another (Wiley, 1981)].

Photo: Neil Hewitt

Figure 1. *Clivia mirabilis*, Oorlogskloof Nature Reserve, Northern Cape.

In terms of morphology, *C. miniata* is distinct and easy to identify, but the morphological differences between the tubular-flowered species are subtle and geographical data have often been favoured in the identification of species.

Based on an earlier study by Conrad *et al.* (2003), the winter rainfall *C. mirabilis* is placed as sister to all of the summer rainfall

species. This prompts questions whether the disjunction to the Northern Cape is due to vicariance or dispersal and whether *C. mirabilis* shows reduced genetic diversity. In addition, partial sympatry between *C. miniata* and the tubular-flowered species also prompts questions about the discreteness of the populations in the areas of overlap.

C. robusta was described from Pondoland, Transkei, along the east coast of South Africa in 2004. Murray *et al.* (2004) used karyological, morphological and distribution pattern data to distinguish *C. robusta* from *C. gardenii*, the species to which it is most closely related. Considering the controversial nature of this taxonomic decision it poses an interesting population level case study.

Given the unusual distribution pattern of *Clivia*, its horticultural importance and its growing commercial market, the aim of this investigation was firstly to elucidate the species level relationships of *Clivia* and secondly to obtain a better understanding of the evolutionary relationships, by exploring the phylogeographic patterns within the *Clivia* species and among them.

Materials and Methods

Sampling

Of the 107 individuals sampled, 89, representing 33 populations across the distribution range, were successfully amplified for three plastid regions: the *rpoB-trnC* intergenic spacer, *trnL* intron and *trnL-F* intergenic spacer. Leaf material from all the localities cited by Murray *et al.* (2004) in their description of *Clivia robusta* was included in the analysis.

Selection of DNA regions for analysis was based on the broader study of the tribe,

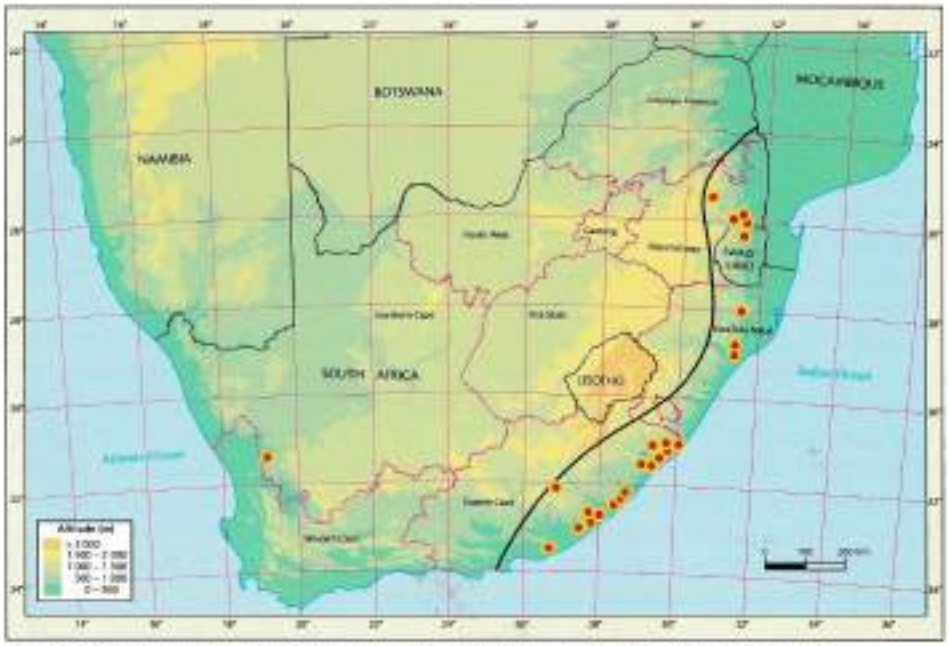

Figure 2. Distribution map of *Clivia*. Blue circles indicate populations included in the study.

where the *trnL-F* region and the *rpoB-trnC* intergenic spacer produced the most variation. Voucher and population locality information are listed in Table 1.

DNA extraction, PCR and DNA sequencing

Standard extraction, PCR and DNA sequencing protocols were followed.

Phylogenetic and phylogeographic analyses

To investigate genetic relationships among the haplotypes, networks were constructed separately for both the individual regions (*trnL-F* and *rpoB-trnC*) and the combined data matrices. Analysis software TCS (Clement *et al.*, 2000) and median joining networks using Network version 4.1.0.0 (Bandelt *et al.*, 1999) were used, and all the individuals (n=89) were included in the analyses.

To allow an assessment of the degree of differentiation among the sampling areas, a spatial analysis of molecular variance (SAMOVA; Dupanloup *et al.*, 2002) was conducted *post hoc* identification of clades in the network. The SAMOVA takes into consideration geographic locations of sampling and sequence data to identify groups of populations that are geographically homogenous and maximally separated from each other. It aims to maximize the proportion of genetic variation due to differences between groups based on simulated annealing procedures (Dupanloup *et al.*, 2002). As such, this analysis was useful for statistically differentiating between historically isolated groups in the network (Tolley *et al.*, 2006). It also incorporates traditional F-statistics (F_{CT}, F_{SC}, F_{ST}) in recognising population substructure. F_{CT} is the proportion of total genetic variance due to the

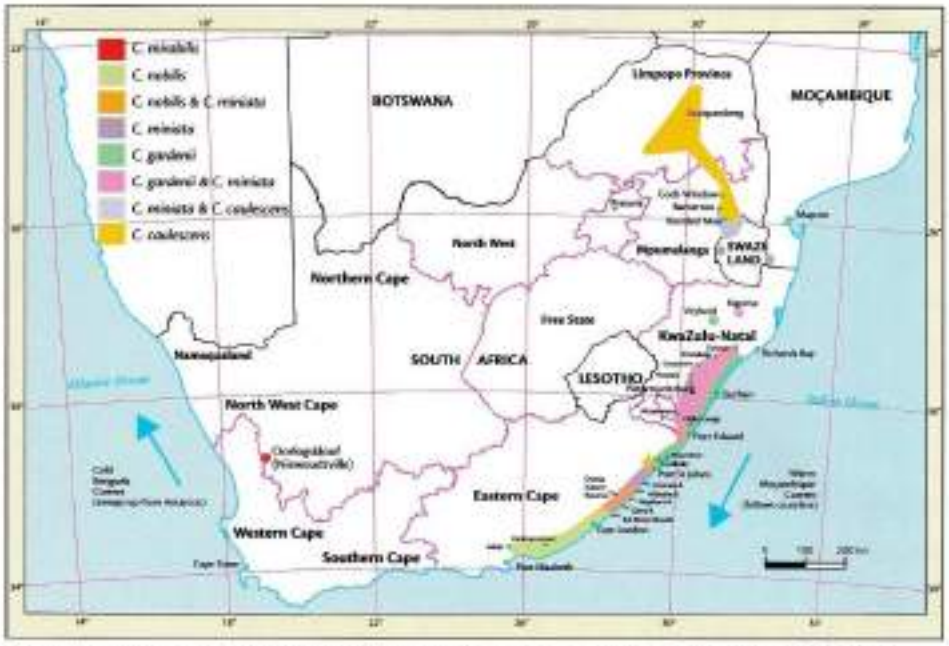

Figure 3. The known distribution of *Clivia* species (orange star = *C. robusta*). The map also shows where the species grow sympatrically. Source: Felbert (2003).

Table 1. Kirstenbosch Botanical Garden (NBG) accession information and population locality details for all *Clivia* individuals used in the analyses.

Population	Haplotype network reference	Individuals in population	Locality	Grid	Collector's details	Kirstenbosch accession number
Clivia caulescens	Caul01-03	3	locality 1 – Mpumalanga, Malandweni, Lobodtlyana Cliffs/ kloofs on Vooruitzicht	2531CC	JWI 569	NBG 441/99
Clivia caulescens	Caul04-05	2	locality 2 – Mpumalanga Elandshoogte Ngodwana Sappi forest station	2530DA	JWI 57	NBG 433/99
Clivia caulescens	Caul06-09	4	locality 3 – Swaziland, Bearded Man	2531CD	JWI 573	NBG 573/99
Clivia caulescens	Caul10	1	locality 4 – Swaziland, Malandzela	2631AB	ALL	NBG 321/00
Clivia caulescens	Caul11-14	4	locality 5 – Mpumalanga, Malandweni in Songimvelo Nature Reserve	2531CC	JWI 567	NBG 439/99
Clivia gardenii	Gard15-18	4	locality 1 – KZN, Ngome Forest	2731CD	JWI 565	NBG 437/99
Clivia gardenii	Gard19-21	3	locality 2 – KZN, Mpushini Falls on the outskirts of Eshowe	2831CD	JWI 559	NBG 430/99
Clivia gardenii	Gard22-25	4	locality 3 – EC, Mkambati Nature Reserve	3129BB	JW557	NBG 517/98
Clivia gardenii	Gard26-29	4	locality 4 – KZN, Entumeni Forest (Ngoye) at the waterfall on Ngoje River	2831CD	JWI 562	NBG 434/99
Clivia gardenii	Gard33-36	4	locality 5 – EC Umtentu Valley, Mkambati side of river	3129BB	Ex hort Fred van Niekerk	
Clivia gardenii	Gard37	1	locality 6 – EC Cultivated from seed from above plants	3129BB	Ex hort Fred van Niekerk	
Clivia gardenii	Gard38	1	locality 7 – EC, Lombazi riverbank, swamp	3129BD	Ex hort Len Chiazzara	
Clivia robusta	Robu30-32	3	locality 1– EC, Dimfi few km east of Ndindindi (locality 5 from Murray et al. paper)	3129BD	JWI 579	NBG 314/00
Clivia robusta	Robu39	1	locality 2 – EC, Mkambati Nature Reserve, cultivated at Kirstenbosch, Prof Kobus Eloff (Locality 1 from Murray et al. paper)	3129BD	JW557	NBG 517/98
Clivia robusta	Robu40	1	locality 3 – EC, Near Lusikisiki at the Fraser Falls S. Venter (Locality 2 from Murray et al. paper)	3129BC	Venter 3864	PRE 556875
Clivia robusta	Robu41	1	locality 4 – EC, Mount Sullivan, Port St John (Holotype from Murray et al. paper)	3129BB	J.T. Truter 4072	
Clivia robusta	Robu42	1	locality 5 – EC, locality 9 Lambasi Village on the Ndindindi Road, Mkambati (locality 4 from Murray et al. paper)	3129BD	JPR 2180	NBG 313/00
Clivia robusta	Robu43	1	locality 6 – KZN, Umtamvuna, Port Edward (locality 6 from Murray et al. paper)	3129BD	JWI 554	NBG 514/98
Clivia robusta	Robu44	1	locality 7 – EC, Mkambati Reserve, 3km west of Umtentu River mouth (locality 3 from Murray et al. paper)	3129BB	JPR 2145	

Continued on page 12

Continued from page 11

Table 1. Kirstenbosch Botanical Garden (NBG) accession information and population locality details for all *Clivia* individuals used in the analyses.

Population	Haplotype network reference	Individuals in population	Locality	Grid	Collector's details	Kirstenbosch accession number
Clivia nobilis	Nobi45-47	3	locality 1 – EC, Bushmans River mouth – on dunes on west bank	3326DA	JWI 525	NBG 598/97
Clivia nobilis	Nobi48-51	4	locality 2 – EC, Nahoon Beach, Cambridge	3227DD	JWI 467	NBG 717/96
Clivia nobilis	Nobi52-54	3	locality 3 – EC, Ngabara River	3228BD	JWI 500	NBG 573/97
Clivia nobilis	Nobi55-57	3	locality 4 – EC, Wembley Farm	3228CC & CD	JW 534	NBG 606/97
Clivia nobilis	Nobi58-59	2	locality 5 – Transkei, Kei mouth collected on sand dunes, Butterworth	3228CB	JWI 485	NBG 715/96
Clivia nobilis	Nobi60	1	locality 6 – EC, Riet River	3326AA	JWI 466	NBG 716/90
Clivia miniata	Mini61-63	3	locality 1 – KZN, Entumeni River	2831CD	JWI	NBG 435/99
Clivia miniata	Mini64-67	4	locality 2 – EC, Umtamvuna Nature Reserve	3129CB	JWI555	NBG 513/98
Clivia miniata	Mini68-69	2	locality 3 – EC, Mbashe	3228BB	JWI 545	NBG 524/98
Clivia miniata	Mini70-73	4	locality 4 – Swaziland, Bearded Man (WEST) Mondi SATICO plantation	2531CD	JWI 570	NBG 442/99
Clivia miniata	Mini74-76	3	locality 5 – EC, Quora River, Mazeppa Bay	3228BC	JWI 470	NBG 720/96
Clivia miniata	Mini77-80	4	locality 6 – EC, Wembley Farm Gagura River and unnamed stream Kei V	3228CA	JPR 2195	NBG 569/00
Clivia miniata	Mini81-82	2	locality 7 – KZN Oribi Gorge, Port Shepstone, KZN- yellow, orange, white centred	3030CB	S Venter	
Clivia mirabilis	Mira83-87	5	Oorlogskloof Nature Reserve, Northern Cape	3119AC	JPR 2228	NBG 35/04

differences between groups of populations; F_{SC} reveals the degree of differentiation between populations within groups; F_{ST} shows the genetic variation between subpopulations relative to the total population. One hundred simulated annealing processes were performed for each possible number of populations, ranging from two through to eight populations for the *trnL-F* and combined datasets.

Haplotype diversity (*h*) and nucleotide diversity (π) among groups were calculated in Arlequin version 2.000 (Schneider *et al.*, 2000). The distribution of variation within and between assemblages was investigated by an analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) as implemented in Arlequin version 2.000.

Figure 4. *Clivia nobilis*, Bushmans River, Eastern Cape.

Figure 5. *Clivia mirabilis*, Oorlogskloof Nature Reserve, Northern Cape.

To assess the genetic divergence among these groups, F_{ST} and Φ_{ST} were estimated. F_{ST} takes into account only the differences in haplotype frequencies in the different populations, while Φ_{ST} takes into account both the haplotype frequency and nucleotide diversity (Hurwood & Hughes, 1998; Beheregaray & Sunnucks, 2001).

To estimate maximum likelihood (ML) migration rates among the populations of the six *Clivia* species we used MIGRATE version 2.1.3 (Beerli, 1997–2004). This approach, based on coalescence using Markov Chain Monte Carlo (MCMC) searches, takes both history and asymmetrical gene flow into account, unlike migration-drift equilibrium classical approaches, and allows simultaneous estimation of population growth or decline (Beerli & Felsenstein, 2001). The analyses were repeated several times with different combinations of short and long chains.

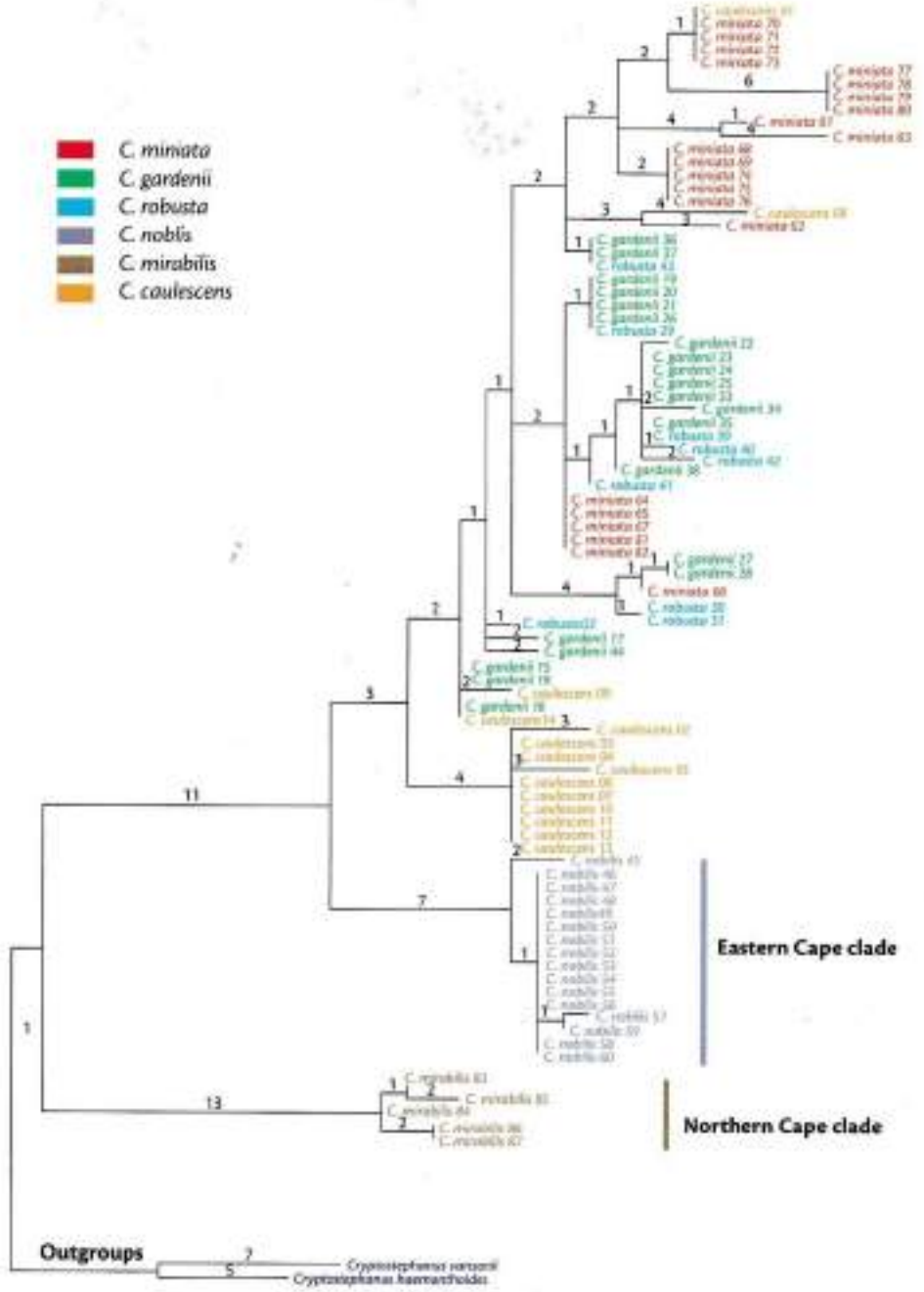

Figure 6. One of 208 equally parsimonious trees from combined analysis with branch lengths indicated above the branches. Numbers at end of species names indicate haplotype network reference numbers.

Results

Parsimony and Network analyses

Although individual datasets were analysed, only results from the combined analysis will be discussed as both the *trnL-F* and *rpoB-trnC* datasets represent chloroplast loci. Since the chloroplast is inherited as a unit, all loci by definition share the same history, and any differences must be due to positional sampling effects or compositional bias. It is therefore appropriate to combine chloroplast loci, as separate analyses are difficult to justify.

Haplotype reconstruction software Network (Bandelt *et al.*, 1999) was unable to execute data matrices consisting of more than 1000 characters. An alternative software TCS (Clement *et al.*, 2000) was utilised, as the combined datasets consisted of more than 1000 characters.

Of the 1732 characters included in the combined analysis 1667 were constant, 15 variable characters were parsimony-uninformative and 50 were parsimony informative. 3780 equally parsimonious trees were recovered with tree length 104, CI 0.644 and RI 0.920. Two clades were recovered in one of the equally parsimonious trees (Figure 6). One clade consisted of a monophyletic *C. mirabilis* and the other is further subdivided into two subclades comprising a monophyletic *C. nobilis*, sister to a clade consisting of the other four species. This split reflects the two lineages of *Clivia*: one lineage representing the winter rainfall/ Northern Cape lineage and the other the predominantly summer rainfall/east coast lineage.

Haplotype network reconstructed for the combined analysis is shown in Figure 7. Haplotypes of *C. mirabilis* occur in close proximity to each other. Sharing of haplotypes occur between *C. miniata and C. caulescens*; *C. gardenii* and *C. robusta*; and *C. miniata* and *C. robusta*.

Molecular Diversity Analyses

The SAMOVA analysis was run on the combined (*trnL-F* and *rpoB-trnC*) datasets to investigate the variation within and between the species. Although Table 2 only shows the structure for eight different groups, 24 groups were analysed in total. SAMOVA did not produce any significant results as the FCT results increased constantly without any noteworthy increase at any particular groupings. This may suggest that no isolation of the lineages has occurred with respect to the sequences used here and that variation in these data between the species is as great as the variation within species. None of the groups proposed by SAMOVA reflect the haplotype networks obtained.

Due to the small sample sizes from many localities and the lack of obvious criteria for the definition of groups of populations, individuals were assigned to groups on the basis of their taxonomic classification, i.e. groups were assigned according to species. For the six groups reflecting the six species, AMOVA revealed variation among populations at 33.92%, half of that of the within population variation (66.08%).

The groups revealed a low degree of genetic subdivision with only 23% genetic variation between the groups (species) for the combined analysis (F_{ST} 0.23424 $p < 0.0001$; Φ_{ST} 0.49653 $p < 0.0001$ respectively). Haplotype and nucleotide diversity are highest for *C. miniata* but this could be caused by sampling bias (Table 3). Nucleotide composition (Table 4) is similar between the different groups for both datasets.

Effective population size, FST values and migration rates obtained are summarized in Table 5 and 6. Several combinations of short and long chains were carried out with

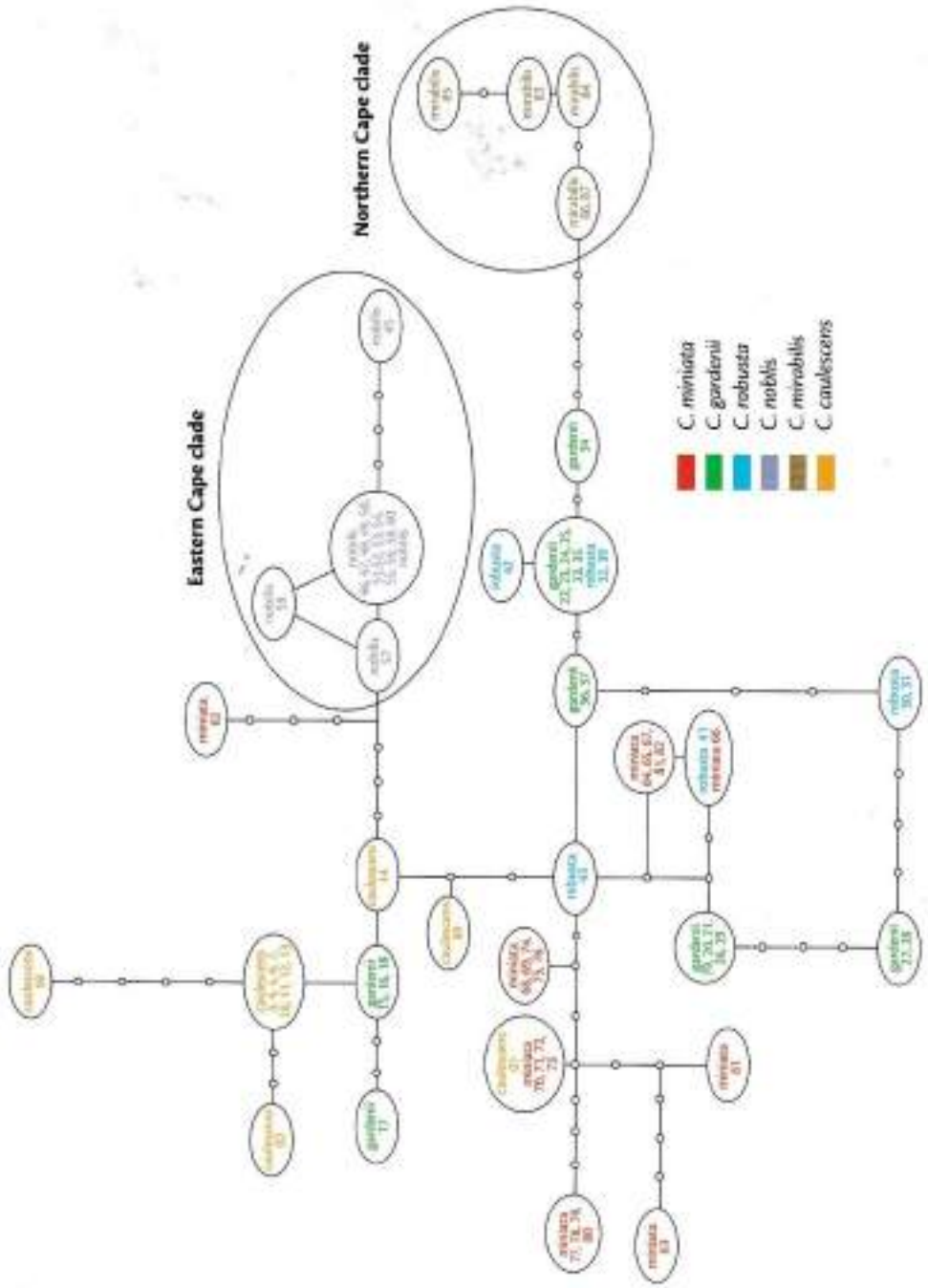

Figure 7. Haplotype network from TCS using combined plastid data sets. Circles surround species. Missing intermediates occur on the branches linking haplotypes. Numbers at end of species names indicate haplotype network reference numbers.

Table 2. Results from a spatial analysis of molecular variance (SAMOVA) showing F values given different numbers of groupings for the combined datasets. Sets of lineages that were combined within the groups are indicated.

2 Groups	
1. caulescens1, caulescens2, caulescens3, caulescens4, caulescens5, gardenii1	F_{SC} = 0.660
2. gardenii2, gardenii3, gardenii4, gardenii5, gardenii6, gardenii7, gardenii8, gardenii9, gardenii10, gardenii11, gardenii12, gardenii13, gardenii14, nobilis1, nobilis2, nobilis3, nobilis4, nobilis5, nobilis6, miniata1, miniata2, miniata3, miniata4, miniata5, miniata6, miniata7, mirabilis1	F_{ST} = 0.886 F_{CT} = 0.664
3 Groups	
1. gardenii2, gardenii3, gardenii4, gardenii5, gardenii6, gardenii7, gardenii8, gardenii9, gardenii10, gardenii11, gardenii12, gardenii13, gardenii14, , miniata1, miniata2, miniata3, miniata4, miniata5, miniata6, miniaca7, mirabilis1	F_{SC} = 0.506
2. nobilis1, nobilis2, nobilis3, nobilis4, nobilis5, nobilis6	F_{ST} = 0.858
3. caulescens1, caulescens2, caulescens3, caulescens4, caulescens5, gardenii1	F_{CT} = 0.714
4 Groups	
1. gardenii2, gardenii3, gardenii4, gardenii5, gardenii6, gardenii7, gardenii8, gardenii9, gardenii10, gardenii11, gardenii12, gardenii13, gardenii14, miniata1, miniata2, miniata3, miniata4, miniata5, miniata7, mirabilis1	F_{SC} = 0.424
2. caulescens1, caulescens2, caulescens3, caulescens4, caulescens5, gardenii1	F_{ST} = 0.853
3. miniata6	F_{CT} = 0.745
4. nobilis1, nobilis2, nobilis3, nobilis4, nobilis5, nobilis6	
5 Groups	
1. mirabilis1	
2. caulescens1, caulescens2, caulescens3, caulescens4, caulescens5, gardenii1	F_{SC} = 0.361
3. miniata6	F_{ST} = 0.845
4. nobilis1, nobilis2, nobilis3, nobilis4, nobilis5, nobilis6	F_{CT} = 0.757
5. gardenii2, gardenii3, gardenii4, gardenii5, gardenii6, gardenii7, gardenii8, gardenii9, gardenii10, gardenii11, gardenii12, gardenii13, gardenii14, miniata1, miniata2, miniata3, miniata4, miniata5, miniata7	
6 Groups	
1. mirabilis1	
2. caulescens1, caulescens2, caulescens3, caulescens4, caulescens5, gardenii1	
3. gardenii2, gardenii3, gardenii4, , gardenii5, gardenii6, gardenii7, gardenii8, gardenii9, gardenii10, gardenii11gardenii12, gardenii13, gardenii14, miniata1, miniata2, miniata3, miniata5, miniata7	F_{SC} = 0.297
4. nobilis1, nobilis2, nobilis3, nobilis4, nobilis5, nobilis6	F_{ST} = 0.839
5. miniata6	F_{CT} = 0.770
6. miniata4	
7 Groups	
1. caulescens1, caulescens2, caulescens3, caulescens4, caulescens5, gardenii1	
2. miniata4	
3. nobilis1, nobilis2, nobilis3, nobilis4, nobilis5, nobilis6	F_{SC} = 0.218
4. mirabilis1	F_{ST} = 0.828
5. gardenii2, gardenii3, gardenii6, gardenii7, gardenii8, gardenii9, gardenii10, gardenii11gardenii12, gardenii13, miniata1, miniata2, miniaca3, miniata5, miniata7	F_{CT} = 0.780
6. miniata6	
7. gardenii4, gardenii5, gardenii14	

Continued on page 19

Continued from page 18

Table 2. Results from a spatial analysis of molecular variance (SAMOVA) showing F values given different numbers of groupings for the combined datasets. Sets of lineages that were combined within the groups are indicated.

8 Groups	
1. gardenii4, gardenii5, gardenii14	
2. mirabilis1	
3. gardenii7, gardenii13	
4. gardenii2, gardenii3, gardenii6, gardenii8, gardenii9, gardenii10, gardenii11, gardenii12, miniata1, miniata2, miniata3, miniata5, miniata7	F_{SC} = 0.180 F_{ST} = 0.826 F_{CT} = 0.788
5. nobilis1, nobilis2, nobilis3, nobilis4, nobilis5, nobilis6	
6. miniata4	
7. miniata6	
8. caulescens1, caulescens2, caulescens3, caulescens4, caulescens5, gardenii1	

Table 3. Molecular diversity indices for the 6 groups (species) for the combined dataset. Significance values (p) are given in brackets.

Group	No. of individuals	No. of haplotypes	Molecular diversity indices	
			Haplotype diversity (h)	Nucleotide diversity (π)
Caulescens	14	6	0.6813	0.002078
			(0.1316)	(0.001247)
Gardenii	21	9	0.8571	0.002874
			(0.0466)	(0.001682)
Robusta	9	8	0.7222	0.000933
			(0.1592)	(0.000886)
Nobilis	16	4	0.3500	0.000388
			(0.1478)	(0.000388)
Miniata	22	9	0.8788	0.003343
			(0.0364)	(0.001843)
Mirabilis	5	4	0.9000	0.001449
			(0.1610)	(0.001082)

Table 4. Nucleotide composition for combined datasets from AMOVA.

Combined datasets		
Caulescens	**Gardenii**	**Robusta**
C:15.20%	C:15.27%	C:15.41%
T:31.88%	T:31.92%	T:31.67%
A:36.50%	A:36.58%	A:36.66%
G:16.42%	G:16.24%	G:16.26%
Nobilis	**Miniata**	**Mirabilis**
C:15.23%	C:15.22%	C:15.27%
T:31.86%	T:31.89%	T:31.88%
A:36.71%	A:36.67%	A:36.69%
G:16.20%	G:16.21%	G:16.16%

Table 5. Effective population size (expressed as $\theta = {}_{f}N\mu$) and FST values.

	θ	FST
caulescens (1)	0.023259	0.00688
gardenii (2)	0.016958	0.00081
robusta (3)	0.007471	0.00326
nobilis (4)	0.001986	0.00091
miniata (5)	0.005124	0.00187
mirabilis (6)	0.006533	0.00156

17 short and 3 long producing the optimal results with no error values reported. The estimates obtained for effective population size indicate *C. caulescens* to be the biggest and *C. nobilis* the smallest. Migration between the species was bidirectional with very high past gene flow rates observed between *C. robusta* and *C. gardenii*, within *C. mirabilis* and within *C. nobilis*.

Discussion

The phylogeny reconstructed from the combined dataset has short internal branches and weak bootstrap support, while most of the polytomies show sharing of haplotypes (Figure 6). One likely scenario is that the genus has recent origins, a phenomenon not uncommon in the region. Research focusing on dating of the tribe Haemantheae, using two different methods [Non Parametric Rate Smoothing (NPRS) and Bayesian Evolutionary Analysis Sampling Trees (BEAST)] suggests 17 Ma and 15.6 Ma for the genus, respectively. Richardson *et al.* (2001) dated the genus *Phylica* using island species and other genera from the same tribe and reported a radiation date of 7-8 Ma and Klak *et al.* (2004), in their study of the family Aizoaceae, reported a radiation date of 3-4 Ma.

Phylogeography relies on interpreting patterns of congruence, or lack of congruence between geographical distribution of the haplotypes and their genealogical relationships. When clades of closely related haplotypes are geographically restricted or occur in close proximity, congruence exists (Schaal *et al.*, 1998). Using parsimony, results from the phylogenetic analysis for the combined datasets (Figure 6), showed two clades. One clade consisted of *C. mirabilis*, one of only two monophyletic species in the genus, the other clade divided into two subclades. One of the subclades comprised a monophyletic *C. nobilis*, sister to a clade consisting of a combination of the other four species. A haplotype network reconstruction showed the same pattern. The incongruence between the phylogeographic patterns, the currently accepted taxonomy and geography suggest that there may be ancestral polymorphisms present or incomplete lineage sorting in the genus.

An alternative explanation for the sharing of haplotypes between species is hy-

Table 6. Migration rates estimated via ML with *MCMC* searches (using *MIGRATE*).

Direction of migration	Migration rate
M21	247.716904
M31	247.716904
M41	247.716904
M51	1057.06
M61	247.716904
M12	4445.21
M32	21508.1
M42	1822.47
M52	1822.47
M62	1822.47
M13	266.551971
M23	1298.19
M43	266.551971
M53	266.551971
M63	266.551971
M14	1234.05
M24	1234.05
M34	1234.05
M54	1234.05
M64	2289.35
M15	302.433968
M25	741.581994
M35	302.433968
M45	302.433968
M65	302.433968
M16	589.847565
M26	589.847565
M36	589.847565
M46	589.847565
M56	1091.98

bridization. References to artificial hybrids are made in the literature by Rourke (2003) and Koopowitz (2002). Natural interspecific hybridization in the genus has rarely been recorded. In 2006, Swanevelder *et al.* formally described a natural *Clivia* hybrid *Clivia* x *nimbicola*, an intermediate between *C. caulescens* and *C. miniata*, growing sympatrically with *C. caulescens* and *C. miniata* and confined to the Barberton area of endemism on the border of South Africa and Swaziland.

In the northern part of the Eastern Cape *C. miniata* and *C. robusta* grow sympatrically and share haplotypes in the haplotype network reconstructed from the combined datasets. However, they do not share the same flowering times; *C. miniata* flowers in June and *C. robusta* in September which makes hybridization unlikely although it cannot be ruled out completely since *C. miniata* has been known to flower sporadically throughout the year. In Mpumalanga *C. miniata* and *C. caulescens* grow sympatrically and they are also observed to share haplotypes but these two species have different pollinators; swallowtail butterflies for *C. miniata* and sunbirds for *C. caulescens*, again making hybridization unlikely, but not impossible. Although the presence of ancestral polymorphisms and incomplete lineage sorting are possible options to explain haplotype sharing for these sympatrically occurring individuals, it is difficult to discern which of the possibilities are likely for *C. miniata*. This aside, haplotype sharing is clearly evident in these sympatrically occurring species.

Haplotype sharing is also observed between *C. gardenii* and *C. robusta* in the Eastern Cape where they occur sympatrically. The interconnectedness between these two species brings into question the recognition of these two elements as discrete species since *C. robusta* was considered a 'robust' form of *C. gardenii* until it was formally described in 2004 by Murray *et al.*

Clivia mirabilis (Northern Cape) and *C. nobilis* (Eastern Cape) show the most discrete haplotypes, probably as a result of highly restricted gene flow. *Clivia mirabilis* occurs in the western most part of the distribution range and *C. nobilis* in the southern most part of the range.

Dupanloup *et al.* (2002) state that the SAMOVA model allows one to define the strongest structure of populations in genetic terms but that the identification of the correct number of groups depends critically on the degree of differentiation between groups. SAMOVA reveals no significant groupings of the populations, suggesting a lack of genetic structure. AMOVA was

Photo: Heidi Hendriks

Figure 8. *Clivia nobilis*, Bushmans River, Eastern Cape.

therefore structured to reflect the six species of *Clivia* as groups. The results showed low genetic variation among groups and high variation within groups and a low degree of genetic subdivision, implying once again a lack of genetic structure and the likelihood that no isolation of the lineages has occurred in these data. All analyses, including the statistical analyses, therefore support the likelihood of incomplete lineage sorting present in *Clivia*.

With the discovery of *Clivia mirabilis* in the Northern Cape in 2002, the question arose whether the genus once occupied a wider range spanning the Eastern and Western Cape. Evidence from the coalescent model, MIGRATE, supports the hypothesis that this is the case, with past flow gene rates recorded between *C. mirabilis* and *C. nobilis*, the most southerly species of *Clivia* and the closest geographically to *C. mirabilis*. Subsequent fragmentation of this distribution range may have been precipitated by the increase in aridity experienced in the Northern and Western Cape during the late Miocene (15-8 Ma), with subtropical elements giving way to fynbos elements. This may have caused the range of *Clivia* to retreat and may account for only one lineage, now represented by *C. mirabilis*, occupying a semi-arid habitat in the Northern Cape.

Although long distance dispersal should be considered, it is highly unlikely for two reasons. Firstly, *Clivia* have heavy fleshy berries that make them unsuitable for wind

Figure 9. *Clivia miniata*.

dispersal, and secondly, while dispersal of seeds by frugivorous birds between adjacent forest patches is likely, dispersal over 800 km of arid country does not appear very probable (Rourke 2002). Moreover, no frugivorous birds are known to migrate between the Eastern Cape and Northern Cape (Snijman, 2002).

An alternate scenario is that *Clivia mirabilis* is a relictual population that successfully colonised a previously unoccupied habitat in the Northern Cape. Tolley *et al.* (2006), in their study of the biogeography of dwarf chameleons, suggested that climatic fluctuations have created islands of differing vegetation types, some of which may have persisted as isolated patches for long periods of time. The considerable climatic fluctuation throughout the Pliocene and into the Quaternary caused vegetation changes in the region which are thought to be more complex than a simple reduction of forest and establishment of fynbos and other mesic and arid vegetation types (Midgley *et al.*, 2001; Barrable *et al.*, 2002).

In 2003, Snijman proposed that recurring intense fires in the fynbos have served to isolate clivias in the Northern Cape from those along the east coast of South Africa. In this scenario it seems that *C. mirabilis* has persisted at Oorlogskloof for hundreds of generations untouched by fires that probably destroyed its ancestors which once occupied the southern Cape during more favourable times (Snijman, 2003).

Conclusion

The debate on the definition of a species and how to determine species boundaries or species delimitations has been waging for decades and is one that will probably never be resolved. Preliminary DNA sequencing results for the phylogeography of *Clivia* suggests that only two species of the six in the genus *Clivia* are 'true species' in this case referring to monophyletic species, namely *C. mirabilis* and *C. nobilis*. The monophyly of *C. caulescens*, *C. gardenii*, *C. robusta* and *C. miniata* was not established using two plastid regions. However, the possibility that the haplotypes which *C. miniata* shares with other taxa (*C. gardenii* and *C. caulescens*) is due to hybridisation where their populations overlap, cannot be eliminated.

Two scenarios have been proposed to understand how *C. mirabilis*, in the Northern Cape, came to be isolated from the other five *Clivia* species which occur along the east coast of southern Africa, the nearest being *C. nobilis* some 800 km away. No records of wild *Clivia* exist in the southern Cape, despite more than a century of botanical exploration. The first scenario was long distance dispersal through seed but this was ruled out by Rourke (2002). Instead he speculated that *C. mirabilis* is relictual, a survivor of the past climatic history when subtropical vegetation covered much of the interior of South Africa. Dating of the tribe revealed the estimated divergence of *C. mirabilis* from the summer rainfall *Clivia* species to be about 16 Ma (17 Ma for NPRS and 15.6 Ma BEAST). This coincides with the Miocene and the increase in aridification that eliminated subtropical vegetation leaving survivors to adapt to the emergence of an increasingly dry climate. A second scenario, proposed by Snijman (2003), is that the impact of fire on the Cape forests since the development of the Mediterranean-type climate in the south-western Cape, and the inability of *Clivia* to cope with fire, have been major factors that led to its current disjunct distribution pattern.

Paper adapted from a chapter of the Ph.D. thesis by F. Conrad.

Photo: Carrie Knight

Figure 10. *Clivia robusta*.

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Glossary

Bootstrap – a statistical way to evaluate the strength of support for nodes on phylogenies. A number is presented by each node, which reflects the percentage of bootstrap trees which also resolve that clade

Clade – a group of organisms which includes the most recent common ancestor of all its members and all the descendants of that most recent common ancestor.

Effective population size – the number of breeding individuals in an ideal population.

Frugivorous – fruit eating.

Haplotype – combination of alleles at multiple loci that are transmitted together on the same chromosome.

Intergenic – a section of DNA sequences located between clusters of genes; portions of a genome that are not considered to lie within defined genes.

Mesic – adapted to moderately moist habitat or habitat which has a moderate amount of moisture.

Monophyly – clade, consisting of an ancestor and all its descendants.

Parapatry/ Parapatric – species whose ranges do not significantly overlap but are immediately adjacent to each other; they only occur together in the narrow contact zone.

Phylogenetics – the study of evolutionary relatedness among various groups of organisms (which is discovered through molecular sequencing data).

Phylogeography – the study of the historical processes that may be responsible for the contemporary geographic distributions of individuals.

Polytomies – a node which has more than two branches

Relictual – a remnant of a formerly widely distributed group in an environment different from that in which it originated.

Sympatry/ Sympatric – species occurring together; areas of distribution that coincide or overlap.

Vicariance – the separation of a large group of organisms from the population due to a geographic barrier.

Barcoding *Clivia* for species identification

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It is assumed that *Clivia* species can be easily and correctly identified morphologically. But what happens if we need to identify a seedling, or a plant that has not yet flowered, based only on morphological traits? What happens if a seedling of *C. mirabilis* is bought from a seller, but the buyer suspects that it is a *C. nobilis* seedling? Are there any alternative methods to identify and classify *Clivia* species correctly?

The answer is yes and no. DNA barcoding is a method where an area of the DNA is amplified (multiplied) with a technique called the polymerase chain reaction (PCR). When enough copies of an area have been obtained, the PCR-products are sequenced and a nucleotide pattern (Figure 1) is generated by an automated sequencer. The piece of DNA strand is represented as an electropherogram and each curve generated, indicates one of the four building blocks of DNA. Different sequences are then compared and the differences can be presented in a form that resembles barcodes (Figure 2 – each colour represents one of the nucleotide bases of DNA [A, T, C or G]). Like a barcode for products in a shop, each species should have a unique nucleotide barcoding pattern and should be identifiable from this unique pattern. But as we know, very few things in life are only black or white, and most things in life come with a challenge. But before we get to the challenges, let's start at the beginning.

The chloroplast is a cellular organelle containing its own set of DNA, which codes for proteins involved in electron transport in photosynthesis. Because a pollen grain mainly contains nuclear DNA, the chloroplast DNA is inherited from the maternal parent and passed on to all the offspring of that maternal plant. If we study the DNA of the chloroplast, we automatically study the maternal lineage of a plant. It is easier working with the chloroplast DNA, because you only have one copy of DNA.

Genes of an organism code for proteins that are important for the survival of the organism. A mutation in a gene can have a lethal effect on the organism, and for that reason, organisms have built-in mechanisms to minimize mutations in genes.

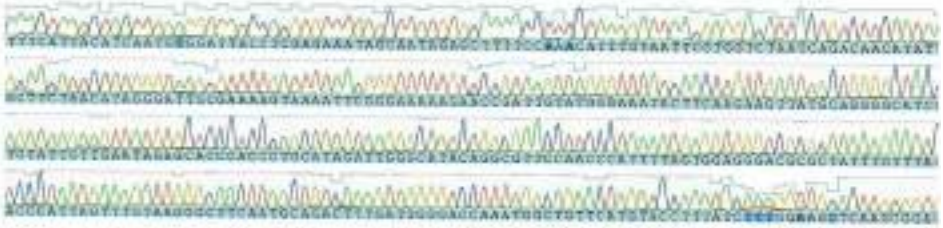


Figure 1. Example of results (called an electropherogram) from an automated sequencer.

When we want to generate a barcode for an organism, we need mutations. We need differences in the DNA so we can distinguish between species. Where do we get DNA that has enough mutations?

"Junk" DNA was considered as pieces of DNA that are removed from the genome during transcription and translation, when DNA is converted to protein. These days the "junk" DNA seems to play a bigger role in the organism than expected. Whether the organism uses this DNA or not, is of little concern to us, the geneticists, working with the DNA. These "junk" DNA apparently does not contribute to the survival of the organism and for that reason has a much higher mutation rate than the transcribed genes. This provides us with the perfect type of DNA we need for barcoding purposes.

Every time a cell divides and the DNA copies itself, there is a possibility that the wrong nucleotide will be incorporated in the new strand. The faster a plant grows, the faster cells divide and the higher the mutation rate will be. Because *C. mirabilis* and *C. nobilis* both have a very slow reproductive and growth cycle, the mutation rate of these two species will be much slower than the rest of the species. We suspect that there should be much less variation within these two species, than within the fast growing species with a higher mutation rate.

What are the different ways for a species to form? There are at least 2 different ways: 1) two species hybridise, the hybrid survives better than the two parents and develops into a new species; 2) a plant mutates. The mutation improves the adaptation of that plant in the environment. That plant and all its descendants survive better and develop into a new species. In whatever way a species develops, the "old" and

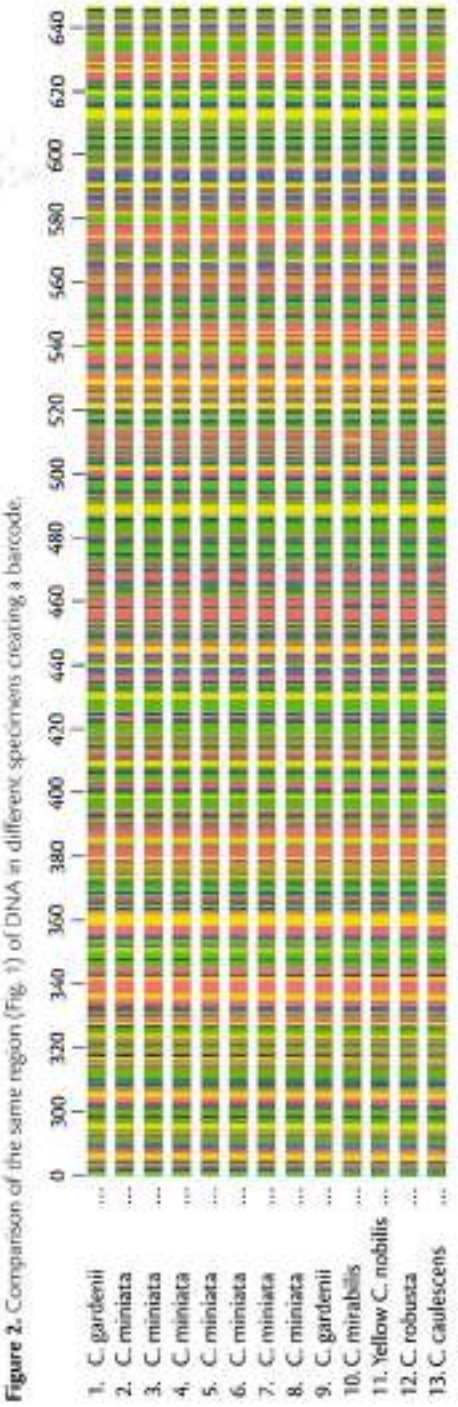


Figure 2. Comparison of the same region (Fig. 1) of DNA in different specimens creating a barcode.

"new" species will share some morphological and DNA characters. The "original" species will not necessarily die, but can co-exist with the "newly developing" species. It is usually only in a severe environmental event such as a drought or virus infection, that the adapted plants will survive and the other plants will die and this can lead to speciation. We have to consider this information when we investigate *Clivia*.

If a new species was formed from a single plant in any of the above mentioned ways, all of the offspring of those plants will contain an exact chloroplast copy of that plant. *Clivia* has a narrow seed dispersal ability since the seeds are too heavy for wind distribution. The seeds of clivias usually get distributed by rodents and by rolling down the slopes. Since the chloroplast, which we are investigating, is in the seeds and not the pollen, we would assume that the distribution area of the maternal DNA is relatively small compared to the distribution area of the nuclear DNA which is found

Figure 3. Approximate geographic distribution of samples used during the preliminary study.

in pollen. But of course, as mentioned, the new species will not have its own unique DNA, and will share most of its DNA with the original species. The earlier the speciation event started, the more time the DNA had to change and the more changes there will be between the two species.

In theory, we should be able to distinguish between populations if the populations are isolated from each other and if the populations adapted to new environments by means of mutation. Pollination from nearby populations, areas or other species should not influence the results, since the DNA in the pollen does not contribute to the DNA studied.

For the study on *Clivia*, the ideal was to find DNA that would distinguish perfectly between the different species. We analyzed five different DNA regions all of which have different mutation rates and which have different numbers of mutation sites. The aim was to find a region with enough mutation sites which could also distinguish between the different species.

The aim of this study was consequently to determine which areas (genes) are suitable for barcoding in *Clivia*. A barcode database will then be set up to which unknown samples can be compared and identified. The Barcode of Life initiative identified two barcoding regions (*matK* and *rcbL*), of which *matK* and four other regions were tested in this study.

Results and discussion

The total number of nucleotides from all five regions combined was 3 641, from which 52 mutation sites varied between the species. Barcoding patterns have been constructed for more or less 50 samples representing all of the species and various geographical distributions in the genus (Figure 3 – Note that the figure does not represent exact geographical positions, but only distribution estimates). *Cryptostephanus vansonii* and several representatives from the family Amaryllidaceae were included as out groups. To simplify the interpretation of the results, only the variable nucleotides were highlighted (Figure 5).

The *Cryptostephanus* barcodes clearly indicate that there is a relationship between *Cryptostephanus* and *Clivia*, but also clearly indicate that *Cryptostephanus* is a separate genus based on the number of unique nucleotides. *Cryptostephanus vansonii* shares 9.6% of the variable nucleotides with mainly *C. nobilis* and/or *C. mirabilis*, indicating that *Cryptostephanus* is closer related to these two species than to the rest of the *Clivia* species. *Cryptostephanus* has 12 unique nucleotide differences, distinguishing it from the rest of the genus *Clivia*.

C. nobilis and *C. mirabilis*

What does the DNA say about identifying *Clivia* species? Barcoding can, with 100% certainty, be used to distinguish between natural growing *C. mirabilis* and *C. nobilis* specimens. *Clivia mirabilis* has 4 nucleotides changes unique to this species whereas *C. nobilis* has 5 unique changes distinguishing it from the rest of the *Clivia* species. These changes can be used to identify both these species by comparing an unknown *Clivia* to the data base. False results can unfortunately be obtained from plants in cultivation. In an event where the pollen of *C. mirabilis* or *C. nobilis* is for instance used to fertilize any other species, a hybrid will be produced. If this hybrid is back-crossed to the pollen donor plant for a few generations, the DNA will indicate that it belong to the same species as the mother plant, even though it might morphologically represent the *C. mirabilis* or *C. nobilis* donor plant.

C. gardenii

The barcoding results show two putative barcoding regions that can identify *C. gardenii* from a 240 km stretch in central KwaZulu Natal (Figure 4). Both of these mutation sites were obtained from the trnL-F region in the chloroplast, which is a region between the coding gene for tRNA leucine and tRNA phenylalanine. More samples have to be included to narrow down this area, but at this stage we will be able to identify *C. gardenii* specimens in the area between Port Shepstone in the South, Entumeni in the North, and as far west as Greytown (Figure 4). An interesting observation is that the *C. gardenii* specimens in these regions share the same nucleotide with the genera *Scadoxus* (2n = 18) and *Brunsvigia* (2n = 22), both of the family Amaryllidaceae. This either indicates that *Scadoxus* and *Brunsvigia* share a unique common ancestor with *C. gardenii* or that there were independent evolutionary events resulting in the same nucleotide change. The probability of the latter occurring is however so small that it cannot even be considered. The ancient relationship that *C. gardenii* has with some of the other genera in the family, cannot be determined

Figure 4. All the samples investigated in this area share two unique nucleotide changes.

Figure 5. Representation of a part of the *matK* region. Grey areas represent similar DNA nucleotides. Coloured "dots" indicate a nucleotide different from the majority of samples.

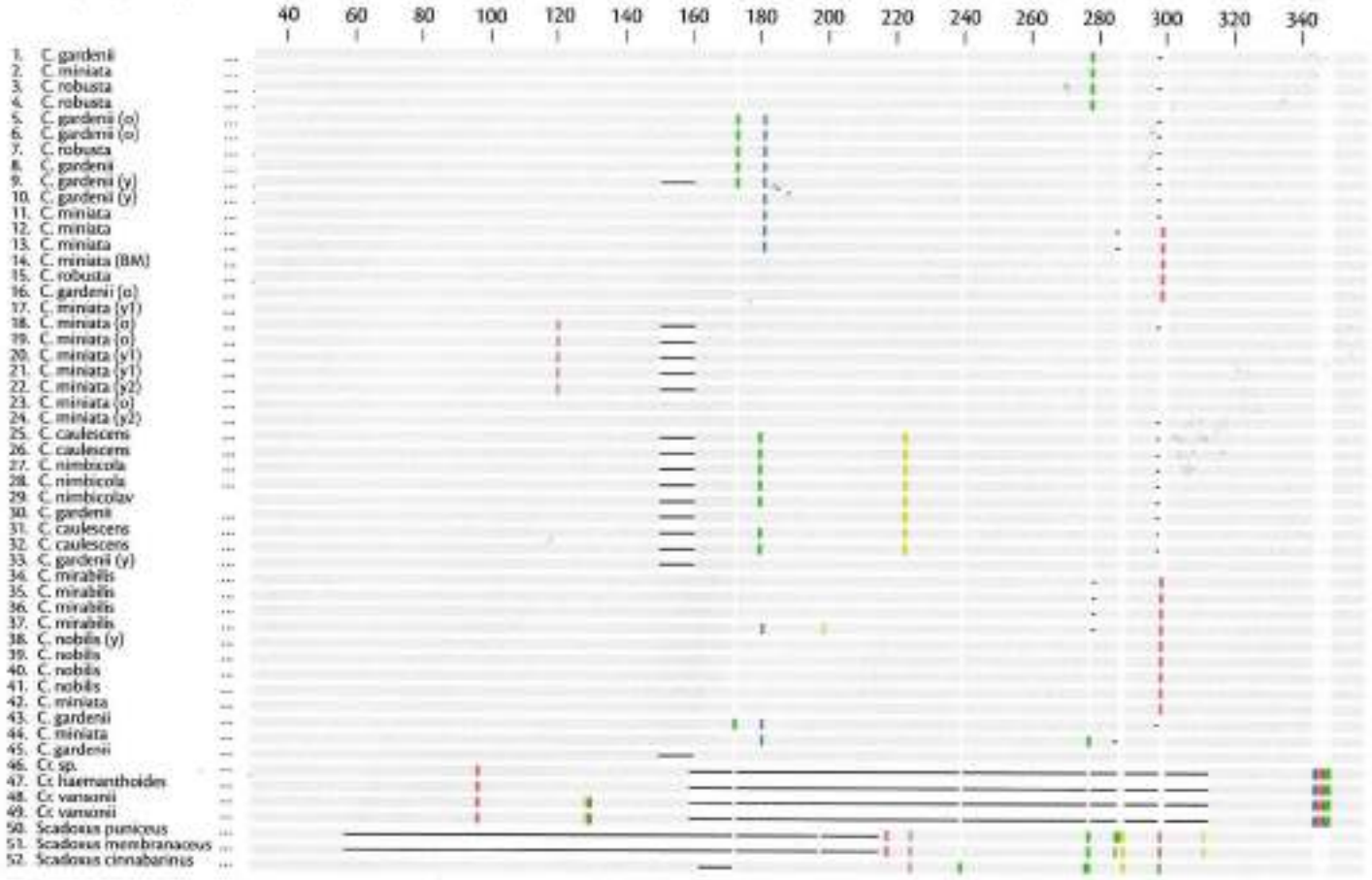
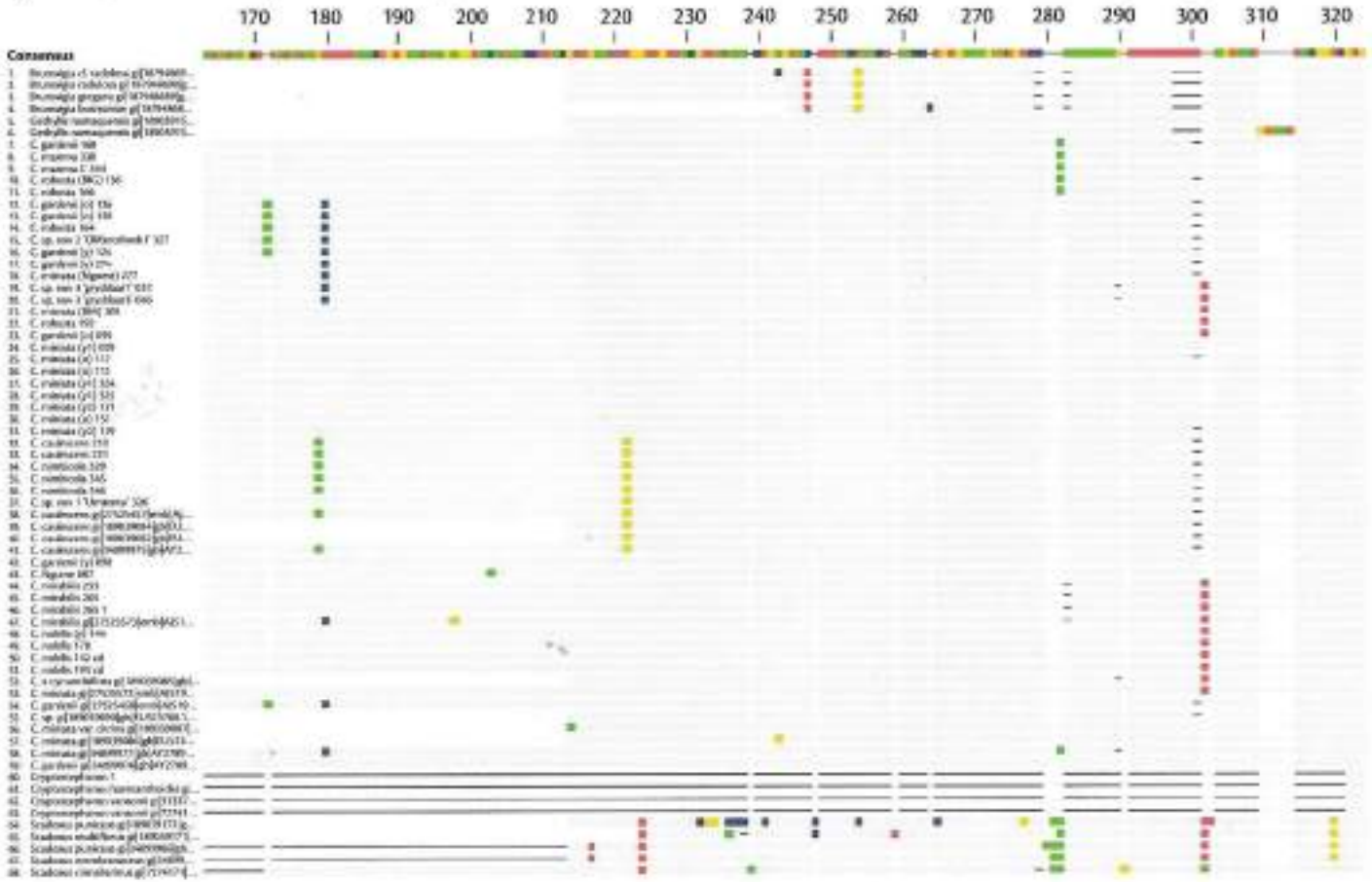


Figure 6. Representation of a part of the *trn*L-F region with barcodes for *C. caulescens* and *C.* × *nimbicola* in the Swaziland/ Mpumalanga area.



from this data. *Clivia*, *Cryptostephanus* and *Scadoxus* are the only genera in the family that have rhizomes and not bulbs, so it is interesting that *C. gardenii* shares a mutation with one of the bulbous genera.

C. caulescens

It was suggested that the natural hybrid, *C.* × *nimbicola*, originated from a *C. miniata* mother, since the flowers of this species is believed to be more susceptible to receiving pollen. In our samples for this pilot study, *C. caulescens* is the pollen receiver (mother plant) in all three cases. We studied a *C. caulescens* specimen which contained a *C. miniata* chloroplast genome. It means that a reciprocal cross with *C. miniata* as the pollen donor is possible and has happened. After a few back-crosses to *C. miniata* plants, the plant has developed a *C. miniata* morphology, but resulted from natural hybridisation. We are still in the process of investigating more chloroplast regions and more samples from the area between Mariepskop to Bearded Man (including samples from Swaziland).

There are two putative regions that could be used as barcodes to identify *C. caulescens* (Figure 6), but this part of the study is still in progress. The number of samples edited is insufficient to determine if these barcodes are linked to specific species/mother plant (i.e. *C. caulescens*), or whether they are linked to specific regions (Swaziland and Mpumalanga).

C. miniata, *C. robusta* and southern *C. gardenii*

We have searched intensively for markers to identify *C. miniata*, *C. robusta* and any *C. gardenii* specimens south of Port Shepstone.

Searching through 5 chloroplast regions, we found seven putative mutation sites that can be used as a barcode to identify *C. miniata* specimens, but found only one of these nucleotide changes (*rpoB* 56 – that is position 56 on the *rpoB* region) present in almost all (23 samples) of the *C. miniata* specimens. A group of *C. miniata* specimens from the Mzamba River and adjacent areas are excluded and do not have this barcode. Only one *C. miniata* from the Mzamba area shares this mutation with the rest of the *C. miniata* specimens. Plants from the Mzamba area are characterised as being morphologically different from the type *C. miniata*. Many of the specimens from the Mzamba river area have *C. miniata* flowers, but similar stem formation to *C. caulescens* and harder leaves than normal *C. miniata*. It seems that "marker" *rpoB* 56 can be used as a guide to identify "normal" *C. miniata* or any other plant that had a *C. miniata* ancestor (Table 1).

All of the other *C. miniata* specimens from the Mzamba and adjacent Umtamvuna area have a unique nucleotide difference (*rpoB* 303), that distinguishes them from the rest of the *C. miniata* specimens and all the other species (Table 2). Also sharing this mutation are three *C. robusta* and one *C. gardenii* specimens, indicating a shared ancestor. Answers we hope to find are whether plants from this area belong to a separate group/species/subspecies/variety. We are investigating more samples and more genes from this region to answer this question.

Evolution and hybridisation

Any species that has the morphology of one species but the chloroplast DNA of another can be considered a hybrid – either recent or ancient. Three plants in our preliminary study could be identified as having a *C. miniata* ancestor but with back-crosses to other species have "non-miniata" mor-

phology. These are a *C. caulescens*, *C. gardenii* and a *C. gardenii x C. miniata* hybrid, all being geographically in the same area as *C. miniata* species.

We found that some plants, for example a *C. gardenii*, share a nucleotide mutation with other *C. gardenii*, but ALSO with *C. miniata* (See Tables 1 & 3). You would think that the plant being investigated is a hybrid between the two species, therefore having DNA from both species. This is impossible through hybridisation, because the chloroplast DNA is only inherited from the mother to the offspring, the hybrids will have only one set of chloroplast DNA – that of the mother. The only explanation is that *C. miniata* and *C. gardenii* shared a common ancestor (i.e. with nucleotide sequence CAAAGC) (Fig. 7). The ancestor evolved through mutation into two predecessor species. The chloroplast DNA of these two predecessors would have differed slightly from each other, for example the last nucleotide mutated from a C to an A to form a *C. gardenii* predecessor (CAAAGA), and the second last nucleotide from a G to C in the *C. miniata* predecessor (CAAACC). However, the predecessors did not die and not all of them mutated further. Another mutation event resulted in plants belonging to the same species, but having slightly different DNA within a species: *C. gardenii* (TAAAGA and CAAAGA) and *C. miniata* (CATACC and CAAACC). If, by chance, we sequence a plant that is a descendant of the predecessor, it might look like one of the modern species, but still contain the "old" DNA. As seen with the arrows in Figure 7, a *C. gardenii* predecessor will share the first nucleotide with *C. miniata*, and the third nucleotide with the rest of the *C. gardenii*. In this manner it is possible for a plant to share nucleotide mutations between two modern species. If we assume that this

Table 1. *C. miniata* specimens sharing a unique nucleotide (rpoB 56), but not the nucleotide found in *C. miniata* species from the Mzamba area (rpoB 303). The two plants in green must have a *C. miniata* ancestor. The four plants in red are all morphologically *C. miniata* but share in other chloroplast regions a nucleotide with other species (indicated in brackets).

Species	No of samples	rpoB 56	rpoB 303
C. miniata group yellow 1	2	✓	✗
C. miniata orange	8	✓	✗
C. miniata group yellow 2	2	✓	✗
C. miniata Swaziland	1	✓	✗
C. miniata Bearded man	6	✓	✗
C. miniata (*C. gardenii*)	1	✓	✗
C. miniata Bearded man (*C. maxima*, *C. caulescens*)	1	✓	✗
C. miniata (*C. maxima*)	1	✓	✗
C. miniata Mzamba (*C. caulescens*)	1	✓	✗
C. gardenii x *C. miniata* natural hybrid	1	✓	✗
C. gardenii	1	✓	✗
C. caulescens	1	✓	✗

Table 2. Samples from the Mzamba area which have a unique nucleotide in position rpoB 303, but do not share the nucleotide with the rest of the *C. miniata* or any of the other species.

Species	No of samples	rpoB 56	rpoB 303
C. robusta	3	✗	✓
C. gardenii	1	✗	✓
C. maxima	6	✗	✓
C. miniata Mzamba	13	✗	✓

Table 3. Two *C. miniata* specimens with neither the *C. miniata* nor the "Mzamba" barcode, but sharing other nucleotides with species in brackets.

Species	No of samples	rpoB 56	rpoB 303
C. miniata (*C. gardenii*)	1	✗	✗
C. miniata Mzamba (*C. gardenii*, *C. caulescens*, *C. x rambicola*)	1	✗	✗

theory is true, then we can also assume that the species or plants with shared mutation between two species are more ancient or contain the more ancient chloroplast.

Without going into too much detail, we can conclude a few things about the evolution in the genus. The DNA indicates that there might have been a split from a common ancestor and two groups formed: Group 1: *C. nobilis*, *C. mirabilis* and *C. caulescens* and the second group being *C. miniata*, *C. gardenii* and *C. robusta* (Figure 8). The fact that *C. caulescens* was previously included with *C. nobilis* and only identified as a new species in April 1943, supports the fact that these species are morphologically similar. *Clivia caulescens* split from the rest, probably due to geographical changes, and

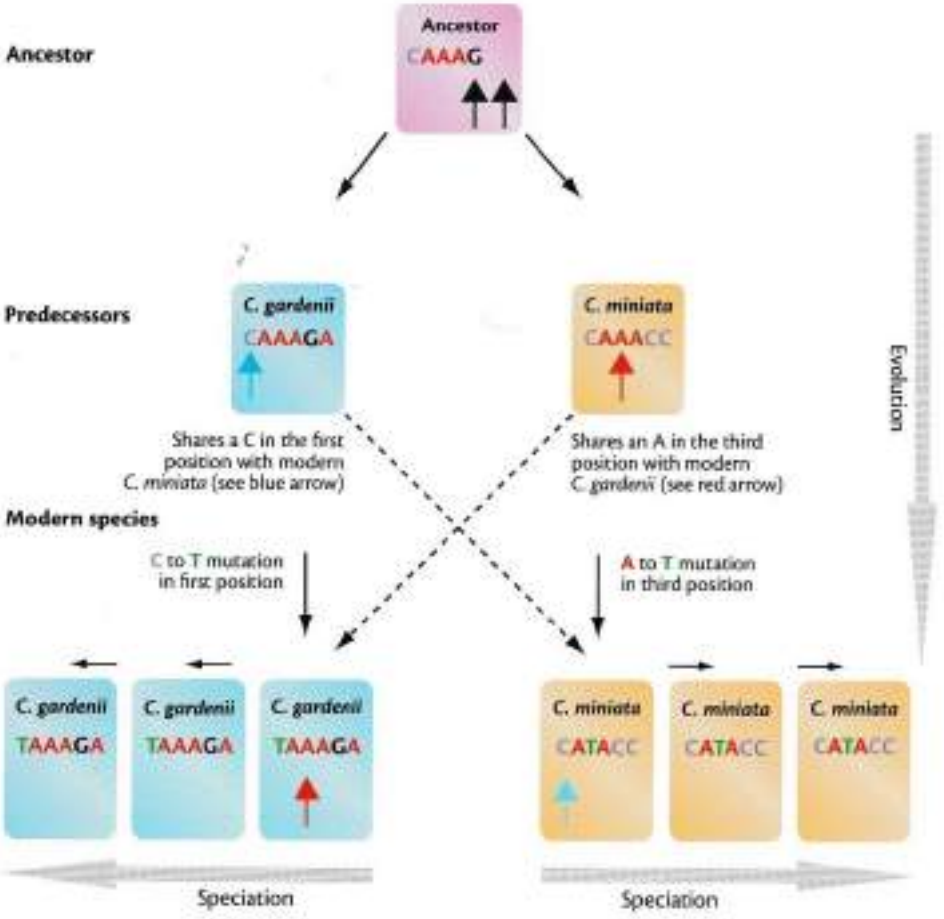


Figure 7. A schematic representation illustrating how two of the *Clivia* species might have evolved from a common ancestor and some specimens can share mutations with other species. *C. gardenii* can share the same nucleotide with some of *C. miniata* specimens, but not with all of them. This is explained by the possibility of predecessor *C. miniata* specimens which did not evolve with the rest of the *C. miniata* specimens.

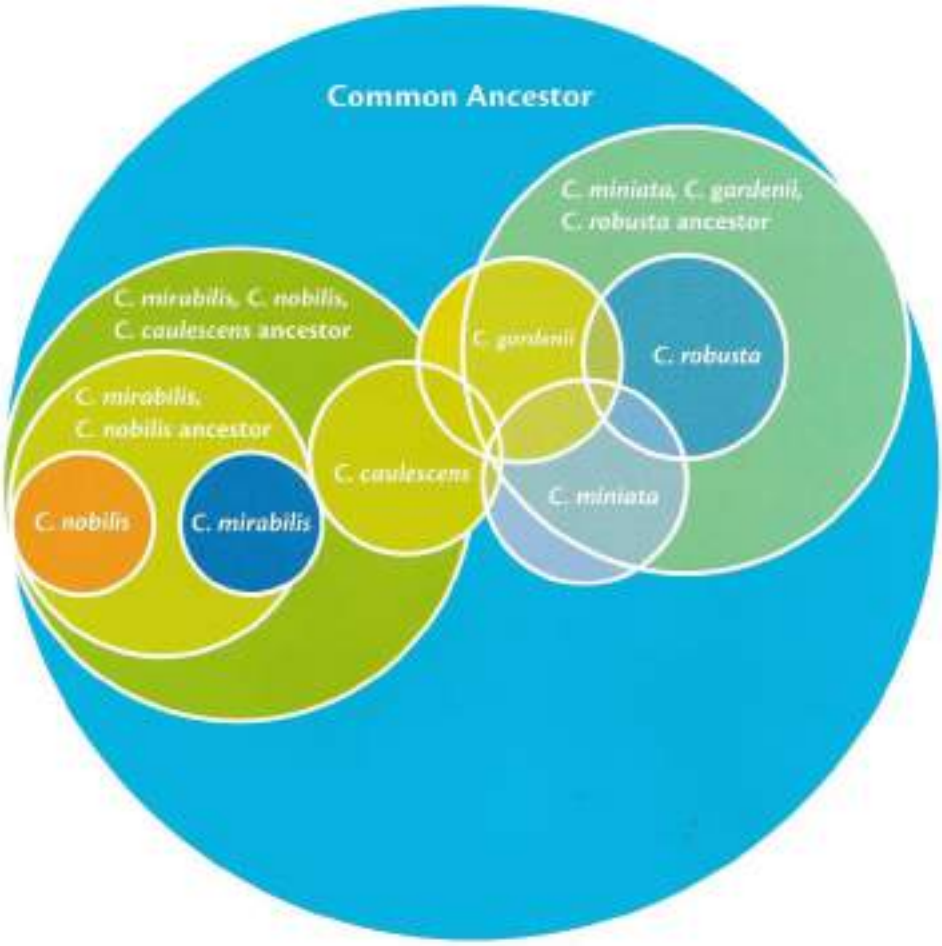


Figure 8. *Clivia* evolved from a common ancestor and split into two groups. Group 1 consisted of a *C. mirabilis*, *C. nobilis* and *C. caulescens* ancestor. Group 2 consisted of a *C. miniata*, *C. gardenii* and *C. robusta* ancestor. Group 1 subdivided into a *C. mirabilis* and *C. nobilis* ancestral group, from where these two species speciated into the modern species we know today. There seems to be ancestral and modern gene flow between the remaining species, explaining the overlapping morphological and DNA characteristics.

C. nobilis and *C. mirabilis* split from each other, also probably due to climatic changes. The second group is much more complicated, since there is very little geographical and reproductive isolation between the three species (*C. miniata*, *C. gardenii* and *C. robusta*) in the group. There is a degree of speciation, where we can clearly see morphological differences in the three species, but due to overlapping distribution areas, it is inevitable that there will be natural hybridisation. Since *C. caulescens* also overlaps geographically with other species, it contains DNA from other species as well.

To summarize, we have identified unique barcodes for *C. mirabilis* and *C. nobilis*.

Clivia caulescens and *C. gardenii* north of Port Shepstone can be identified with almost a 100% certainty. *Clivia miniata* also has one nucleotide mutation that can be used as a reference to identify a *C. miniata* or a plant which had a *C. miniata* mother as ancestor. Because of the gene flow through ancient hybridisation and evolutionary events in the rest of the species, 100% identification based on DNA barcoding is impossible. It is possible to identify plants from certain areas, for example if a plant originates from the Mzamba-Umtamvuna area or in the area between Port Shepstone and Entumeni. Ancient hybridisation can also be detected in some *Clivia* samples.

Some clivias can easily be identified based on morphology and DNA testing, but other plants test our patience and knowledge. The genus *Clivia* remains an interesting and challenging genus. This study has proved to us that while we try and fit all species in their own containers, nature is alive, has its own way and is constantly developing and changing. We humans have a huge need to identify, describe and name species. Maybe one day we will be able to do it to our own satisfaction, but in the meantime, we have this gift to humanity – called the *Clivia* – that is there for us to enjoy and study.

Photo: Rina van der Merwe

Figure 9. *Cliva miniata* with a strong green throat.

Identifying *Clivia nobilis* and *C. mirabilis* in the laboratory

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Clivia nobilis and *C. mirabilis* share a number of morphological traits that are unique to these two species. Both have a rough, tooth-like texture on the edge of the leaves and both may or may not have a median line on the leaves. Some of the leaves of both species have a notched (indented) tip that is mostly found in these two species. They are geographically separated in nature – *C. nobilis* is found in the Eastern Cape and *C. mirabilis* grows on the border between the Northern Cape and the Western Cape. Many enthusiasts, however, grow both species in almost every province of South Africa. Vegetative material (leaves) of these species is often difficult to identify, especially for such laymen as plant protection inspectors.

Photo: M. Coetzee

Figure 1. *C. mirabilis* in Oorlogskloof Nature Reserve, Northern Cape.

Clivia nobilis has a larger distribution range than *C. mirabilis* and many more *C. nobilis* plants are found in nature. Although both species are described as vulnerable in the Red Data List of southern Africa, the conservation strategy for *C. mirabilis* is currently enforced more strictly. To date there has been no effective way to distinguish between some specimens of the two species. Unscrupulous people sell (export) *C. mirabilis* plants as *C. nobilis*.

From a conservation perspective it is essential to find a way to safeguard against this practice. A study was consequently done to find a way to correctly identify these species from the DNA of a very small leaf sample. A technique called DNA barcoding was employed to correctly identify these species (Van der Westhuizen, 2011).

Over the last three years an in-depth study has focused on *C. nobilis* and *C. mirabilis*. In the process unique barcodes were developed for these two species.

A barcode is obtained when the DNA (nucleotide – AGCT) sequence is determined. If the sequence is the same for all the plants in a specific species and the sequence differs from the one obtained for the other species, it is considered a barcode.

During this study, seven different regions of DNA were tested. The DNA regions investigated include the *atpH-I*, *rpoB*, *rpoC1*, *matK*, *rpl16* and *tmL-F* regions in the chloroplast, and the *ITS1* region in the nucleus. The other five taxa of *Clivia* (*C. caulescens*, *C. miniata*, *C. gardenii*, *C. robusta* and *C.* × *nimbicola*) were used as so-called out groups to ensure that the barcodes obtained for *C. nobilis* and *C. mirabi-*

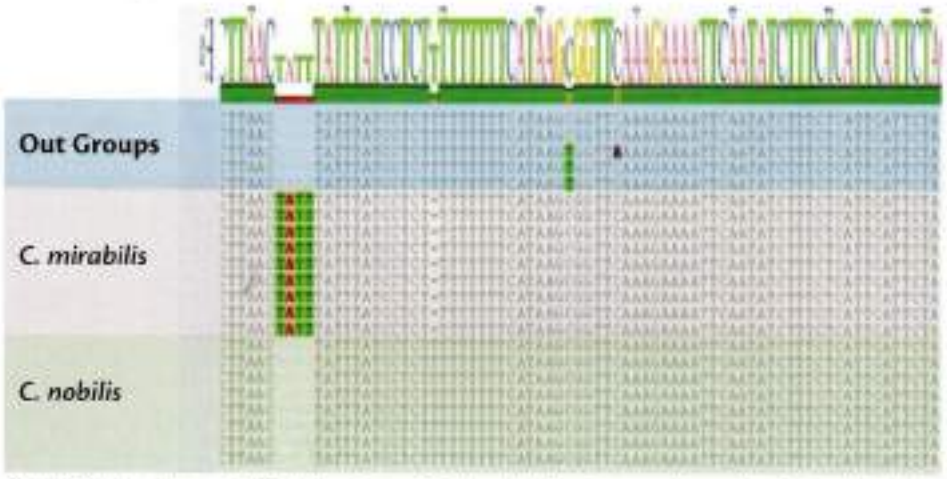

Figure 2. A four base pair (TATT) insertion within the *tmL-F* region was observed in *C. mirabilis* which was absent in all other species. In addition a single nucleotide (T) was missing from all *C. mirabilis*.

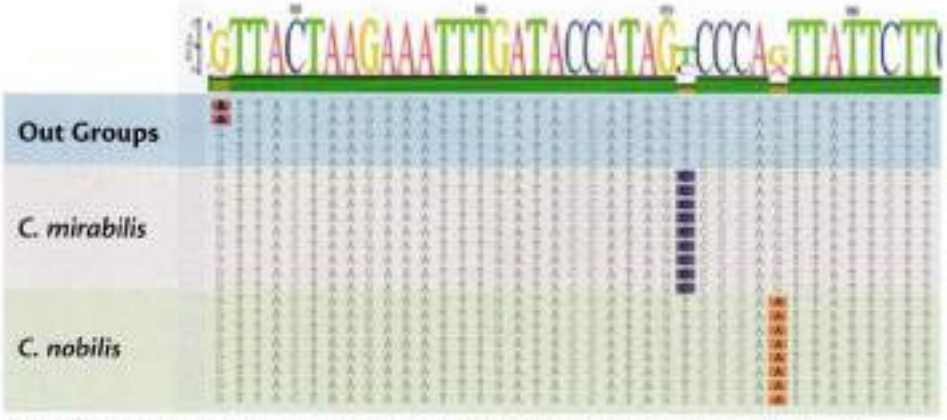

Figure 3. Two unique one base pair differences (SNP – single nucleotide polymorphism) were obtained respectively for *C. nobilis* (A instead of the G present in all other species) and *C. mirabilis* (C instead of T) within the *matK* region.

lis were unique for these species. We found a total of twenty-two variable sites between *C. nobilis* and *C. mirabilis*. Four of these differences are shown in Figures 2 to 5. The out groups consisted of one specimen of each of *C. caulescens, C. miniata, C. gardenii, C. robusta* and *C. × nimbicola*.

All seven gene regions showed a distinct difference between *C. nobilis* and *C. mirabilis* on the one hand and the other *Clivia* species on the other hand. The results showed that the *atpH-I, rpoB, rpoC1* and *ITS1* regions were not as informative as the *matK, rpl16* and *trnL-F* regions. These three regions showed a high number of polymorphic and parsimony informative sites, as can be seen in Table 1. A site is parsimony informative if it contains at least two types of nucleotides and each of those nucleotides occurs in at least two of the sequences.

All seven regions can be used together

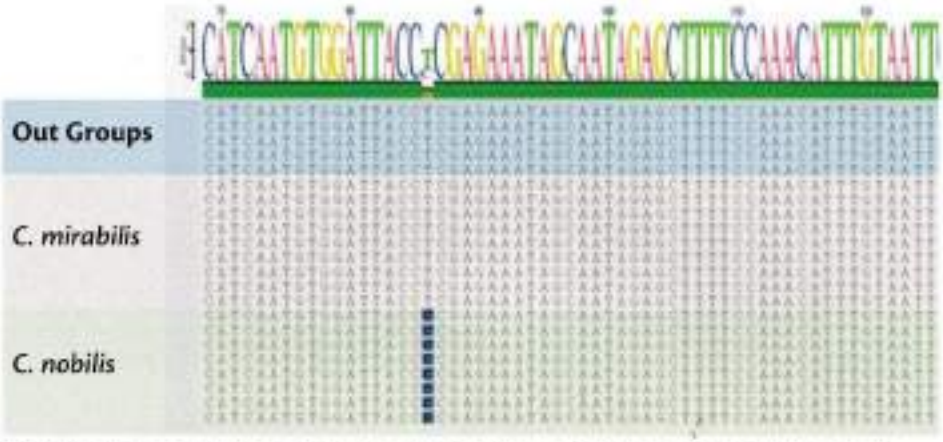

Figure 4. A unique one base pair difference (C replacing T in the other species) within the *rpoC1* region for all *C. nobilis* samples.

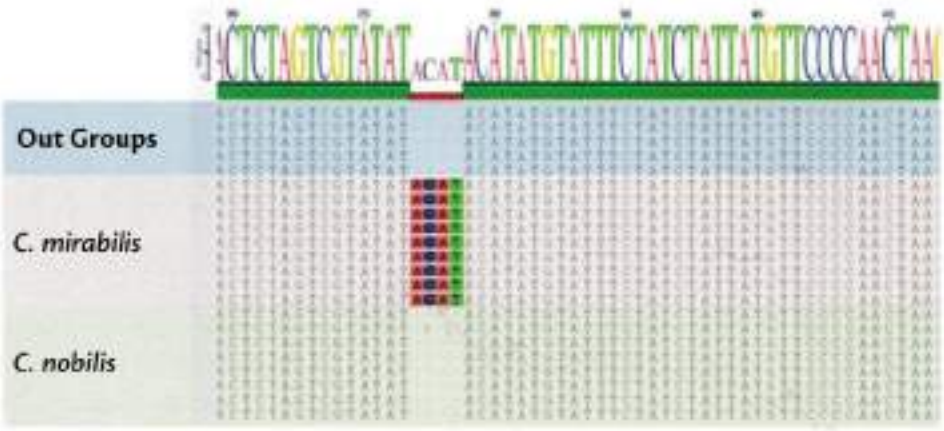

Figure 5. Another four base pair insertion in *C. mirabilis* (ACAT) in the *atpH-I* region.

Figure 6. *C. mirabilis*, Oorlogskloof Nature Reserve, Northern Cape.

for bar-coding purposes or different combinations can be used. Individually, none of the regions provided strong enough species discrimination and, therefore, more than one region should be used. We propose that *matK*, *rpl16* and *trnL-F* are used together as a barcode in the genus *Clivia*. Bar-coding is quite expensive and, therefore, it would be ideal to limit the different regions which must be analyzed without compromising the reliability of the results. The results from these three regions will be able to identify any *C. nobilis* and *C. mirabilis* plants that were removed from their natural environment with an accuracy level of 100%, provided that the plant investigated is not a hybrid of *C. nobilis* or *C. mirabilis* from a private collection or nursery.

This study has thus conclusively provided a tool to distinguish *C. nobilis* and *C. mirabilis* from one another and from all other *Clivia* species.

Table 1. Summary of the results of the seven *Clivia* taxa for the seven gene regions.

Region	Length in bp	Variable (polymorphic) sites	Parsimony informative sites
matK	651	15	8
rpoB	488	3	1
rpoC1	329	1	1
atpH-I	444	0	0
rpl16	916	11	11
trnL-F	725	14	8
ITS1	284	3	3
Total	**3837**	**47**	**32**

The authors wish to thank the University of the Free State for facilities and financial aid, as well as the Clivia Society for financial aid during this study. We thank Willem van Zyl, Mias Vollgraaff, John Roderick and Stella van Gass for supplying the plant material.

Reference

Van der Westhuizen, H.M., (2011). *Genetic variation in the most primitive Clivia species*. M.Sc. thesis, Department of Genetics, University of the Free State.

The identification of genes involved in colour formation in *Clivia miniata* flowers

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Members of the genus *Clivia* (family Amaryllidaceae) are native to countries in southern Africa, including South Africa and Swaziland (Koopowitz, 2002). For many years, clivias have received considerable attention worldwide as cultivated ornamental crops (Duncan, 2008). Interest in these plants lies particularly in their flowers that display a diverse array of colours and variable forms (i.e. shapes). *Clivia* flower colour typically comprises orange, red, yellow and green. However, different combinations and intensities of various colours may also occur. For example, some flowers may display pastel colours such as peach and apricot. The demand for certain colours (e.g. almost white) is very high, such that the plants and seed stocks command exorbitant prices in the market. Thus, clivias are among the commercially important flower species in the floriculture industry.

Flower colour (also referred to as 'flower pigmentation') is a highly variable characteristic that is important to plants, where it attracts flower pollinators and seed dispersers (Weiss, 1991). In human terms, flowers are particularly important in the floriculture industry where they are sold as cut flowers, potted plants, or as ornamental bedding plants (i.e. garden plants). The floriculture industry is highly commercial such that, in 2007, it was projected to generate a global consumption value, at

Figures 1–3. *Clivia miniata* flowers.

consumer level, of between €100 – 150 billion (Chandler & Tanaka, 2007). The biggest challenge facing commercial flower growers and breeders is to produce cultivars with novel characteristics, such as flower colour and shape. So far, this has been achieved by applying classical plant breeding approaches that enable the assessment of characteristics (e.g. flower colour) to be performed only in mature plants. However, the approaches can be improved by incorporating information on the biology (read 'genetics') of characteristics of interest into the breeding strategy. In particular, knowledge of the genetics of flower pigmentation and shape will be relevant to approaches that aim to improve flower cultivars. Also, the availability and use of genetic information resources from currently commercialized and well-studied flower species will further aid efforts toward producing cultivars with novel characteristics.Studies in various plant species have enabled an understanding of the biochemistry and genetics of flower pigmentation (Grotewold, 2006; Chandler & Tanaka, 2007; Tanaka *et al.*, 2008). In general, there are three main pigments that occur in flowers. These include betalains, carotenoids and flavonoids. The latter two pigments are broadly distributed in the majority of flowering plants whereas betalains are restricted to plants in the order Caryophyllales, which includes carnations and catchfly (Steglich & Strack, 1990). Among the three types of pigments, flavonoids are the most abundant in flowering plants where they occur in almost all vascular plants. Flavonoids are responsible for a variety of floral colours including orange, yellow, red, purple, violet and blue. This array of colours is made possible by the coexistence of different classes of flavonoids including chalcones, aurones, anthocyanins, flavones,

Figure 4. *Clivia miniata* flowers used in the experiment.

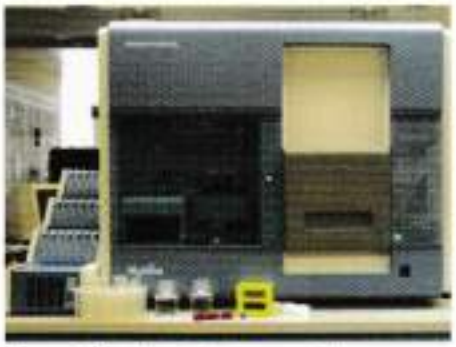

Figure 5. The Illumina Genome Analyzer on which DNA isolated from the flowers was used to perform DNA sequencing experiments.

and flavonols. Unlike flavonoids, the colour spectrum of carotenoids in flowers is typically yellow to orange, but coexistence with red or purple anthocyanins may result in brown and bronze colours (Forkmann, 1991). In addition to flower pigmentation, carotenoids also serve as precursor molecules for the formation (also termed 'biosynthesis') of vitamin A (Fraser & Bramley, 2004). Further, flavonoids and carotenoids are able to absorb UV-light, thus protecting plants and humans from UV damage (Winkel-Shirley, 2002). Considering the

functional importance of flavonoids and carotenoids. It is therefore not surprising that most of the genes (i.e. DNA sequences that carry information responsible for the formation and variation of characteristics) that produce biochemical agents (termed 'enzymes') involved in flower pigmentation have been identified in various plant species (Grotewold, 2006; Chandler & Tanaka, 2007; Tanaka *et al.*, 2008).

In spite of the vast amount of biochemical and genetic resources available on the biosynthesis of flavonoid and carotenoid pigments, few studies have been conducted in monocotyledonous (i.e. monocot) plants. In particular, very limited information is available on the biosynthesis of flavonoid and carotenoid pigments in monocot flower species including clivias. Nonetheless, previous studies in orchids (Hieber *et al.*, 2006; Chiou *et al.*, 2008; Ma *et al.*, 2009; Albert *et al.*, 2010; Chiou *et al.*, 2010), lilies (Nakatsuka *et al.*, 2003; Nakatsuka *et al.*, 2009; Yamagishi *et al.*, 2010) and tulips (Momonoi *et al.*, 2009; Shoji *et al.*, 2010) have identified a few genes involved in flower pigmentation in these species. In addition, monocot food crops including maize (Paz-Ares *et al.*,

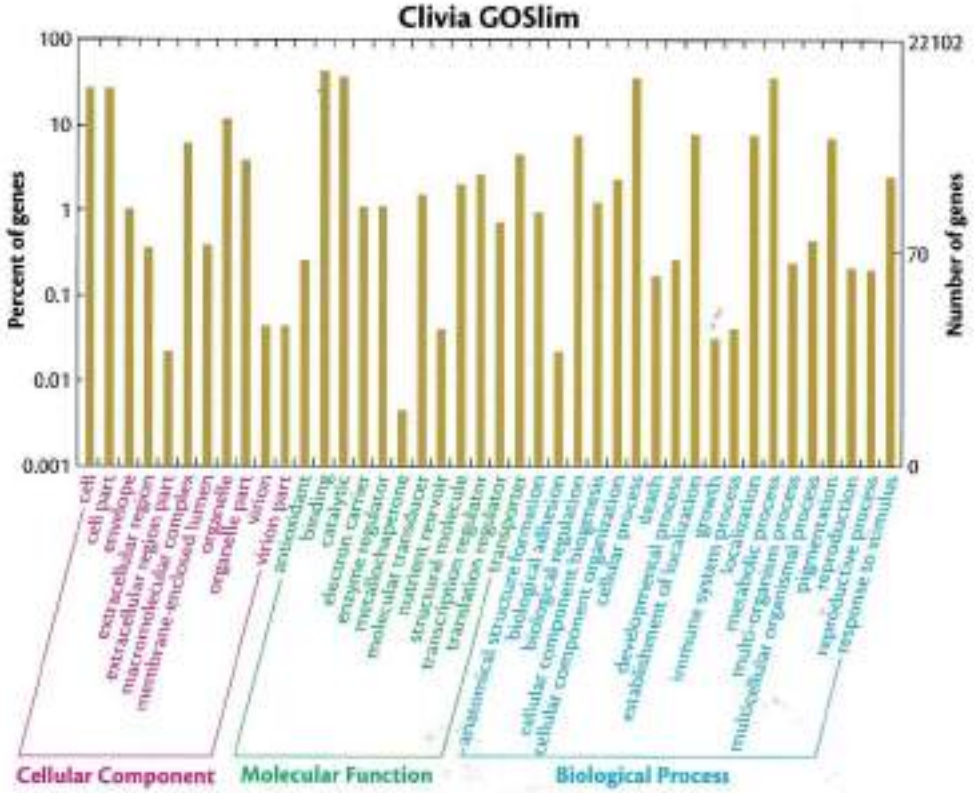

Figure 6. A summary of clivia genes that were assigned specific cellular functions (vertical bars) and grouped according to the functions they perform – Cellular Component, Molecular Function or Biological Process.

1986; Bernhardt *et al.*, 1998), onions (Kim *et al.*, 2004a, b, c, 2005) and rice (Reddy *et al.*, 1996; Bong *et al.*, 2007; Jeong *et al.*, 2008) have also been used to identify genes involved in the biosynthesis of flavonoid (particularly anthocyanin) and carotenoid pigments. This information, coupled with information reported for dicotyledonous (i.e. dicot) plant species, will be useful for further identification of flower pigmentation genes in other plants including clivias.

Rationale

The discovery of flower pigmentation genes in clivias will greatly aid efforts toward producing cultivars with novel characteristics in terms of colour. That is because the information on genes that produce enzymes involved in flower pigmentation can be used to genetically modify flower colour. Such studies have already been performed in other plants with varying level of success. In 1987, *Petunia* became the first plant to have its flower colour genetically modified (Meyer *et al.*, 1987). Subsequently, in the 1990s, the company Florigene (http://www.florigene.com/) released the 'Moon' series of genetically modified carnations. Recently, efforts were made to produce genetically modified blue roses (Katsumoto *et al.*, 2007) and tulips (Shoji *et al.*, 2010), but the results were not completely convincing.

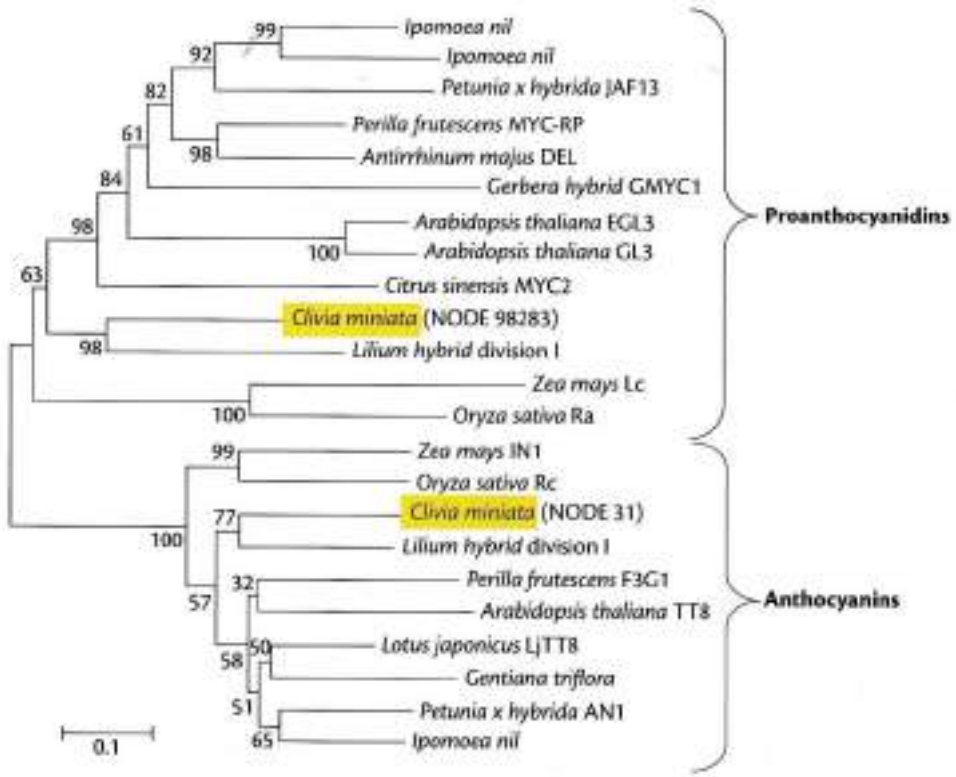

Figure 7. Phylogenetic analysis of plant bHLH genes. The genes are clustered into two main groups according to their function, namely, genes that activate the biosynthesis of proanthocyanidins and anthocyanins. Two *Clivia* genes were identified, one belonging to each of the groups.

In other cases, information on genes that produce enzymes involved in carotenoid pigmentation has been used to genetically modify the nutritional value (and colour) in food crops. In 2000, a genetically modified cultivar of rice (subsequently named 'Golden Rice') was produced that contained higher levels of provitamin A, which is the precursor molecule used in vitamin A biosynthesis (Ye & Beyer, 2000). Alternatively, studies on gene sequences have lead to the identification of mutations (i.e. changes in the DNA sequence information) that are responsible for variation in specific characteristics. For example, the variation in flower colour (Morita *et al.*, 2006; Choi *et al.*, 2007), flower colour patterning (Habu *et al.*, 1998; Itoh *et al.*, 2002; Koseki *et al.*, 2005; Saito *et al.*, 2006), and even fruit colour (Espley *et al.*, 2007; This *et al.*, 2007) have been determined through the analyses of various genes involved in the biosynthesis of pigments that occur in flowers and fruits. These reports, including those not reported herein, are important as they suggest that *Clivia* can possibly be improved as an ornamental crop in the floriculture industry.

Figure 8. *Clivia* 'Rouge Magic'.

Photo: Chris Wiegerinck

Figure 9. *Clivia* 'GT Pink'.

Results

We recently initiated studies aimed at the identification of genes involved in flower pigmentation in clivias. Our efforts were boosted by the availability of new technology that enables the analyses of many (ca. thousands) genes, simultaneously. In our studies, we harvested orange flower tepals of *Clivia miniata* (Fig. 4) and immediately froze the tissues using liquid nitrogen. The purpose of the latter step was to "freeze" all cellular activity including the biochemical processes that lead to flower coloration. Subsequently, DNA was isolated from the tissues and prepared for DNA sequencing experiments essentially aimed at determining the order of letters (namely A, C, G and T and these are collectively termed 'nucleotides') that make up genes. A high-throughput system (the Illumina Genome Analyzer, Fig. 5) was used to determine the DNA sequence of genes that were 'selectively' obtained from the *C. miniata* flowers. Overall, more than 23 million DNA sequences (each being 76 nucleotides in length) were generated. Various computer-based analyses were applied that joined the DNA sequences into 'recognized' genes comprising longer DNA sequences. Following this, a total of 37 014 gene sequences were generated. Of these genes, only 22 102 were successfully assigned specific cellular functions and ultimately grouped according to the functions they perform in the flower tissues (Fig. 6). Approximately 10% of the 22 102 genes, roughly 2 200 genes, were assigned functions related to one or more processes leading to flower colour. In addition, two other categories ('transcription

regulator' and 'transporter activity') were identified which, based on previous studies, should include genes that are involved in controlling various processes leading to flower pigmentation (Fig. 6).

Considering that each category of genes (i.e. the vertical bars in Fig. 6) included many sequences, further analyses were necessary to identify key genes that are particularly involved in flower colour in *C. miniata*. The analyses were based on the assumption that genes producing similar enzymes (hence, functions) in various species will tend to have similar DNA sequences. Therefore, the analyses compared *Clivia* genes with counterpart genes already known to be involved in flower pigmentation in other plant species. Herein, we report on the analyses of a specific group of genes (termed bHLH genes) that are known to control processes leading to flower pigmentation, thus, genes in the 'transcription regulator' category. Similar genes have previously been shown to activate the flower pigmentation processes in snapdragon (Goodrich *et al.*, 1992), petunia (Spelt *et al.*, 2000) and lily (Nakatsuka *et al.*, 2009). Overall, two *Clivia* genes were identified (Fig. 7). The analyses further revealed that not only were the *Clivia* gene sequences highly similar to counterpart genes obtained from other plant species, but they also tended to cluster/group with gene sequences obtained from other monocot plant species (Fig. 7). The same approach is currently being used to identify more genes that are involved in floral pigmentation in clivias. Once identified, *Clivia* genes will be studied further to better understand their specific roles during flower pigmentation. The results will guide future efforts aimed at *Clivia* cultivar improvement.

Photo Chris Wolgroved

Figure 10. *Clivia* 'Raspberry Sauce'.

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Photo: Chris Wellgreen

Figure 11. *Clivia* 'Pico'.

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The identification of genes for flower colour in *Clivia miniata*

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The basis of colour variation in *Clivia* is locked up in its genes. The first study to elucidate the formation of colour in *Clivia* was made by studying the different genes responsible for colour. This paper describes the process followed and summarises the results obtained.

Photo: Carine Knight

Figure 1. *Clivia* 'Estelle'

We know from the literature that anthocyanin occurs in *Clivia* and it is responsible for the formation of colour. Thus the first step of this study was to analyse all the available DNA sequences from the sequence database GENBANK, of different plants, for every gene involved in the anthocyanin pathway. The sequences of specific genes from all available plants was aligned (compared) using computer software and areas in common for these genes in all plants were identified. DNA was then extracted from open flowers to determine whether the specific gene was present and expressed. A laboratory technique known as the PCR reaction was then used to produce millions of copies of these genes using a piece of synthetic DNA (known as a primer). The millions of copies of DNA were then used to determine the specific sequence of the genes responsible for colour in *Clivia* as well as the level of expression of these genes.

By using this method primer sets were designed to amplify portions of the *CHS* (chalcone synthase), *CHI* (chalcone isomerase), *F3H* (flavanone 3-hydroxylase) and *DFR* (dihydroflavonol 4-reductase) genes. The amplified DNA was then sequenced to determine the order of the nucleotides. The DNA sequence of the *Clivia* DNA was then compared to the DNA from other plants. This resulted in a cladogram, indicating the position of *Clivia* among the monocots (Figure 2). It is interesting to note that the *F3H* gene of *Clivia miniata* corresponds most to *Allium cepa* (the onion). Similar trees were obtained for all the genes. Unfortunately the sequence data could not be combined

because different species were included in the different trees based on available sequences.

The sequence of a gene can be presented in a graph format that shows different coloured peaks for each nucleotide. This representation of the data is called an electropherogram. The electropherograms of a short section of the *CHS* gene is shown in Figure 3. Note the two instances where two peaks are shown in the same location in two different plants (an orange [A] and yellow [B]). This is an indication that two different alleles of a gene have been sequenced. The alleles of a gene are similar in almost all respects but occasionally a twin peak is observed. This observation strengthens the hypothesis that *Clivia* is an ancient polyploid and one copy differs slightly from the other in both plants.

So now we know that these genes (or to be precise, parts of these genes) are present. However, we have to do further tests to determine whether these genes are active.

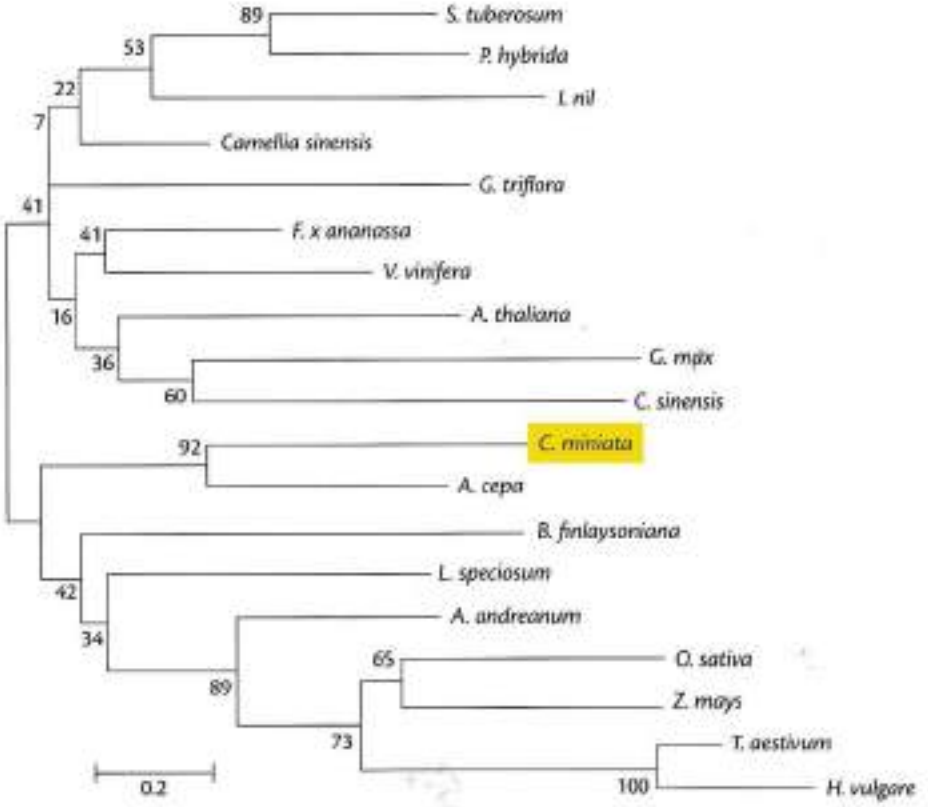

Figure 2. Cladogram determined by minimum evolution, based on the relationship between F3'H genes in different organisms. The numbers next to the branches represent the bootstrap value (based on 500 replicates).

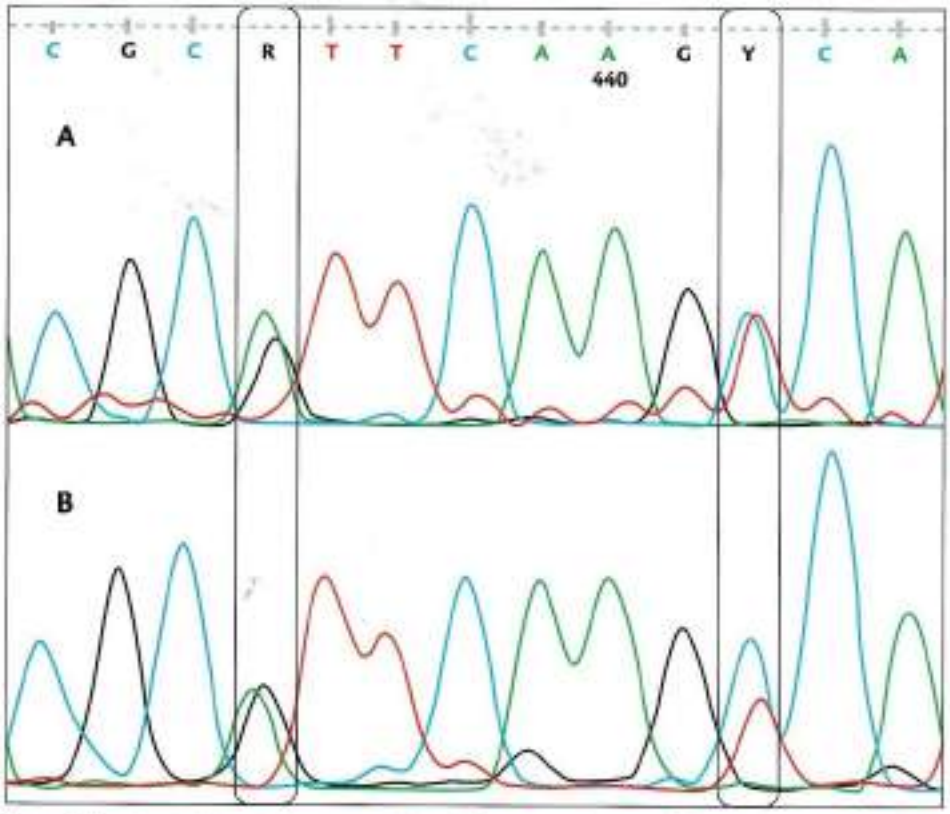

Figure 3. Sections of electropherograms obtained after sequencing part of the CHS gene in orange (A) and yellow (B) *Clivia miniata* specimens. Ambiguities are present at position 435 (R) with a G or A, as well as position 442 (Y) with a T or C.

Let us get back to our basic biology. The DNA is in the nucleus of the cell (with a few exceptions). If a gene is active it will form mRNA (messenger RNA). The mRNA will leave the nucleus to produce the product (a protein) according to the specifications of the DNA in the nucleus. So the next step was to determine whether the different genes were active (i.e. do they form mRNA?) and whether this activity differed through different developmental stages of the flower. Since we only wanted to determine whether the genes were active, we did not study all stages, only those up to the opening of the flower (Figures 4 & 5).

Three different types of tissue (tepal, stamen and carpel) were used for mRNA extraction. The RNA was converted to a more stable substance, cDNA (copy DNA). cDNA only represents the portion of the RNA that determines the composition of proteins since introns (sections of RNA that are excised from mRNA) are excluded. Another gene, the 18S rRNA, which is constantly expressed in all tissues, regardless of the conditions, was used to standardize the expression of the four most important genes in the anthocyanin pathway.

The expression of the genes was measured by a technique called RQ PCR (real-

time quantitative polymerase chain reaction). This technique accurately measures the quantity of mRNA formed from a specific gene. From the results of this study it has become apparent that the transcription of the *CmCHS* and *CmDFR* genes increased as tepals grew and peaked at stage 3 just before anthesis. However, the transcription of both genes decreased as the flower was opening (between stage 3 and 4), after which their transcription increased drastically towards the end of flower development. In the carpel both *CmCHS* and *CmDFR* genes had very similar levels of

Figure 4. The different stages (1–5) of flower development used for the orange *Clivia miniata*.

Figure 5. The different stages (1–5) of flower development used for the yellow *Clivia miniata*.

expression, especially from stage 1 to stage 4. Transcription of the genes peaked during the third developmental stage and then gradually decreased through stage 3 up to stage 4 where the genes were the least expressed. Further expression of CmCHS increased considerably from stage 4 up to the point where the carpel was fully developed, while transcription of *CmDFR* only increased slightly (Figure 6).

The target genes in the stamens showed similar trends regarding their temporal expression, although *CmCHS* was expressed at much higher levels than *CmDFR* (Figures 6C & 6D). Transcription of both genes decreased during stage 1 and was very low at stage 2, followed by a slight increase towards the middle of stage 3 with a decrease again during stage 4 to reach the equally low levels found during stage 2. Thereafter the same steep up-regulation as in the tepals and carpel could be observed until flower development was completed (full bloom).

There was a strong positive correlation in expression trends, for *CmCHS* and *CmDFR* during the different developmental stages in the tepal and stamen. When comparing stages 1 to 4 for *CmCHS* and *CmDFR* in the carpel, a high correlation was also observed (R = 0.996). The high correlation of gene expression may be an indication that these genes are regulated simultaneously.

From the transcriptional changes in *CmCHS* and *CmDFR* in the different flower tissues, two phases of temporal expression can be distinguished. 1) transcription

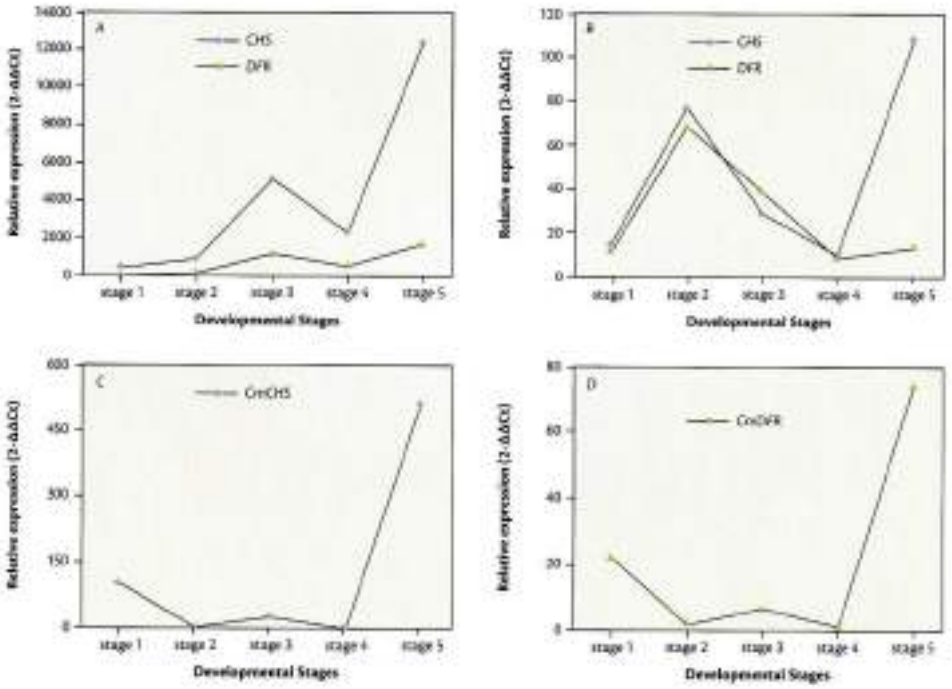

Figure 6. Relative expression of *CmCHS* and *CmDFR* in flower tepals (A), carpel (B) and stamens (C&D) from development stages 1 to 5 of an orange *Clivia miniata*.

of both genes increased as the flower bud grew, then decreased to very low levels before entering the second phase where 2) anthesis was activated and transcription of the genes increased drastically until the flower was in full bloom.

Transcription of *CmCHS* and *CmDFR* genes was constant from stage 1 to 2 in the tepals of yellow flowers. There was an increase in expression from between stage 2 and 3 just before the flower opened (Figure 7A). During stage 3 (anthesis) both genes were highly expressed, followed by down-regulation towards the end of tepal development. The trend in expression of the two target genes was very similar with a positive correlation ($R > 0.950$, $p \leq 0.05$).

In the carpel *CmCHS* and *CmDFR* showed a similar trend in their temporal expression with higher levels of *CmDFR* compared to *CmCHS* (Figure 7B). Transcription of both genes increased from stage 1 and peaked during stage 2, followed by a decrease in expression until stage 3 with an increase that peaked during stage 4. Thereafter the expression of both genes was down-regulated towards the end of carpel development. In the stamens of the yellow *Clivia miniata* no similarities were seen in the transcription of the two genes. *CmDFR* was mostly present at higher levels compared to *CmCHS* during stages 1 and 2, and especially during stages 4 and 5 (Figure 7C). Based on these observations it appears that transcription of *CmCHS* and *CmDFR* tends to decrease from the onset of anthesis (during stage 4) until completion of yellow flower development (stage 5). A decrease in transcription of these genes is expected to affect the production of anthocyanin derivatives, ultimately leading to lower anthocyanin concentration in yellow *Clivia miniata* flowers.

In the present study, *CmCHS* and *CmDFR* were transcriptionally active throughout flower development in pigmented tissue of both the orange and yellow flower varieties, suggesting co-ordinate regulation as a single module for anthocyanin biosynthesis in *Clivia miniata*. As mentioned before, each type of flower tissue exhibited similar

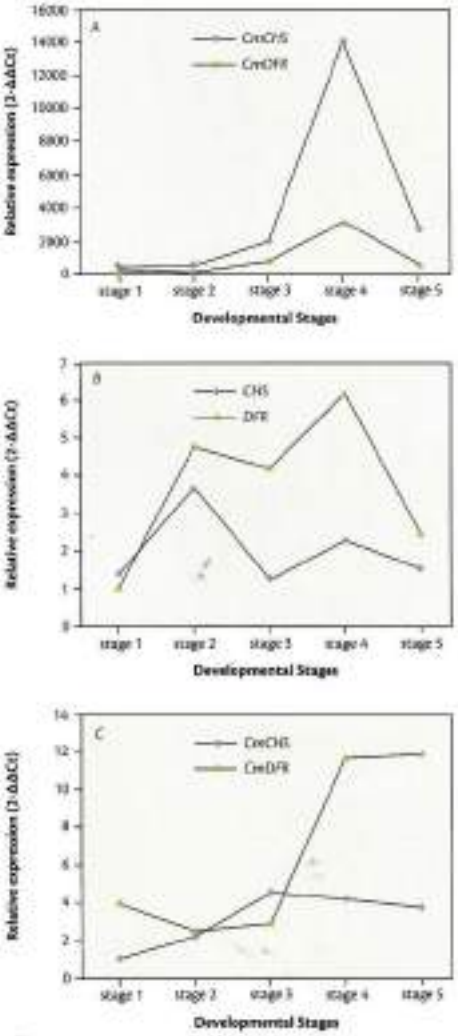

Figure 7. Relative expression of *CmCHS* and *CmDFR* in the tepal (A), carpel (B) and stamens (C) of a Group 2 yellow *Clivia miniata*.

temporal expression for *CmCHS* and *CmDFR* genes, except in the stamens of variety 'Giddy' where each gene was expressed differently. These observations support the possibility of co-ordinate regulation by either the same or alternative transcription factors, depending on the tissue type.

A further test was done to see whether the transcription of the genes correlates with amount of anthocyanin produced. This was done by determining the absorbance of ultra-violet (UV) light. An instrument called a spectrophotometer was used for this purpose.

The anthocyanin concentrations at each developmental stage in both colour forms of *Clivia* were plotted on a bar chart (Figure 8). At stage 5 the anthocyanin content in the orange tepals of the orange plant had increased by almost 16-fold compared with that in the yellow tepals. The results indicate that colour development in the orange tepals is strongly correlated with the accumulation of anthocyanins. The absence of orange colour in yellow tepals can only be caused by the very low anthocyanin concentration, overshadowed by the high concentration of carotenoids and, to a lesser

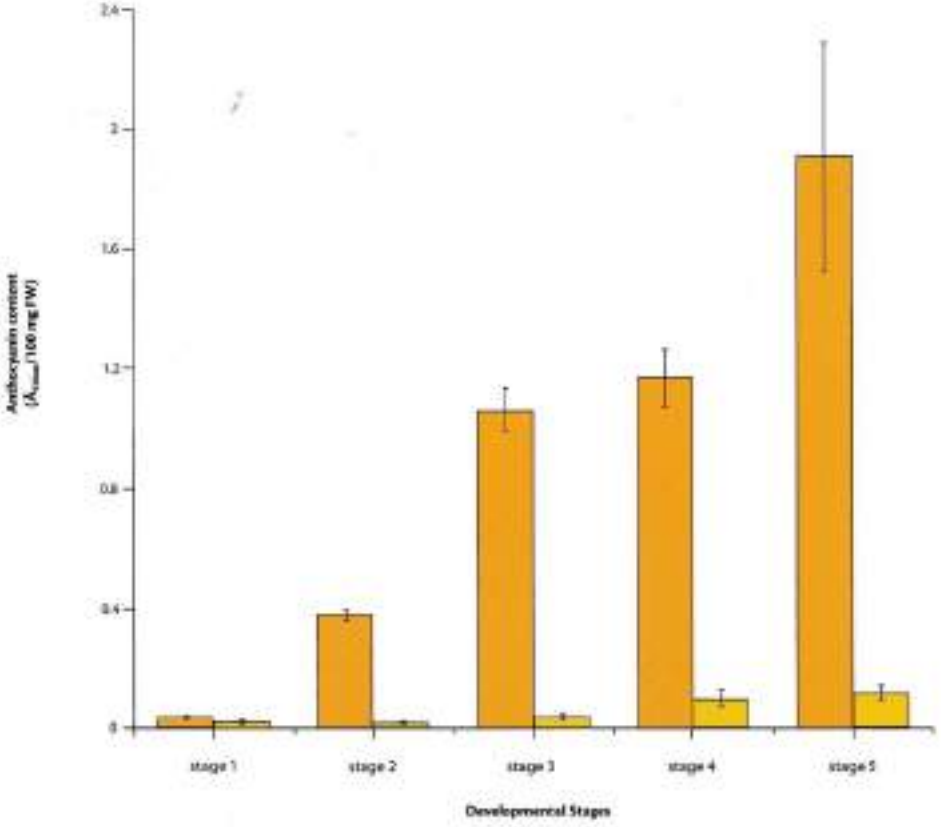

Figure 8. Anthocyanin accumulation at five developmental stages of tepals in *C. miniata* orange and yellow forms respectively. Vertical bars indicate the standard error of the mean of three absorbance readings.

extent, the presence of chlorophylls.

There was a semi-linear increase of anthocyanins in orange tepals, whereas the increase in yellow tepals displayed a sigmoid pattern (Figure 9). When the temporal expression of *CmCHS* and *CmDFR* in the tepals was compared with the anthocyanin accumulation at each stage, a clear trend was visible. In the orange tepals a decrease of gene expression occurred between stages 3 and 4, while a drastic increase of gene expression appeared between stages 3 and 4 in yellow tepals. Both cases coincided with the changes in anthocyanin content depicted in Figure 9. Furthermore, a slight decrease in anthocyanin content in the yellow tepals was observed after stage 4, which could be explained by the down-regulation of *CmCHS* and *CmDFR* expression that was observed between stages 4 and 5.

This study has determined that the genes required for anthocyanin formation are present in both orange and yellow forms of *Clivia miniata*. These genes are expressed in both colour forms. However, the expression is much lower in yellow plants compared to orange plants. Further studies are needed to determine how these genes are regulated in terms of anthocyanin production that ultimately results in orange versus yellow flower colour.

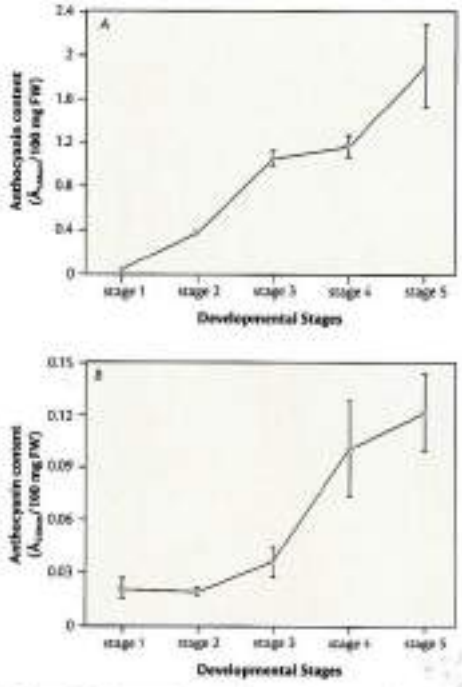

Figure 9. Changes in anthocyanin accumulation at five developmental stages of tepals in *C. miniata* orange (A) and yellow (B) flowers. Vertical bars indicate the standard error of the mean of three absorbance readings.

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Clivia with variegated flowers

Hein Grebe

"From our acquaintance with this abnormal metamorphosis, we are enabled to unveil the secrets that normal metamorphosis conceals from us, and to see distinctly what, from the regular course of development, we can only infer." *J.W. von Goethe – 1790*

The first time I learned about variegated flowers in clivia was in 2008 when I visited a well known property developer in Beijing, Mr. Zhang Jinxiang, who is a keen collector of clivia with unusual flowers. He has written a book called *Junzilan Shiyang Zhishi Wenda* – "Growing and Caring for Clivia" of which he has given me a copy. One of the photos in the book is of a clivia with variegated flowers. The photo was taken of a plant of a fellow Beijing clivia enthusiast. My search for this plant came to a dead end when the owner of the plant informed me that he sold it, because the flowers were not stable. More searches of similar plants were unsuccessful.

When I returned to South Africa beginning October 2009, I was surprised to find a Chinese plant with variegated flowers in my shade house. The wind damaged the few flowers that were still open, but I took a few photos to keep as a record, as I was very excited to find a plant that I spent so much time searching for in China in my collection. It flowered again in October

Figure 1. Almost all the flowers open.

Figure 2. Variegation and mature colours on petals.

2010 and this time I was lucky to take more photos and watch the flowers developing and opening up.

From the photos you will notice that the variegated flowers are from a short-leaf variegated plant. Most variegated plants will carry flowers on variegated flower stems. It is seldom that variegated flowers are produced. However when the flowers die, beautiful variegated berries are formed. The question is why do we not see more variegated flowers on variegated plants? An internet search did not give me any clues.

Variegated flowers take longer to open and can stay open for 3 weeks or longer if not pollinated. This specific plant of mine produces viable pollen, but does not self easily. If the flowers are exposed to direct sunlight, orange pigmentation will develop on sections of some petals. The variegation patterns on the tulip-shaped flowers

Figure 3. Colour comparison.

Figure 4. Colour comparison.

are usually the same as the variegation of the leaves and peduncle. The section of the flower stem with yellow lines will produce yellow or white flowers and those with green lines will produce flowers with similar green variegation on the petals. Sometimes a few normal petals will be formed in a flower. I have noticed that these petals will open first and are also slightly longer than the variegated petals. The colour and pattern on the flowers will vary from year to year on the same plant.

What is causing this abnormal behaviour? Most fellow clivia enthusiasts have identified the condition as phyllody. Phyllody is the development of floral parts into leafy structures, generally caused by virus or phytoplasma. Evidence suggests that the phytoplasma down-regulates a gene involved in petal formation, instead causing leaves or leaf-like structures to form. Phyllody causes the affected plant to become partially or entirely sterile, as it is unable to produce normal flowers. Many clivia enthusiasts have similar plants in their collections that produce green flowers on orange or yellow flowering clivia. Variegation is defined as having marks or patches of different colours [syn. varicoloured].

According to Shige of Japan, phyllody in *Clivia* is caused by inbreeding and carries recessive genes. Selfing the flowers might result in seedlings with the same behaviour. From the 2009 flowers, I harvested one seed and from the 2010 flowers I harvested 6 seeds. It will be a few years

Figure 5. First flower open.

Figure 6. Flower colours after two days in direct sunlight.

Figure 7. Note the orange at the bottom of some flowers.

Figure 8. Variegated flowers of raised vein "Mandarin Duck".

Figure 9. Compare the size difference of the orange-coloured petals.

Figure 10. Note the position of the white flowers on the white side of the flower stem.

before any results will be known. This year a semi-miniature "Mandarin Duck" has produced variegated flowers. Unlike the other plant it produced no pollen; flowers were deformed with almost no flower stem. Unlike the other plant, the flowers opened up unexpectedly quickly and when exposed to direct sunlight, many of the white and yellow coloured flowers changed to orange.

Research that has been done shows an alternative approach for generating new varieties of floriculture plants is to engineer plants with variegated flower colour patterns. Sometimes they carry unstable mutants. Variegated mutants might have defects in anthocyanin genes or genes that control intracellular pH of petals. These defective genes can change the colour of the flower and the leaves of plants. It will thus be interesting to see what happens when line breeding with variegated flowers is done.

Figure 11. Outside of first flowers to open.

Relationships in *Clivia*

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Introduction

The genus *Clivia* was erected by John Lindley in 1828, and the type species is *Clivia nobilis* Lindl. (Lindley, 1828). In 1854 *C. miniata* (Lindl.) Bosse was described (Lindley, 1854; Bosse, 1859). These two plants were introduced to horticulture in Europe and there was a great amount of cultivation and hybridisation between these two taxa in the period 1850 to 1900. In 1856 another taxon, *C. gardenii* Hook., was described (Hooker, 1856). There matters rested until *C. caulescens* R.A.Dyer was described in 1943 (Dyer, 1943) and half a century later *C. mirabilis* (Rourke, 2002) and *C. robusta* (Murray *et al.*, 2004) were added to the genus.

Due to the appeal of the plant, and especially its floral beauty, new varieties were constantly being sought to increase the range of shape and colour. A yellow-coloured form of *C. miniata* was described as a horticultural variety – *C. miniata citrina* or and also *C. miniata* var. *citrina* (Rogers, 1897). The same plant was later illustrated (Watson, 1899). A century later *C. gardenii* var. *citrina* (Swanevelder *et al.*, 2005) and *C. robusta* var. *citrina* (Swanevelder *et al.*, 2006) were described, elevating yellow-flowering forms to taxonomic status. *C.* × *nimbicola*, a natural hybrid between *C. caulescens* and *C. miniata* (Swanevelder *et al.*, 2006), has also been described.

With the increasing upsurge in interest in *Clivia* cultivation in the last 30 years, and the progress in breeding and hybridising, many questions have been raised about the variations and unexpected results obtained by various crossings, and many contradictory observations have been made about genetic traits and origins of different strains and forms.

Figure 1. *Clivia nobilis*. This illustration is from Hooker's 1828 description in Curtis Botanical Magazine of *Imatophyllum Aitoni* or Handsome-flowered Imacophyllum.

The dramatic upsurge in interest in *Clivia* since the formation of the Clivia Club in 1992, which has since become the

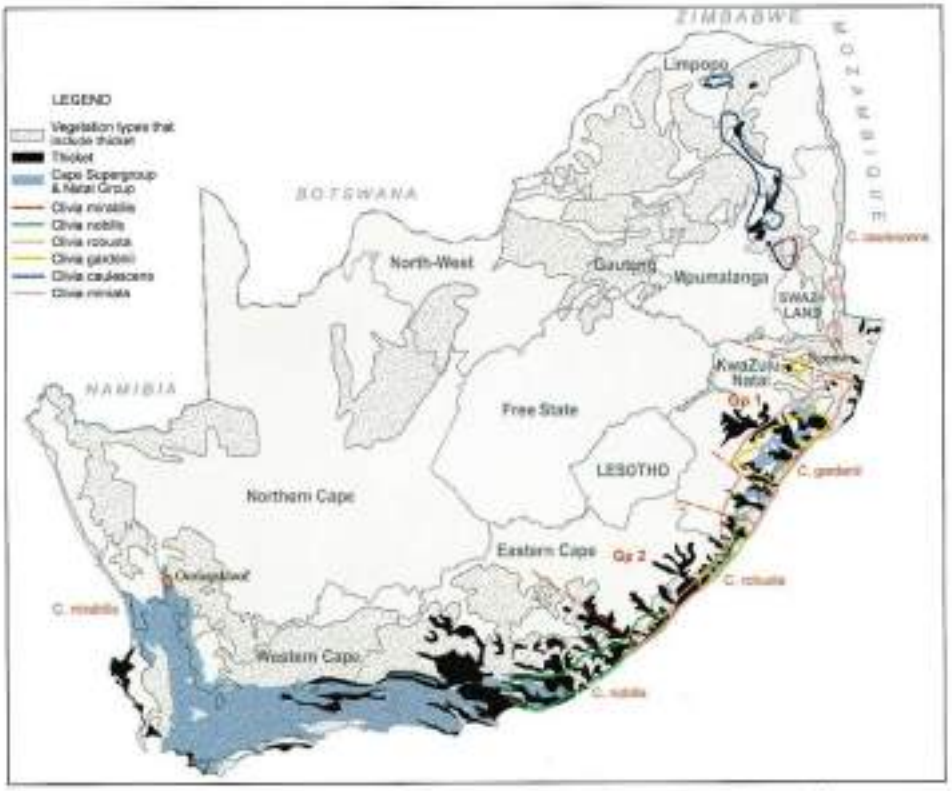

Figure 2. Distribution map of the genus *Clivia*, showing its relationships with southern African subtropical thicket vegetation and the sandstones of the Cape Supergroup and Natal Group. The range limits of Groups 1 and 2, two yellow-flowered forms of *C. miniata*, are also shown. Cartography: Elmi Dixon.

Clivia Society, and the further formation of Clubs and Societies devoted to the genus in a number of countries, has increased the amount of published information and observations on the various taxa in the genus and their hybrids. In addition, with the interest in the horticultural and medicinal promise of *Clivia*, and the quest to understand the origins of the genus, much research has been undertaken by a variety of interest groups, including those of medicinal, horticultural, taxonomic and botanical bent. The improvement of analytical methods in the fields of chemistry and genetics has also broadened our understanding of the genus, although much of this understanding is fuzzy and isolated.

Molecular phylogenetic approaches, combined with analyses of ecological and biogeographical information, both published and anecdotal, have provided sufficient information to propose a speciation model for *Clivia*, and give some insights into the observed behaviour of the genus in cultivation. In order to put all this information in perspective, it is necessary to relate the distribution of *Clivia* to geographic and ecological factors, and then look at the genetic information to understand why we have the variation in the genus.

Photo: Roger Elliott

Figure 3: *Clivia caulescens* in habitat in the Songimvelo Nature Reserve in the Barberton Mountainland.

What is a species in Clivia?

There is no general agreement as to what is the definition of a species, and this is highly variable as one looks at different types of organisms. A good generalisation is that each species is a breeding population that has taken an evolutionary course of its own, separate from related populations, and has consequently developed a set of distinguishing features which makes it identifiable from other species (Waters, 2011).

It may be that the differences between some species are obvious, but often distinguishing between several species is problematic, and a variety of criteria, not always obvious, have to be used to discriminate between them. As populations evolve, the features of the plants differ and change, but the exact point where the accumulated differences amount to the existence of a new species is quite arbitrary. This is because the knowledge of the distribution and appearance of the plants is never absolutely complete. In an ideal world naming a new species would mean that all the details of the plants' distribution, ecology, range of forms, genotype and evolution are known, as well as all the related species. In practice, a new species may be described on the basis of a single specimen with minimal information. At a later stage as more information is obtained the status of the plant may be confirmed or changed – this is what keeps taxonomists employed!

Some taxonomists – the "splitters" – divide plant populations into many species, based on small differences, whereas others – the "lumpers" – focus on similarities.

Photo: Roger Deal

Figure 4. *Clivia coulescens* in habitat at Songimvelo Nature Reserve in the Barberton Mountainland. The low canopy allows in plenty of light, and the festoons of moss and lichens on the tree trunks are indicative of the frequent mists experienced in the area. The substrate upon which the plants are growing is very rocky.

Photo: Roger Dixon

Figure 5. *Clivia miniata* in habitat at Songimvelo Nature Reserve in the Barberton Mountainland. These plants were growing very near to the *C. caulescens* shown in Figures 3 & 4, but the substrate here was less rocky and with deeper soil. The stellate white throat of these flowers is quite distinctive for plants from the area, as is the large flower size.

which can result in a number of different geographical populations being grouped under a single broadly-defined species. Looking at *Clivia*, we can see that during the heyday of botanical exploration, botanists tended towards the second group, with three morphologically distinct and/or geographically separate taxa being identified and described – *C. nobilis*, *C. miniata* and *C. gardenii*. There matters stood for almost a century until, amid a resurgence in botanical exploration, the taxonomic pendulum swung to the other side, and new taxa were identified within the pendulous flowering group – *C. caulescens*, *C. mirabilis* and *C. robusta* – delineating the geographical spread of the pendulous group in a fairly even range around South Africa, between the coastline and the escarpment.

In *Clivia* we find two ranks of classification, as exemplified in *C. miniata* and *C. miniata* var. *citrina*. What does this mean? In botanical classification there are three levels or ranks of a species – subspecies (abbreviated as ssp.), variety (Latin *varietas*, abbreviated as var.) and form (Latin *forma*, abbreviated f.).

Simply put, subspecies are populations of a species that have become reproductively isolated from each other and are evolving in different directions, usually as a result of a geographical barrier, such as a mountain range where the species cannot grow. If the barrier were to be removed, the subspecies (the separated populations) would interbreed again and merge back together. No subspecies have been named in *Clivia*.

Below that is the variety. A botanical variety is a population that has some dis-

tinguishing qualities, but is not as isolated as a subspecies. An example might be a population growing in a more exposed environment resulting in a more compact habit and darker colored flowers. A continuous variation may exist between the compact variety and larger plants growing in a moister, more wooded habitat. In *Clivia* there are three described taxa at this level – *C. miniata* var. *citrina*, *C. gardenii* var. *citrina* and *C. robusta* var. *citrina*. According to the botanical definition described above, all three of these taxa are not justified, as they are based on individuals from within populations which show a range of flower colour. The botanical definition should not

Photo: Rudi Lotter

Figure 6. An umbel of large flowers of *C. miniata* in the Bearded Man area.

Photo: Roger Dixon

Figure 7. *Clivia miniata* in thicket downslope from *C. caulescens* below Bearded Man Mountain in the Barberton Mountainland, where it occurs in soil and leaf-litter, and not on rocks as does *C. caulescens*.

be confused with the horticultural word "variety", which usually refers to a named cultivar.

Below variety is the form (Latin forma, abbreviated f.). A form is any plant or group of plants showing a difference in some character or other, for example, a yellow flowering plant in a population of more typical orange flowering plants. This does not imply that each form is a discrete breeding population, just that they are noticeably different in some way.

In the latter part of the 19th century, when hybridisation of *Clivia* was burgeoning (van der Linde, 2008), there was little distinction between the terms "variety" and "form", and they appear to have been used interchangeably. Also, botanical nomenclature had not yet been as rigidly defined as it is today, and botanical literature was not as clearly divided, as it is today, into the "scientific" and "horticultural" areas. The following admonition appeared in *The Gardeners' Chronicle* in 1891: "CLIVEIA: C.S. – Imantophyllum, Himantophyllum and Clivea are all synonymous. Cliveia has the preference as being the most correct name, but if you prefer to write Clivea there is no power on earth to prevent you."

Yellow flowering *Clivia* – a botanical or horticultural "variety"?

Yellow *C. miniata* had already been described as the variety 'Sulphurea' in *The Gardeners' Chronicle* in 1891 (Douglas, 1891) as "a distinct variety of a very pale yellow colour". Douglas further noted that: "There is a wide field for the hybridists in this genus of plants. Not more has been done than to breed in and in from the specific form, or a variety of it with broader foliage, named robusta."

The variety 'citrina', first described by Mrs Powys Rogers in *The Garden* in 1897.

Figure 8 *Clivia miniata* 'Mme Le Grelle d'Hanis', from the *Revue de l'horticulture belge et étrangère*, 1881.

The same plant was described in two horticultural magazines in 1899, in articles written by a certain W. Watson. The plant was illustrated in *The Garden* in Plate 1246, and Watson had this to say:

> "If the introduction of such a slight variation of the type as superb is resulted in the breeding of such varieties as Marie Reimers..., much more may be expected from crosses between variety citrina, and some of these."

He also discussed it in *The Gardeners' Chronicle*, as follows:

> "CLIVEIA MINIATA CITRINA. – This well-marked, beautiful variety is now in flower in the T range at Kew. It is said to have been collected wild in Zululand by Captain Mansell, and first flowered in the garden of Mrs. Powys Rogers, Perrenwell, Cornwall, in April, 1897, when flowers of it, and subsequently a little plant, were sent to Kew. An example of the same variety had, however, already been added to the Kew collection by the Rev. W. H. Bowden, Bow, North Devon, who sent it along with some other plants which had been collected in Zululand, and it is this plant which is now in flower. It resembles a good form of

Photo: Roger Dixon

Figure 9. *Clivia gardenii* from Ngome Forest in Northern KwaZulu-Natal.

Figure 10. Deep yellow *Clivia gardenii* from Ngome Forest in Northern KwaZulu-Natal.

the type in every particular except colour, in which it differs widely from all the forms hitherto raised in gardens, and popularly known as Imantophyllums. These are all more or less of a reddish orange colour, but the variety citrina is coloured a clear pale cream with a faint tinge of orange at the base of the segments. This variety ought to prove valuable to breeders of Clivelas, whose efforts so far have produced exceptionally little colour variation in the seedlings raised."

Other references to yellow *C. miniata*, in the *Journal of the Royal Horticultural Society*, include:

1908, vol. XXXIII, Page liv: "Award of Merit – Clivia miniata citrina from the Hon. Mrs Evelyn Cecil, 10 Eaton Place, S.W. A pure yellow-flowered form of the well-known C. miniata, and was found growing wild near Eshowe, Zululand, by Lady Saunders."

1910, vol. XXXVI, Page cxxxiii: "Award of Merit – Clivia 'King George V' Mr Miller, Wisbeck. A honey-yellow flowered variety."

1911, vol. XXXVII, Page xxxiii: "Yellow clivia – Mr R Hooper-Pearson showed a flower of Clivia citrina apparently a form of C miniata which Mr Worsley said occurred wild in South Africa."

From the above, it is abundantly clear that, botanically speaking, the yellow-flowered *C. miniata*, which occurs sporadically amongst orange-flowered plants in habitat populations, can be accorded no more than the taxonomic status of form – *C. miniata* f. *citrina*.

Plants occurring in cultivation, which are most probably the offspring of individuals from a variety of origins, selected for specific features, get the horticultural name more correctly referred to as a cultivar, for example *Clivia* 'King George V' cited above.

Photo Anne le Roux

Figure 11. A pink form of *Clivia* × *nimbicola* from Bearded Man Mountain from Area A.

The yellow-flowered forms of *C. gardenii* and *C. robusta*, which apart from their colour are otherwise indistinguishable from the populations from which they have been collected, similarly only warrant this status – *C. gardenii* f. *citrina* and *C. robusta* f. *citrina*.

This digression is warranted, in that it is necessary to be very clear when giving a name to a plant, as a name has meaning beyond that of the immediate label – it tells the history of the plant and its origins. In order to determine the origins of *Clivia*, we must ensure that our groundwork is soundly based, and not the construct of unfortunate interpretations of imprecise and often limited information.

Distribution

Clivia belongs to the Order Asparagales, which contains both the Amaryllidaceae family and the Orchidaceae family, which date from the early Cretaceous, around 100 Ma (million years ago). By 65 Ma all families within the Asparagales were present. The genus *Crinum* is the oldest and originated in south-western Africa.

The genus *Clivia* is found associated with a type of vegetation known as southern African subtropical thicket, which has been a major component of southern African vegetation for at least 60 million years (Cowling *et al.*, 2005), when it was globally widespread (Schrire *et al.*, 2005). The leaves of *Clivia* are net-veined, which indicates a forest origin, and the age of the genus. The roots of orchids as well as clivias are similar, and are adapted for a forest environment in trees, on rocks and in leaf litter, and not in soil.

Thicket is a dense formation of evergreen and weakly deciduous shrubs and low trees (2–5m), often spiny and festooned with vines, which derived from elements in the forests that prevailed on the subcontinent prior to that. The arid spiny forests of southern and western Madagascar are also thicket, and in some areas experience a very low rainfall. They are very old, and contain a number of plant genera which are also present in the thickets of southern Africa, showing that thicket was an established vegetation type before Gondwana broke up.

The distribution of thicket has contracted and expanded repeatedly in response to Neogene and Quaternary climatic oscillations. From the end of the Palaeogene at around 25 Ma till about 17 Ma, the southern African climate was quite tropical, with a high land mass (altitude c 2000 m) ringed by the sandstones of the Cape Supergroup and the Lesotho Highlands. At around 20 Ma there was an uplift that was greatest in the eastern part of southern Africa where it approached 300 m. This event correlates with the retreat of thicket to the coastal margins

Figure 12. A Google Earth image of the area just below Bearded Man Mountain, in the Barberton Mountainland, showing the abrupt transition between the thicket vegetation and the grasslands. The thicket catches moisture from mists and clouds, and protects the understorey from frosts.

Photo: Roger Deane

Figure 13. While *Clivia* stayed in the protection of the fire free thickets, other amaryllids adapted to the grassland and savanna biomes where they were subject to fire by becoming geophytes. *Scadoxus puniceus* in the Pilanesberg Game Reserve.

and escarpment of southern Africa. After that until around 8 Ma the climate was more variable, fluctuating from temperate to cold (in the Namib), with the tropics going northwards. From 8 Ma till present we have had the development of Berg winds, and the return southwards of the tropics. A major factor that has affected the climate in southern Africa for the last 4 million years has been the large amount of uplift on the south-eastern side of the subcontinent (up to 900 m) (Partridge & Maud, 1987). The steeper slopes of the escarpment resulted in the creation of incised valleys, and a great diversification of habitat types. The creation of these new habitats as a result of these uplift and erosion events provided a great stimulus for the diversification of southern African plant lineages, as witnessed by the number of centres of endemism.

The ancestral amaryllids came from West Africa, with net-veined leaves developed for forest conditions, thick roots and fleshy seeds. *Clivia* evolved towards the south-east and *Cryptostephanus*, its closest relative in the Amaryllidaceae, in the east, when the central part of southern Africa became hot and dry, and the grasslands developed, around 20 My ago. Amaryllids adapted to arid savannas with the retreat of thickets, with many becoming geophytes to adapt to the dry conditions and fires, while *Clivia* stayed in the retreating thickets.

It can be seen from the present-day distribution of thicket, shown greatly simplified in Figure 2, that there is a strong correlation between it and the sandstones of the Cape Supergroup and the Natal Group, and that in the south-eastern part of the subcontinent the distribution of thicket is

strongly correlated to the deeply incised valleys which are a result of the dramatic uplift experienced in the last 4 million years. The southern distribution of thicket corresponds with the long parallel valleys in the Cape Fold Belt, which have been stable for a long period, in stark contrast to the incised valleys of the south east.

Clivia has been a member of thicket for a long time, and its distribution is mirrored by a number of other very old plant lineages, such as *Encephalartos*, *Strelitzia* and *Cussonia*. In many of these genera, there is an increase in diversity of species in the major diversity centres, or centres of

Photo: Andy Forbes-Hastings

Figure 14. *Clivia robusta* in habitat, growing in quartz sand in a shallow braided stream flowing through thicket near the coast. Many of these plants, which are growing semi-aquatically, have buttress roots and can reach 2 m in height.

Photo: Roger Dixon

Figure 15. *Strelitzia* and *Encephalartos altensteinii* in thicket in the Kap River valley near Port Alfred in the Eastern Cape.

Photo: Roger Dixon

Figure 16. *Clivia nobilis* growing in thicket in the Kap River valley near Port Alfred in the Eastern Cape.

endemism, from the Eastern Cape up into Limpopo (Cowling *et al.*, 2005; van Wyk & Smith, 2001), which corresponds to the areas of major uplift of the land mass in the south-eatern part of the sub-continent over the past few million years.

Clivia and climate

The *Clivia* anatomy shows a number of features which have developed to survive arid environments and which are more pronounced in the taxa from the south and south-west – *C. nobilis* and *C. mirabilis*. These features include leaves which are stiff and leathery and semi-succulent, and roots which are absorbent and water storing, adapted for aerated leaf litter, not soil. The leaves contain cutan, which is a biopolymer found in plants adapted for surviving drought conditions (Boom *et al.*, 2005). These features indicate that the plants are adapted for distinct wet and dry periods.

A distinguishing feature of thicket climates is bimodal rainfall, with peaks in late spring and early autumn, although copious rain may fall at any time of the year. These peaks are mirrored in the flowering times of the various species – *C. mirabilis*, *C. nobilis* and *C. caulescens* usually flower in October to November, while *C. gardenii* and *C. robusta* flower in April to June. *C. miniata* usually flowers before the first rains, in August to October, depending on locality.

The areas of highest endemism among plant taxa, which are also those areas where *C. robusta*, *C. gardenii* and *C. caulescens* are found (see Figure 2), are also the areas of highest rainfall and are associated with sandstones and quartz-rich rocks and soils.

Speciation of *Clivia*

Speciation can be defined as the splitting of one ancestral species into two (or more) daughter species. This splitting is caused by a change in existing conditions which promotes the independent evolution of two parts of a formerly contiguous population.

The change could be an event creating geographical isolation, such as the origin of a barrier to migration (the incised valleys in the south-eastern part of southern Africa, separated by grasslands, exemplified by the development of *C. gardenii* and *C. robusta* in centres of endemism (van Wyk, 1990; van Wyk & Smith, 2001) or fragmentation of the original range due to local extinction – separation of *C. mirabilis* and *C. nobilis* due to climate change and subsequent loss of habitat (Snijman, 2003).

It can also be triggered by a change leading to the onset of divergent selection, for example a shift to a new range that straddles an ecological gradient (the open-flowered mutation of *C. miniata* in response to pollinator availability at the margins between grassland and thicket (Manning, 2005). Other modes of speciation exist, for example hybridisation between two parent species – *C.* × *nimbicola*, found at the interface between two species *C. caulescens* and *C. miniata*, with a very limited distribution (Swanevelder *et al.*, 2006; Spies *et al.*, 2011; Truter *et al.*, 2006), however isolation and ecological divergence are required for such a new taxon to survive and persist.

It can be seen from the discussion above that there are a number of factors which determine why, when and how species may develop. These can be divided into intrinsic (biotic) and extrinsic (environmental) factors. Extrinsic factors include the numbers of

(Photo: Roger Dixon)

Figure 17. A view of the thicket rolling over the hills along the Kap River valley near Port Alfred in the Eastern Cape.

potential geographical isolating barriers, i.e. mountain ranges, rivers, etc., or the strength and number of ecological gradients.

Intrinsic factors might be the typical dispersal distance of the lineage, which would affect gene flow and how severely they are affected by extrinsic barriers, or generation time, which could affect speeds of evolutionary divergence between populations (Barraclough, 2006). As a consequence the speciation rate of a particular genus, in this case *Clivia*, will depend on the interaction between a number of intrinsic and extrinsic factors.

What the DNA reveals

There is a large body of literature relating to the genetics and DNA of *Clivia*, which has resulted in a number of conclusions being drawn, at different times, as to the relationships between the different taxa described in the genus. Being restricted to the thicket, the study of the genetic evolution of *Clivia* can act as an independent measure of how the geological and climatic changes have affected the development of the flora of southern Africa over the past 20 million years or more. Such population genetic data for thicket taxa has previously been lacking (Cowling *et al.*, 2005).

Studies by Ran *et al.*, (2001), Swanevelder (2003), Gagiano (2006) and Conrad (2008), and the papers in this volume – Conrad & Snijman (2011), Spies *et al.*, (2011) and van der Westhuizen *et al.* (2011), have shown that *C. mirabilis* and *C. nobilis* are distinct species, clearly separated by both geography and genetics, but that the other taxa are more difficult to define precisely. What is even more puzzling is that *C. miniata* with its open flowers is, depending upon what particular part of the genome

Figure 18. *Clivia* × *nimbicola* growing on rocks on a steep south-facing slope at Bearded Man.

Photo: Roger Dixon

Figure 19. An atypical form of *Clivia miniata* with highly reduced tepals. This tepal reduction can reach the extreme of no tepals at all, a feature found in other members of the Haemanthae such as *Scadoxus*. This feature in *Clivia* is thought to be a possible result of the reduction of genetic diversity within small iisolated populations.

you use, indistinguishable from its pendulous flowering counterparts which occur nearby. Spies *et al.*, (2011) consider *C. caulescens* to be also a distinct taxon, and its geographical separation from the *C. gardenii* – *C. robusta* distribution is on a par with that of *C. nobilis*.

Within *C. miniata* there are distinct geographic groupings of genetic markers. Swanevelder (2003) showed that different haplotypes for orange-flowered plants were located in different geographic areas, and some were only found in one area. Gagtano (2006) looked only at the yellow-flowered forms and showed that what had been called Group 1 and Group 2 yellows, on the basis of their breeding characteristics, were due to different mutations, and were also geographically constrained (see Figure 2).

DNA also shows that *C. mirabilis* is the oldest of the *Clivia* taxa, and diverged at about 16 – 17 Ma (Conrad & Snijman, 2011), which correlates with the retreat of thicket to the coastal margins and escarpment of southern Africa, as described above, and the beginning of the aridification of central southern Africa. The relative ages of the various taxa can also be estimated from the amount of nuclear material in the cell, and a study by Zonneveld (2005) showed that this correlated directly with the distribution of the pendulous species from *C. mirabilis* having the least nuclear material, to *C. caulescens* having the most. The position of *C. miniata* was nearest to *C. caules-*

cens, however this is contradicted by other studies which showed it to be closest to *C. gardenii*. The position of *C. robusta* is discussed by Hammett (2005), who likens the speciation in *Clivia* to the formation and break up of clouds. The latest DNA studies clarify this and it becomes obvious that the genetic variation in *C. miniata* is as broad as that of the pendulous species whose distribution it mirrors.

Discussion

C. mirabilis and *C. nobilis* would appear to have been surviving relatively unchanged over the past 10 or more million years, sheltered by thicket, which has been reduced in distribution over time by a changing climate driven by, amongst others, uplift of the continental land mass and aridification of the central area of southern Africa.

The uplift of the escarpment and a changing, dissected landscape in the south-eastern part of the sub-continent in the last 4 million years resulted in a myriad of individual habitats and isolated populations, resulting in the need for the original *Clivia* populations to move and adapt to new or changing habitats, as the escarpment rose. This pressure was greatest in the Eastern Cape and KwaZulu-Natal, and resulted in the development of new taxa with thinner leaves and in some areas thinner roots, as the rainfall increased and the plants were forced to adapt to different soil types. *C. caulescens*, the northernmost taxon, is most probably the youngest as well.

Photo Roger Dixon

Figure 20. Another atypical form of *Clivia miniata* with highly modified tepals. This tepal modification (phyllody) can reach the extreme where flowers consist of solid green leaf-like tepals, with non-viable pollen. This feature in *Clivia* is another possible result of the reduction of genetic diversity within small isolated populations.

Photo: Roger Deane

Figure 21. Thicket at the top of a rock outcrop in the Kap River valley near Port Alfred in the Eastern Cape. Typical plant genera include *Strelitzia*, *Euphorbia* and *Encephalartos*.

having spread northwards from its traditional habitat on sandstones onto other rock types with similar properties.

The open-flowered mutation, which has been described as one taxon, *C. miniata*, developed independently at least two areas and most probably in more, corresponding to its range, as the climate changed and more different pollinators were available. It is now necessary to look more closely at the genetics of the open-flowered form of *Clivia* to see whether it merits the status of a single species, *C. miniata*, or whether it is in fact necessary to do the same to it as has been done to the pendulous forms.

It can be seen that as the populations of the open-flowered form became more common, and more of the form spread, it hybridised back to its pendulous correlate. This introgression can clearly be observed in the habit of the pendulous species going northwards, and is demonstrated when hybridising pendulous forms across the range with a single open form – the resulting plants show a range of habits which vary from pendulous umbels in the south to round umbels in the north.

As *Clivia* moved northwards its variation and gradation increased, due to adaptive change & habitat islands. Each incised valley which was suitable for *Clivia* had a reduced and restricted gene pool – a reproductive island, and gene flow between these populations was limited due to the size of the seed and its dispersal by birds. In-breeding has resulted in the great variety of shapes and sizes we see today. The reduction in diversity in local populations has resulted in the distinct differences we observe when hybridising plants of different phenotype and geographic origin.

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PHOTOGRAPHIC

OVERALL WINNER

&

Best Photograph: **Trumpet Flower** Category

C. miniata 'Errol of Ankh-Morpork'
Photographer & grower: Ken Rosling
Breeder: Val Thurston, F1 plant bred from Val Thurston's
C. miniata 'Kaleidoscope'

COMPETITION

Best Photograph: **Tubular Flower** Category

Interspecific *Clivia* 'White Pico'
Photographer, grower & breeder: Shigetaka Sasaki

PHOTOGRAPHIC

Best Photograph: **Single Flower** Category

C. miniata 'Green & Gold'
Photographer: Gordon Fraser
Grower: Kobus Kearny
Breeder: Hirao

COMPETITION

Best Photograph: **Novelty** Category

Red-flushed foliar tepals
Photographer: Joubert van Wyk

PHOTOGRAPHIC

Best Photograph: **Art** Category

Photographer: Joubert van Wyk

COMPETITION

Special Mention

C. miniata 'Carmen Miranda'
Photographer & grower: Gordon Fraser
Breeder: John Handman

PHOTOGRAPHIC

Special Mention

Clivia 'Dreaming'
Photographer, grower & breeder: Carrie Kruger

Clivia 'Two-to-Tango'
Photographer, grower and breeder: Carrie Kruger

COMPETITION

Special Mention

C. miniata var. *citrina*
Photographer & grower: John Hunter

PHOTOGRAPHIC

Special Mention

Clivia 'Dainty Ballerina'
Photographer & grower: Ken Rosling
Breeder: Rudo Lotter

COMPETITION

Special Mention

C. miniata 'Joubert's Vesuvius'

Photographer: Joubert van Wyk

PHOTOGRAPHIC

Special Mention

C. miniata 'Harvest Moon'
Photographer & grower: Ken Rosling
Breeder: Gerhard Malan [*C. miniata* 'Vico' giant yellow
(Cape Show Champion 2001) × *C. miniata* Best 'Vico' yellow]

COMPETITION

Special Mention

C. miniata 'Tiffany Aching of Nkutu'
Photographer, grower & breeder: Ken Rosling
[Pastel *C. miniata* 'Helen's Dawn' × Pat Benett *C. miniata* yellow]

PHOTOGRAPHIC

Special Mention

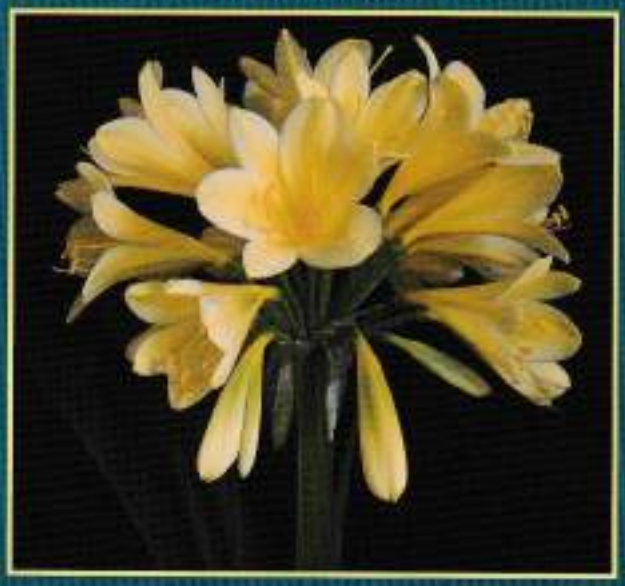
C. miniata 'Voné's Cheer'
Photographer & grower: Ken Rosling

C. miniata 'Flamenco Flame'
Photographer & grower: Ken Rosling

COMPETITION

Special Mention

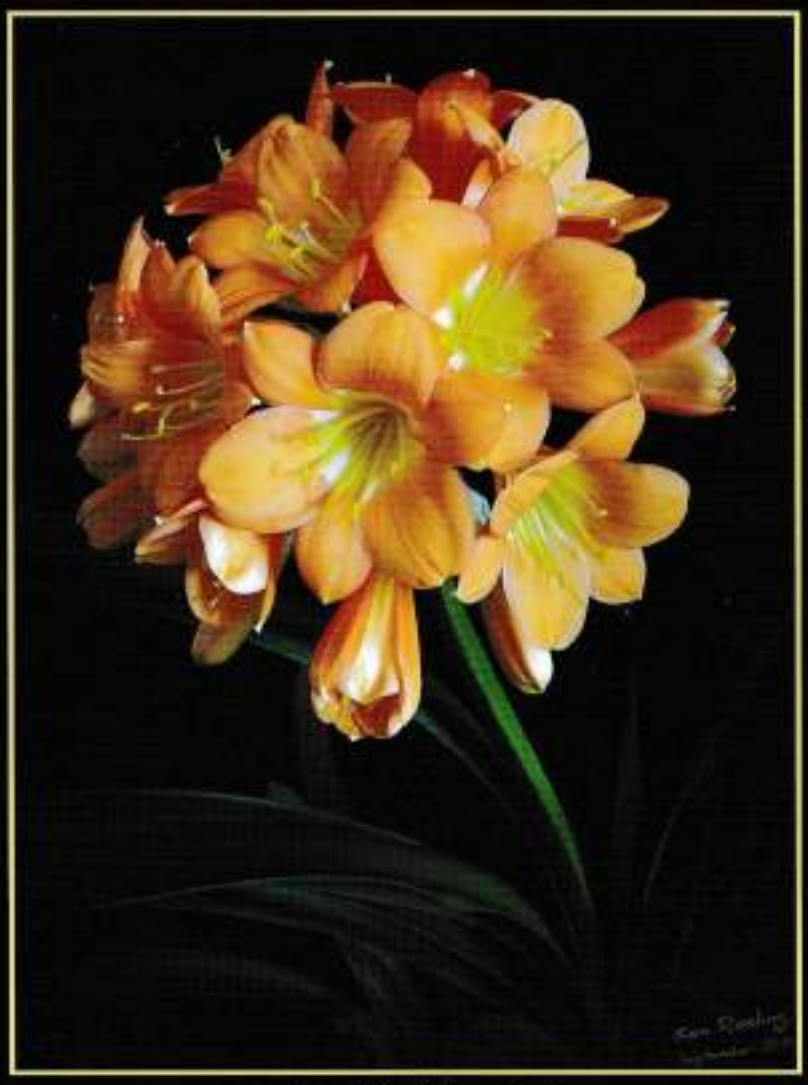
C. minutcia 'Orange Marmalade'
Photographer & grower: Ken Rosling
Breeder: Dr Bing Wiese



C. minnata 'Gail's Peach'
Photographer & grower: Ken Rosling

PHOTOGRAPHIC COMPETITION

Special Mention



C. miniata 'Nkutu's "Nanny Ogg"'
Photographer, grower & breeder: Ken Rosling

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