GENETIC VARIATION IN

CLIVIA MINIATA VAR. CITRINA

By

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DECLARATION

I declare that the thesis hereby submitted for the Magister Scientiae degree at the University of the Free State is my own work and has not been previously submitted by me at another University for any degree. I cede copyright of the thesis in favour of the University of the Free State.

Anthia Gagiano

June 2006

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Instead of shame and dishonor I have given you a double portion
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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ATP	Adenosine 5'-triphosphate
bp	Base pair(s)
^{o}C	Degree Celsius
cpDNA	Chloroplast DNA
CTAB	Hexadecyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DTT	Dithriothreitol
EC	Eastern Cape
EDTA	Ethylenediaminetetraacetate
FvN	Fred van Niekerk
g	Relative centrifugal force
GS	Genetic similarity
IGS	Intergenic Spacer
ITS	Internal transcribed spacer
kb	Kilobase(s)
KZN	KwaZulu-Natal
M	Molar
MAS	Marker-assisted selection
MD	Mick Dower
mg	Milligram(s)
ml	Millilitre(s)

mm	Millimetre(s)
mM	Millimolar
mtDNA	Mitochondrial DNA
µg	Microgram(s)
µl	Microlitre(s)
µM	Micromolar
ng	Nanogram(s)
NTSYS	Numerical taxonomy and multivariate analysis system
PAGE	Polyacrylamide gel electrophoresis
PAUP	Phylogenetic analysis using parsimony
PCR	Polymerase chain reaction
PG	Pat Gore
pmol	Picomole(s)
r	Correlation coefficient
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SEC	South Eastern Cape
SNP	Single nucleotide polymorphism
sp.	Species
SSR	Simple sequence repeat
Taq	*Thermus aquaticus*
TBE	Tris. HCl / Boric acid / EDTA
TBR	Tree bisection and reconnection

Tris	Tris(hydroxymethyl)aminomethane
TE	Tris.HCl / EDTA
U	Unit(s)
UPGMA	Unweighted pairgroup method using arithmetic averages
UV	Ultraviolet
var.	Variety
v/v	Volume/volume
W	Watt
w/v	Weight/volume

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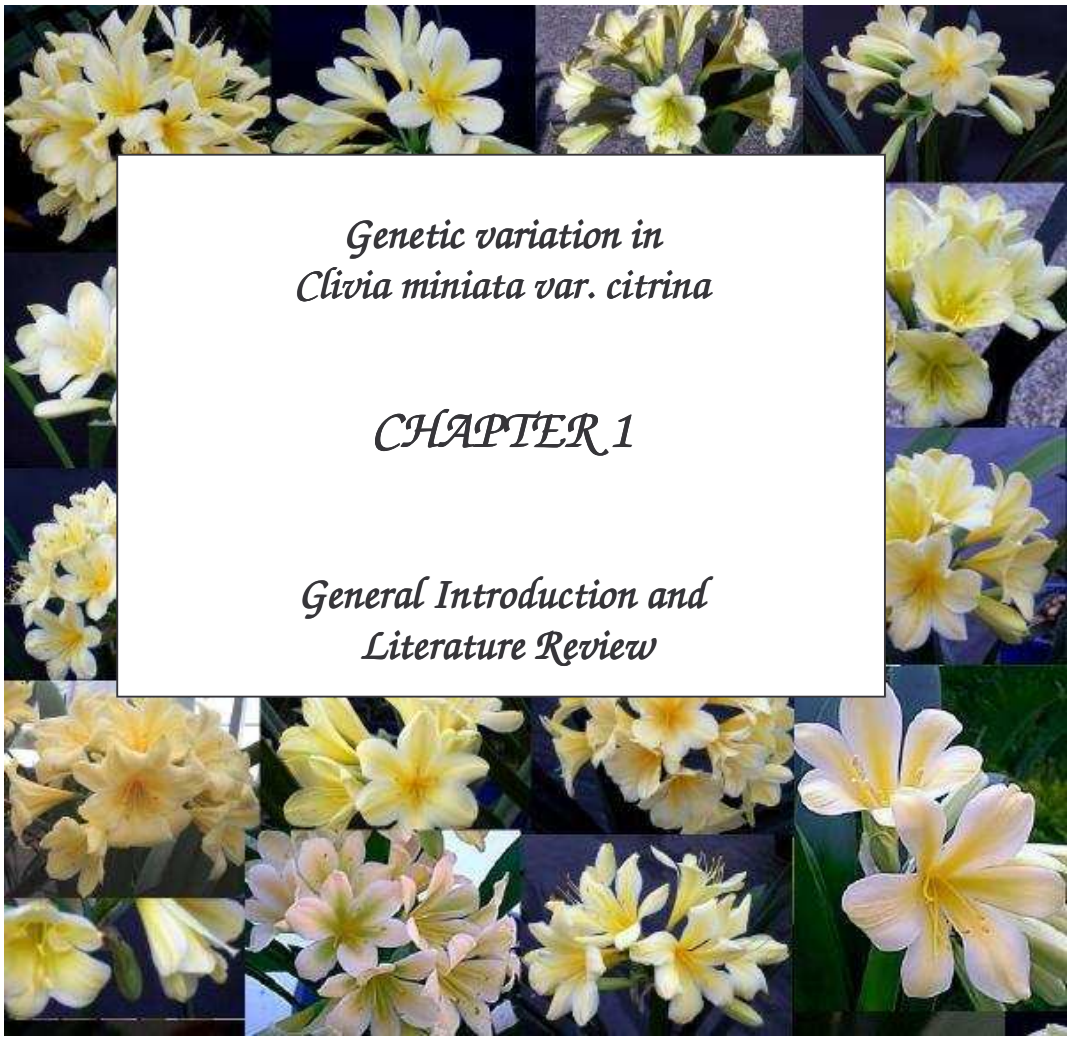


Genetic variation in
Clivia miniata var. citrina

CHAPTER 1

General Introduction and Literature Review

1.1 Introduction

A population is a group of individuals of the same species sharing certain traits and occupying a given area (Starr & Taggart, 1995). Yet, details of a trait vary from individual to individual. Inherited characteristics of an individual are a reflection of the structure and organisation of its genes (Dale & von Schantz, 2002). Within populations there are several alleles for most genes resulting in an almost unlimited cache of genetic variation (Winter *et al*., 2002). It is important to realise that this includes an extremely complex set of interactions between different genes and their products, as well as environmental factors. The gene(s) directly responsible for the observed characteristic may be identical but effects may be different because of variation in other genes that affect their expression. Alteration in other cellular components that affect the activity of proteins encoded by those genes may be influenced simultaneously. Mutation forms the basis of all genetic variation (Winter *et al*., 2002). Environmental influences that cause mutations will have a major role to play in determining the observed characteristics of organisms. Ascribing every change to a single gene is an over simplification as many traits are much more complex (Starr & Taggart, 1995; Winter *et al*., 2002). The study of genetic variation can be used to examine differences between species and different individuals within a species (Mohan *et al*., 1997; Dale & von Schantz, 2002; Francia *et al*., 2005).

When considering the horticulture industry many of the currently important bulb species e.g. tulips, daffodils etc., have been highly developed by decades of selection and breeding, resulting in big differences from the original wild form or forms from which they were derived. Other bulb species however, are very similar to their wild ancestors which still grow in their original habitats. Information on the development

of modern cultivars is somewhat uneven in quantity and quality; some genera and species have been studied in detail, others lack comprehensive investigation (Rees, 1992).

Clivia miniata also known as 'Boslelie' (Afrikaans), 'Bush lily', 'Orange lily' and 'Umayime' (Zulu), has recently received considerable horticultural attention (Swanevelder, 2003). The genus *Clivia* belongs to the family Amaryllidaceae. Used as a medicinal plant by traditional healers long before its colonial discovery, the Bush lily has waxed and waned in the view of European horticulturists during the previous century (Duncan, 1985).

In 1992 the establishment of the Clivia Society in South Africa heralded in a new age of interest in these extraordinary plants. Traits of interest for the South African market include flower form, flower colour, leaf width, leaf variegation and interspecific hybrids. In Europe commercial interests in *Clivia* have been renewed in recent years, especially in Belgium, Denmark, Finland, France, Germany, Italy, Netherlands, Portugal, Spain, Sweden and the United Kingdom. Asia has had a fascination with *Clivia* from the time when Japan invaded China after the Opium war. Selection of plants in Japan is based mainly on foliage features as flowers are considered a bonus (Swanevelder, 2003).

1.2 The Family Amaryllidaceae

There are 61 genera in the family Amaryllidaceae (Meerow *et al*., 2000). Some of the most important ornamental genera found in southern Africa include *Brunsvigia* Heist., *Crinum* L., *Cyrtanthus* Aiton., *Nerine* Herb. and *Clivia* Lindl. (Germishuizen &

Meyer, 2000). The family is mostly concentrated in southern Africa and the Mediterranean (Duncan & Du Plessis, 1989). The genus *Clivia* is a member of the Amaryllidaceae, from the African tribe Haemantheae that includes the baccate-fruited genera *Scadoxus* Raf., *Haemanthus* L., *Clivia*, *Cryptostephanus* Welw., *Gethyllis* L., *Apodolirion* Baker and *Cyrtanthus* (Meerow, 1995; Germishuizen & Meyer, 2000). Lack of a true bulb occurs in three genera of this tribe namely *Clivia*, *Cryptostephanus* and *Scadoxus* (Meerow, 1995).

1.2.1 The Genus *Clivia* Lindl.

The genus *Clivia* is endemic to southern Africa and includes six species, *Clivia nobilis* Lindl., *C. miniata* (Lindl.) Regel, *C. gardenii* Hook., *C. caulescens* R.A. Dyer, *C. mirabilis* Rourke and *C. robusta* Murray, Ran, De Lange, Hemmett, Truter & Swanevelder (Murray *et al*., 2004). *Clivia nobilis* (Figure 1.1a) was first discovered in 1815 near the mouth of the Great Fish River in the Eastern Cape (Duncan, 1999).

Discovery of the spectacular *C. miniata* in KwaZulu-Natal (Figure 1.1b) followed in the early 1850s (Duncan, 1985). In 1856, *C. gardenii* (Figure 1.1c) was collected in Natal. *Clivia caulescens* (Figure 1.1d) was the first *Clivia* to be described scientifically in South Africa in 1943. *Clivia mirabilis* (Figure 1.1e) was found in the Oorlogskloof Nature Reserve, in South Africa in February 2001 by a game guard, Mr. J. Afrika. Based on studies by Ran *et al*. (1999, 2001a, b) and Swanevelder (2003) *C. robusta* achieved species status in 2004 (Figure 1.1f). This species was initially classified as *C. gardenii* with differences in morphology attributed to natural variation (Swanevelder, 2003; Murray *et al*., 2004).

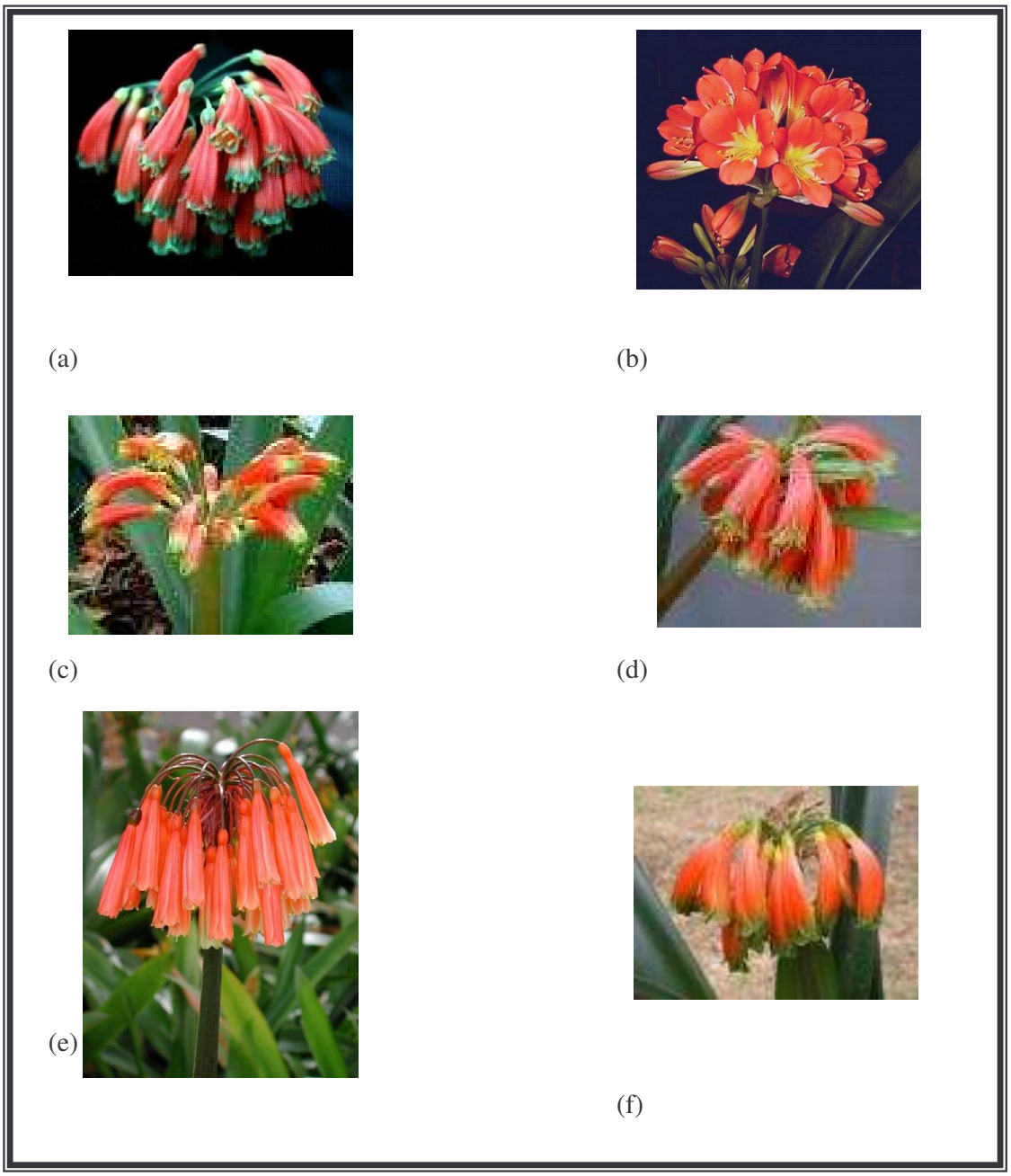


Figure 1.1 **Photographs of different *Clivia* species: (a) *C. nobilis*, (b) *C. miniata*, (c) *C. gardenii*, (d) *C. caulescens*, (e) *C. mirabilis* and (f) *C. robusta***

Clivia robusta is known in horticulture as the 'robust form' of *C. gardenii* or 'Swamp Forest *Clivia*' or 'Robust gardenii' (Ran *et al.*, 2001a, b). Key diagnostic characters for the identification of *Clivia* species (Swanevelder, 2003) are presented in Table 1.1.

Clivia plants thrive in semi-shade, preferring well-drained, shaded habitats that are located in summer rainfall areas (Swanevelder, 2003). Of the known six species, only *C. mirabilis* has a localised distribution in semi-arid areas with a Mediterranean climate and accompanying winter rainfall. *Clivia* is ideally suited to permanent positions under deciduous or evergreen trees, shady garden corners or in large container pots on a porch. The evergreen foliage, flowers and even decorative berries all attribute to the attractiveness of this ornamental species (Duncan & Du Plessis, 1989).

Clivia is an evergreen genus with a rhizomatous rootstock (Duncan & Du Plessis, 1989; Duncan, 1999). The rhizome is a modified stem that grows horizontally at or just below the soil surface (Koopowitz, 2002). The terminal bud on the rhizomes grows in a horizontal direction and lateral buds can develop into rhizomes behind the terminal bud. The adventitious roots close to the terminal bud grow actively but become less vital the further away they are from it. Typically, both roots and foliage arise at right angles to the rhizome which has a uniform unjoined appearance. Like a corm, the storage tissue is stem-like in the rhizome (Duncan & Du Plessis, 1989).

Table 1.1 Key diagnostic characters for the identification of *Clivia* species (Swanevelder, 2003)

Character	*Clivia nobilis*	*Clivia miniata*	*Clivia gardenii*	*Clivia caulescens*	*Clivia mirabilis*	*Clivia robusta*
Flowering time	August – January (Spring – Summer)	August – November (Spring – early Summer)	May – July (late Autumn – mid Winter)	September – November (Spring)	October – mid-November (late Spring)	Late March – Early August (Autumn – Winter)
Flower number	20 – 50	10 – 40	10 – 20	14 – 50	20 – 48	15 – 40
Umbel form	Dense, compact, round umbel	Forming big, round umbels, almost globose	Usually loose, flattened to one side, slightly rounded on other side	Usually tight and flattened on one side	Forming a tight umbel (as seen from photographs)	Variable, usually loose, slightly globose
Distance stigma protrudes from tip of perianth tube	< 6 mm	Variable	Prominent, > 7 mm	< 7 mm	Slight (as seen from photographs)	Variable, pushed out beyond anthers
Degree anthers protrude from tip	Variable	Variable	Always	Slight	Slight	Slight – prominent
Flower length (Perianth and ovary length)	24 – 40 mm	Variable, depending on flower shape	40 – 52 mm	30 – 35 mm	35 – 50 mm	30 – 55 mm
Pedicel orientation	Slightly curved along length / drooping	Stiff and erect		Stiff, erect, drooping near flower	Drooping	Stiff, erect / sub - erect

Table 1.1 (continued)

Character	*Clivia nobilis*	*Clivia miniata*	*Clivia gardenii*	*Clivia caulescens*	*Clivia mirabilis*	*Clivia robusta*
Pedicel colour	Usually green	Green	Usually tinged red or orange	Usually green	Red / orange during flowering, green when fruiting	Variable
Pedicel length	20 – 40 mm	30 – 70 mm	20 – 40 mm	15 – 35 mm	25 – 40 mm	15 – 60 mm
Flower orientation	Drooping	Erect	Drooping on stiff pedicels	Drooping	Drooping	Drooping on stiff pedicels
Flower perianth shape	Tubular and linear with straight inner petals	Open, funnel – shaped with spreading flower segments	Tubular and curved (falcate) downward; inner petals re-curved	Tubular and curved; inner petals re-curved	Tubular, linear to curved, tubular with increasing flaring at the apex	Tubular, somewhat falcate with an increasingly flaring apex
Leaf sheath colour	Purplish	Green – light red	Green – light red	Green – light red	Prominent, flushed deep carmine maroon	Green – light red
Leaf orientation	Stiff, sub – erect	Arching	Recurved	Arching	Stiff, erect	Arching – erect
Leaf length x width (mm)	300 – 700 or 1000 x 25 – 45	400 – 500 or 900 x 25– 65 or 50 - 70	300 – 400 or 900 x 35 – 50 or 70	350 – 450 or 900 x 25 – 50 or 60	400 – 500 or 900 x 25– 65 or 50 - 70	300 – 800 or 1200 x 30 – 70 or 90
Leaf margin	Rarely serrated	Cartilaginous, minutely toothed	Usually entire	Entire, cartilaginous, usually smooth	Serrated	Cartilagenous and dentate
Leaf apex	Obtuse – acute	Obtuse – acute	Acute	Obtuse – acute	Retuse and oblique	Abruptly rounded / retuse

Table 1.1 (continued)

Character	*Clivia nobilis*	*Clivia miniata*	*Clivia gardenii*	*Clivia caulescens*	*Clivia mirabilis*	*Clivia robusta*
Leaf special characteristics	White stripe absent or present	-	-	-	Prominent white stripe in centre of leaf	White stripe absent or present
Aerial Stem	Absent	Rarely present; very old specimens	Rarely present; very old specimens	Usually present when mature; up to 3m long	Not yet reported	Usually present for swamp forms
Seed number	1 or 2 or 1 – 6	1 – 4 or 1 - 25	Usually 1 or 2	1 – 4	1– 4 or 2 – 7	1 or 2 or 1 – 4
Seed maturation time (months)	± 9 – 12	± 9 – 12	± 9 - 12	± 9	± 4 – 6	± 9 - 12
Seed size (diameter in mm)	Small, ± 9 mm	Medium, ± 12 mm Transkei and Eastern Cape forms larger	Large, ± 18 mm	Medium, ± 12 mm	Small, ± 10 mm	Large, 10 – 18 mm
Endocarp colour	Colourless	Colourless	Colourless	Colourless	Red – pigmented	
Distribution	Eastern Cape Province	Eastern Cape Province (Transkei) KwaZulu – Natal Province, Swaziland, Mpumalanga Province	KwaZulu – Natal Province	Limpopo Province (Soutpansberg) Mpumalanga Province and Swaziland	Northern Cape Province	Southern KwaZulu – Natal Province, Eastern Cape Provence (Pondoland Centre of Endemism)

7. The strap-shaped foliage is borne in an erect or spreading position. New leaves are produced annually from the centre of the growing shoot, with the outer older leaves dying off each year (Duncan & Du Plessis, 1989). These luscious leaves of *Clivia* are covered with a waxy cuticle on the dorsal side, with stomata present ventrally only (Koopowitz, 2002).

The inflorescence is a dense or sparse umbel of tubular, pendulous or open-faced semi-erect flowers in shades of yellow, red or orange. The fruit is a red or yellow berry containing few large, hard seeds (Duncan & Du Plessis, 1989; Duncan, 1999; Koopowitz, 2002).

1.2.1.1 Flower colour in *Clivia*

Colour mutations deviating from orange, although rare, occur naturally in *Clivia* populations (Koopowitz, 2002; Chubb, 2005). Mutations have been found in wild populations as well as in 'chance' seedlings in commercially grown *Clivia* populations. These mutations incorporate a large variety of colours including Apricot, Blush (a pinkish colour), Peach, Yellow and colour related features such as Bicolour and Picotees (Chubb, 2005; Van Niekerk, 2005).

Yellow flowered *Clivia* result from mutations that occur in the biochemical pathways responsible for the manufacturing of anthocyanins. Many genes underlie the synthesis of anthocyanins therefore one of any number of different mutations could eventually result in flowers having yellow colouring (Koopowitz, 2002) (the exact pathways and biochemical assessment of these changes lie beyond the scope of this study).

1.2.1.2 Classification of *C. miniata* var. *citrina*

Classification of *C. miniata* in its yellow form has an interesting history. From an extract in a book "Flower Paintings of Katherine Saunders" in 1893, *Imantophyllum* was the genus name initially given to *Clivia* (Koopowitz, 2002; Swanevelder, 2003; Van Niekerk, 2005). The flower was described as 'Yellow *Imantophyllum* from Eshowe, flower withering after being two days in post bag. Most lovely, delicate, peculiar shade of yellow, not orange but like straw-colour mixed with pink, quite inimitable by me…'. The first recorded history of a yellow flowered *Clivia* obtained from a natural population in Eshowe, KwaZulu-Natal was described as *Clivia miniata* var. *citrina*. Later the genus name *Imantophyllum* was replaced by the genus name *Clivia* as this was the oldest recorded genus name (Koopowitz, 2002; Swanevelder, 2003; Van Niekerk, 2005). *Clivia miniata* var. *citrina* is the name that appears in modern literature. Koopowitz (2002) regards a more appropriate designation to be *C. miniata* 'Citrina'. Modern yellow plants have been referred to as *C. miniata* var. *citrina*, *C. miniata* var. *aurea* and *C. kewensis* var. 'Bodnant' (Koopowitz, 2002; Swanevelder, 2005). However, *C. miniata* var. *citrina* is commonly used to refer to the yellow flowered *Clivia*.

1.2.1.3 The relevance of the term 'variety' to *Clivia miniata* var. *citrina*

A species embraces the phenotypic variation within its populations (Starr & Taggart, 1995). The inherent variation at infraspecies categories are dealt with by assigning the terms 'subspecies' or 'varieties'. These terms are applied to populations of species in various stages of differentiation (Jones & Luchsinger, 1987). In taxonomy today, the yellow flowered form of *Clivia miniata* is known as *C. miniata* var. *citrina*.

Gradual divergence from a homogenous species or population into more than one population is seen to be the result of evolution and / or speciation (Winter *et al*., 2002). Divergence is usually related to adaptation to differing geographical areas or climates or to differing ecological habitats (Starr & Taggart, 1995). In the process of becoming adapted, populations may become genetically distinct (Winter *et al*., 2002). Such ecotypes occupy adjacent ranges where they may interbreed and integrate at the point of contact, forming one population (Jones & Luchsinger, 1987). Discontinuities in the variation patterns between divergent populations may occur. Ecotypes often form the basis of varieties or subspecies (Winter *et al*., 2002).

Variety was the first infraspecific category used in plants (Jones & Luchsinger, 1987). Linnaeus viewed this term as primarily an environmentally induced morphological variation (later known as variation in phenotype). In taxonomy varieties and subspecies are recognisable morphological phenotypic variations within species. Their populations have own patterns of phenotypic variation correlated with geographical distributions or ecological requirements (Starr & Taggart, 1995).

Although there exists rumours of populations of *C. miniata* that have only yellow flowers, no strong evidence exists that justifies a separate taxa (Koopowitz, 2002). Yellow mutants are rare in the wild, however, several distinct clones have been discovered and described. *Clivia nobilis* and *C. gardenii* have also been reported to occur in yellow forms in the wild and are presently under cultivation (Koopowitz, 2002; Swanevelder, 2003).

Many of the yellow clones of *C. miniata*, obtained from naturally occurring populations or cultivated by enthusiasts from clones from natural populations or cultivated plants, have been passed on from person to person and breeder to breeder. Along the way these plants have acquired different names, their true origin and history somewhat less than presenting a clear picture. For the purposes of this study, we will refer to different yellow plants of *C. miniata* as *C. miniata* var. *citrina*. Some names of modern forms of *C. miniata* var. *citrina* are presented with their synonyms (if synonyms exist) in Table 1.2.

1.2.1.4 Yellow strains, clones and cultivars of *C. miniata*

Demand for yellow forms of *C. miniata* exceeded supply at one stage. Nurserymen in different parts of the world realised the market potential and succeeded in producing seed strains that guaranteed yellow flowers (Koopowitz, 2002). Therefore, a strain refers to plants that were produced from seed that produce similar phenotypes. Names of strains developed are presented in Table 1.2.

Clone refers to offshoots of plants (originally collected from habitat) that are believed to be genetically identical to the parent plant that produced the offshoot. Individual, specially selected clones have high value. This resulted in individual clonal and cultivar names (Koopowitz, 2002). The name 'cultivar' can be applied to an assemblage of cultivated plants that is clearly distinguished by any characters and that following reproduction (sexual or asexual), retains its distinguishing characters. Cultivars are written with a capital initial letter (Jones & Luchsinger, 1987).

Table 1.2 Names of modern *Clivia miniata* var. *citrina* plants with synonyms of clones, 'Group' designation, cultivar and strains (if known) (Koopowitz, 2002; Van Niekerk, 2005)

Name	Clone/Strain/Cultivar	Synonym
Centani Yellow [Group 2]	Clone	Similar to Natal Yellow
Dwesa Yellow [Group 2]	Clone	Bashee Yellow, Transkei Yellow, Smith's Yellow, Tsolo Yellow, Floradale Yellow
Eshowe Yellow [Group 1]	Clone	Saunders Yellow
Mare's Yellow [Group 1]	Clone	Howick Yellow
Natal Yellow [Group 2]	Clone	Giddy Yellow, Fred Gibello Yellow, Jardine Yellow, Swellendam Yellow, Holl Yellow, Stella Parish Yellow
Port St John Yellow [Group 2]	Clone	Similar to Dwesa Yellow
Vico Yellow	Cultivar	Smither's Yellow
Aurea	Cultivar	-
Blinkwater Yellow [Group 1]	Clone	-
Byrne Valley Yellow	Clone	-
Cape Butterfly	Cultivar	-
Cape Snowflake	Cultivar	-
Celtis Kloof [Group 3]	Cultivar	-
Citrina	Cultivar	-

Table 1.2 (continued)

Name	Clone/Strain/Cultivar	Synonym
Col Pitman	Cultivar	-
Crookes Yellow	Clone	-
Cynthia's Best	Cultivar	-
Gold Star	Clone	-
Green Bird	Cultivar	-
Green Grace	Cultivar	-
Green Scene	Cultivar	-
King Hamelin Yellow [Group 1]	Clone	-
Kirstenbosch Yellow	Clone / Cultivar	-
Leiden	Cultivar	-
Lemon Chiffon	Cultivar	-
Lemon Cloud	Cultivar	-
Lessa	Cultivar	-
Megan	Cultivar	-
Mpumulo Yellow [Group 1]	Clone	-
Mvuma Yellow	Clone	-
New Dawn	Strain	
Ndwedwe [Alpha]	Clone	-
Oribi Yellow	Clone	-
Qora Yellow	Clone	-
San Diego Yellow	Cultivar	-
Sir John Thouron	Cutivar	-
Solomone Yellow	Strain	-

Different names for plants from common origin or different names given to clones of cultivated plants have given rise to much confusion, especially when directed breeding using these yellow forms of *C. miniata* have been attempted. Different mutations are responsible for producing yellow *C. miniata*, referred to as 'Group 1', 'Group 2' Yellows etc. (Van Niekerk, 2005). This has been one of the ways employed by *Clivia* breeders in attempts to understand yellow flower colour heritability.

Self-pollination in *Clivia* generally leads to self-incompatibility over time. Therefore, no homozygotic lines exist in *Clivia*. Group 1 Yellows make out the majority of yellow flowered forms of *C. miniata* in breeder's collections. Plants in this group are usually self-compatible and produce seed that is true breeding for yellow flower colour. Berries containing seeds are usually yellow or green. Group 2 Yellows are mostly self-sterile, being self-incompatible. When a Group 1 Yellow is crossed with another Group 1 Yellow, true breeding yellow offspring are produced. When a Group 2 Yellow is crossed with another Group 2 Yellow, yellow offspring are produced. The pattern changes when a Group 1 Yellow is crossed with a Group 2 Yellow. Orange offspring are produced. Groups 3 and Alpha are not widely in known. Group 3 consists of a yellow *Clivia* named Celtis Kloof, from Celtis Kloof in KwaZulu-Natal. The Alpha group contains some Ndwedwe Clivias. No pedigree information is available at present.

Unfortunately, classification based on the 'group'-system requires the presence of flowers and some knowledge regarding pedigree data (although Group 2 Yellows can be identified by pinpricking the flower: If a plant is a Group 2 Yellow, an orange border will form around the punctured area) (M. Dower, personal communication).

When breeding is attempted, it is important to identify similar plants and dissimilar plants to incorporate as broad a genetic base as possible.

1.2.2 Interspecific hybrids

Clivia nobilis and *C. miniata* received much horticultural attention since their introduction to Britain during the early and mid 1800s (Swanevelder, 2003). Not long after *C. miniata* had been described as a new species it was crossed to *C. nobilis* (Koopowitz, 2002). The first hybrid established between these two species, *C. miniata* x *C. nobilis*, became known as *C. cyrtanthiflora* (Koopowitz, 2002; Swanevelder, 2003).

Clivia miniata appears to cross easily with other *Clivia* species, notably *C. nobilis*, *C. gardenii* and *C. caulescens*. Natural hybrids between *C. miniata* and other *Clivia* species are known where *C. miniata* occurs together with other *Clivia* species, notably *C. nobilis* and *C. caulescens* (Winter, 2000). All *Clivia* species can cross and produce vigorous, fertile progeny, suggesting a close relationship (Ran *et al*., 2001a, b).In contrast to Winter (2000), Swanevelder (2003) suggested that due to geographical distances between these individuals, such hybrids would probably not occur in nature, however they are reported to occur in natural populations (F. van Niekerk, personal communication). The extent to which interspecific hybridisations have been attempted on numerous individuals from different localities are normally not indicated and reports are anecdotal mostly (Duncan, 1999; Ran *et al*., 2001a, b; Swanevelder, 2003). Hybrids may be produced between very fertile individuals of different species and can be identified using molecular techniques (Ran *et al*., 2001a, b).

1.3 Molecular studies

Molecular phylogeny is the study of evolutionary relationships among organisms or genes by a combination of molecular biology and statistical techniques, known as molecular systematics if the relationships of organisms are concerned (Li & Graur, 1991; Li, 1997). It is one of the areas of molecular evolution that have generated much interest in the last decade mainly due to the difficulty to assess phylogenetic relationship in any other way. The study of phylogeny began at the turn of the century even before Mendel's laws were rediscovered in 1900. Since the 1950s various techniques have been developed in molecular biology and this started off the extensive use of molecular data in phylogenetic studies. Particularly in the 1960s and 1970s, the study of molecular phylogeny using protein sequence data progressed tremendously. The rapid accumulation of DNA sequence data since the late 1970s has already had a great impact on molecular phylogeny. Since the rate of sequence evolution varies extensively with gene or DNA segments, one can study the evolutionary relationships at virtually all levels of classification of organisms (Nei & Kumar, 2000).

Since the 1980s there has been a blossoming of molecular biological approaches to the study of angiosperm phylogeny (Olmstead & Palmer, 1994). A diverse array of molecular approaches is now available to the plant systematist for use in phylogenetic inference, including restriction site analysis, comparative sequencing, analysis of DNA rearrangements (e.g. inversions), gene and intron loss and various PCR (Polymerase Chain Reaction) based techniques (Soltis & Soltis, 1998).

There are several reasons why molecular data, particularly DNA sequence data, are more powerful for evolutionary studies than morphological and physiological data. Firstly, DNA and protein sequences can provide a clearer picture of relationships between organisms independent of morphological and physiological characters. Secondly, sophisticated mathematical and statistical theories have already been developed for analysing DNA sequence data. Thirdly, molecular data are more abundant. Of course, we should not abandon traditional means of evolutionary enquiry such as morphology, anatomy, physiology and palaeontology. Rather, different approaches provide complementary data. Morphological and anatomical data are necessary for constructing a time frame for evolutionary studies (Olmstead & Palmer, 1994; Li, 1997; Soltis & Soltis, 1998).

With the development of molecular systematics, restriction site analysis of the chloroplast genome was initially the molecular tool of choice for inferring phylogenetic relationships (Soltis & Soltis, 1998). In recent years DNA sequencing has steadily replaced chloroplast DNA (cpDNA) restriction analysis for phylogenetic inference, even at lower taxonomic levels (Olmstead & Palmer, 1994). Until recently, most plant systematists reserved DNA sequencing for phylogenetic analysis of taxa with sequences thought to be too divergent to be easily interpreted by restriction mapping. Consequently only moderately to slowly evolving DNA sequences have been used widely in plant phylogenetics (Soltis & Soltis, 1998).

1.3.1 DNA sequencing

DNA sequencing provides a means for direct comparison. Once considered too time-consuming a process for the comparison of many taxa, DNA sequencing with the advent of PCR technology, has rapidly become a major source of comparative molecular data. A number of DNA sequencing studies in plants have been reported to allow a pragmatic look at DNA sequencing in plant phylogenetic studies (Olmstead & Palmer, 1994; Bayer & Starr, 1998; Fennel *et al*., 1998; Stedje, 1998; Meerow *et al*., 1999; Molvray *et al*., 1999; Fay *et al*., 2000; Asmussen & Chase, 2001). The primary challenge in using nucleotide characters for lower-level phylogenetic studies is the identification of easily amplifiable and relatively rapid evolving but unambiguously alignable DNA regions that can provide sufficiently suitable variation within a short sequence segment (Baldwin *et al*., 1995). Several criteria should be met in the choice of a sequence for phylogenetic analysis:

- The sequence should be of sufficient length to provide enough phylogenetic informative nucleotide positions. In addition, it is necessary that the rate of sequence divergence be appropriate to the phylogenetic question being addressed. A short sequence with a high substitution rate will not necessarily be comparable to a long sequence with a low substitution rate because the chance of a substitution along a branch of a tree must be relatively low for parsimony to succeed.
- Sequences must be readily aligned. Sequence alignment is essential for correct assessment of character homology. Coding sequences exhibiting divergences in the range suggested will usually prove readily alignable.

- Sequences must have evolved orthologous. A serious problem with the phylogenetic analysis of many nuclear genes is distinguishing orthology (genes derived from a speciation event) from paralogy (genes related by gene duplication within a genome). This is not a problem with chloroplast genes which all evolved as single-copy genes, as long as these genes remain within the chloroplast genome (Olmstead & Palmer, 1994; Soltis & Soltis, 1998).

DNA sequence data are not only more abundant but have been used on the one hand to infer phylogenetic relationships among closely related species and on the other hand, to study very ancient evolutionary occurrences (Li & Graur, 1991). DNA sequencing has to resolve some of the long-standing problems in phylogenetic studies. Molecular data have proven useful for studying phylogenetic relationships among closely related populations or species e.g. relationships among human populations and those between humans and apes, ancient evolutionary occurrences (the origin of mitochondria and chloroplasts) and the divergence of phyla and kingdoms. The purpose of phylogenetic studies are to reconstruct the correct genealogical ties between organisms and to estimate the time of divergence between organisms since they last shared a common ancestor (Li, 1997).

The present universal use of PCR for comparative sequencing means that only a minute amount of template DNA is required. Whole genome restriction site studies require more DNA and tissue, although the increasing use of PCR in restriction site studies ameliorates this distinction. Badly degraded DNA can be used as a template for PCR amplification of relatively small fragments of DNA, thereby enabling the use of herbarium specimens and even fossils as sources of DNA. All areas of restriction

site studies require relatively high molecular weight DNA. DNA sequencing examines each base pair individually thereby minimising the multiple hit problem inherent in restriction site analysis where six or four base pair 'words' provide inferential comparison of DNA sequences. Likewise, insertions and deletions that are too small to be detected in restriction site analysis can be identified and used as characters in phylogenetic analysis (Soltis & Soltis, 1998).

For distantly related taxa, highly conserved coding sequences allow accurate assessment of character homology enabling distant comparisons. For closely related taxa, rapidly evolving non-coding sequences in the nucleus should provide informative nucleotide variation at a proportion of sites greater than the random subset sampled by restriction site analysis. At any level of divergence, sequencing more DNA will help achieve an adequate level of character sampling (Soltis & Soltis, 1998).

One last and not insignificant advantage to DNA sequencing is that additional taxa may be added to an existing data set simply by entering the aligned sequence. Computer phylogenetic programmes e.g. PAUP (Swofford, 2002) can read the amended sequence set and identify any new informative nucleotide positions without the researcher having to re-examine the entire data set (Olmstead & Palmer, 1994).

The two primary sources of molecular variation tapped for phylogenetic purposes have been chloroplast genome and ribosomal DNA repeat regions (Olmstead & Palmer, 1994). The mitochondrial genome in plants has been little used for phylogenetic studies, in contrast to animal systematics, where the mitochondrial

genome has played a central role. Singular structural rearrangements, e.g. inversions and intron losses in the plant chloroplast genome have served as markers to identify monophyletic groups but their occurrence is too infrequent to provide sufficient data to construct the phylogeny of most groups (Olmstead & Palmer, 1994).

The chloroplast genome varies little in size, structure and gene content among angiosperms. The typical chloroplast genome in angiosperms ranges from 135 to 160 kb and is characterised by a large 25 kb inverted repeat which divides the remainder of the genome into one large and one small single copy region (Olmstead & Palmer, 1994). Chloroplast DNA sequence variations are now widely used to investigate interspecific relationships among angiosperms and other plants (Taberlet *et al*., 1991). The chloroplast genome in plants and mitochondrial genome in animals are natural counterparts in the phylogenetic study of their respective groups. The more rapid rate of silent substitution in animal mitochondrial DNA (mtDNA) relative to cpDNA offers an advantage to zoologists interested in molecular approaches to population genetics. Despite this, the chloroplast genome has provided useful intraspecific variation in some, but not all, species (Taberlet *et al*., 1991).

In comparison, three features of the chloroplast genome offer distinct advantages for phylogenetic studies at species level and above. First, the approximately tenfold larger size of the chloroplast genome and sixfold greater number of protein genes provide a much larger database for restriction site studies and greater choice of sequence comparisons. Second, the greater than tenfold lower silent substitution rate in cpDNA than in animal mtDNA makes the direct comparison of nucleotide sequences for higher level phylogenetic studies more feasible for cpDNA than animal mtDNA.

Third, structural rearrangements, although infrequent in both cpDNA and animal mtDNA, are somewhat more common in cpDNA, with many inversions and gene or intron deletions characterised in angiosperms (Olmstead & Palmer, 1994).

There are 20 genes in the chloroplast genome that are sufficiently large (> 1 kb) and widespread to be generally useful in comparative sequencing studies. These genes encompass a wide range of evolutionary rates and are suitable for a wide range of taxonomic levels (Olmstead & Palmer, 1994). Non-coding regions display the highest frequency of mutations (Taberlet *et al*., 1991). Chloroplast genes code for diverse functions such as photosynthesis, transcription and respiration, implicating that they are unlikely to be functionally correlated in their evolution. A comparative sequencing strategy that may be powerful both for phylogenetic purposes and for what can be learned about gene evolution is one in which more than one chloroplast gene of differing function is sequenced for a set of taxa. This strategy will yield two sets of data that are relatively free of functional correlations, but all cpDNA sequences exhibit the characteristic of being inherited as a single linkage group (Olmstead & Palmer, 1994).

In the early 1990s most molecular phylogenetic studies relied on *rbcL* (Kass & Wink, 1995; Fay & Chase, 1996; Soltis *et al*., 1996; Plunkett *et al*., 1997; Lledo *et al*., 1998; Meerow *et al*., 1999; Gracia-Jacas *et al*., 2001; Michelangeli *et al*., 2003; Salazar *et al*., 2003; Saunders *et al*., 2003; Van den Heede *et al*., 2003; Whitlock *et al*., 2003) sequences and to a much lesser extent on 18S ribosomal RNA or DNA or ribosomal deoxyribonucleic acid (rDNA) sequences (Soltis & Soltis, 1998). The field of plant molecular systematics has progressed so rapidly that several of the genes mentioned

only recently as new 'alternatives' to *rbcL* for comparative sequencing are now widely sequenced, e.g. the rDNA internal transcribed spacer or ITS (Baldwin, 1992; Suh *et al*., 1992; Manos, 1993; Baldwin *et al*., 1995; Campbell *et al*., 1995; Bogler & Simpson, 1996; Bateman *et al*., 1997; Eriksson & Donoghue, 1997; Jeandroz & Bousquet, 1997; Pridgeon *et al*., 1997; Douzery *et al*., 1999; Schultheis & Baldwin, 1999; Meerow *et al*., 2000; Gracia-Jacas *et al*., 2001; Ran *et al*., 2001a; Cubas *et al*., 2002; Koehler *et al*., 2002; Valiejo-Roman *et al*., 2002; Wedin *et al*., 2002; Carlsward *et al*., 2003; Salazar *et al*., 2003; Samuel *et al*., 2003; Schnabel *et al*., 2003; Van den Heede *et al*., 2003), *ndhF* (Whitlock *et al*., 2003; Olmstead & Sweere, 1994; Kim & Jansen, 1995; Olmstead & Reeves, 1995; Scotland *et al*., 1995; Neyland & Urbatsch, 1996), *matK* (Steele & Vilgalys, 1994; Johnson & Soltis, 1995; Johnson *et al*., 1996; Soltis *et al*., 1996; Plunkett *et al*., 1997; Hilu *et al*., 1999; Ito *et al*., 1999; Ge *et al*., 2002; Carlsward *et al*., 2003; Muellner *et al*., 2003; Salazar *et al*., 2003; Samuel *et al*., 2003; Saunders *et al*., 2003) and the entire 18S rRNA gene (Kron, 1996; Soltis *et al*., 1997).

1.3.1.1 DNA sequencing in *Clivia*

For *Clivia* the *trnL-F* and *matK* regions were used to obtain sequencing data (Booysen, 2003). The *trnL-F* region includes the *trnL* (UAA) intron and the intergenic spacer between the *trnL* (UAA) 3' exon and the *trnF* (GAA) gene. These non-coding regions have phylogenetic potential. Comparisons suggested that non-coding regions might evolve at rates similar to as much as three times faster than *rbcL*, depending on the study group (Soltis & Soltis, 1998). Non-coding regions are easily amplified and sequenced (Taberlet *et al*., 1991) and relatively small with the *trnL* intron ranging from 350-600 bp and the *trnL-F* spacer ranging from roughly 120-350 bp in monocots

and dicots initially sampled. The *trnL* intron, *trnL-F* intergenic spacer (IGS) and whole *trnL-F* region were used to resolve phylogenetic relationships within the Amaryllidaceae (Gielly & Taberlet, 1994, 1996; Meerow *et al*., 1999; Cubas *et al*., 2002; Fujii *et al*., 2002; Hodkinson *et al*., 2002; Koehler *et al*., 2002; Carlsward *et al*., 2003; Fukuda *et al*., 2003; Mayer *et al*., 2003; Perret *et al*., 2003; Salazar *et al*., 2003; Samuel *et al*., 2003; Van den Heede *et al*., 2003).

Among protein-coding regions in the chloroplast genome, *matK* is one of the most rapidly evolving. *MatK* is located in the large single-copy region of the chloroplast genome and is approximately 1 550 bp in length and encodes a maturase involved in splicing type II introns from RNA transcripts (Wolfe, 1991). In all photosynthetic land plants so far examined, *matK* positioned between the 5' and 3' exons of the transfer RNA gene for lysine, *trnK*. *MatK* as well as non-coding regions that flank it are easily amplified using the highly conserved flanking coding regions that include the *trnK* exons and the genes *rps16* and *psbA*. The evolution rate of *matK* makes this gene appropriate for resolving inter-generic or inter-specific relationships in seed plants (Soltis & Soltis, 1998). Most studies have obtained well-resolved phylogenies using approximately two-thirds (~1 000 bp) of the 1 550 bp *matK* gene, whereas some studies used considerably less (Steele & Vigalys, 1994). In Saxifragaceae sp., *matK* sequences provided a level of resolution comparable to that achieved with cpDNA restriction sites. *MatK* sequences were used to discern the maternal parent of allopolyploids in *Saxifraga* (Johnson & Soltis, 1995). Well-resolved generic and species-level phylogenies have been obtained using *matK* sequences in Saxifragaceae sp. and Polemoniaceae (Johnson & Soltis, 1995), Apiales (Plunkett *et al*., 1997) and

many more (Hilu *et al*., 1999; Ito *et al*., 1999; Ge *et al*., 2002; Carlsward *et al*., 2003; Muellner *et al*., 2003; Salazar *et al*., 2003; Samuel *et al*., 2003; Saunders *et al*., 2003).

According to Meerow *et al*. (1999) and Ito *et al*. (1999) the family Amaryllidaceae forms a monophyletic clade and Agapanthaceae is likely to be its sister family. Both of the tribes Amaryllideae and Haematheae are well-supported tribal clades based on *rbcL* and *trnL-F* sequences (Meerow *et al*., 1999). Based on the *matK* sequences, the Amaryllidaceae is a well-supported tribe (Ito *et al*., 1999). The tribe Amaryllideae is a sister clade to the rest of the tribes based on *rbcL*, *trnL-F* (Meerow *et al*., 1999) and *matK* sequences (Ito *et al*., 1999). The *matK* results of Ito *et al*. (1999) indicated that *Clivia* is a sister clade to *Haemanthus* and *Scadoxus*. *Crinum, Brunsvigia, Strumaria* and *Nerine* formed a clade and *Amaryllis* a sister clade to these four species (Ito *et al*., 1999). *RbcL* results of Meerow *et al*. (1999) indicated that *Clivia* is a sister clade to *Apodolirion, Gethyllis, Haemanthus, Scadoxus* and *Cryptostephanus*.

1.3.2 DNA fingerprinting

1.3.2.1 Random amplified polymorphic DNA analysis

Random amplified polymorphic DNA analysis (RAPD) (Welsh & McClelland, 1990; Williams *et al*., 1990) is a PCR-based molecular marker technique (Mohan *et al*., 1997) that is simple, sensitive and relatively cheap in comparison to RFLPs (Restriction Fragment Length Polymorphisms) (Thottappilly *et al*., 2000). Advantages in using RAPD markers are that no prior sequence information is required, small amounts of DNA are required for analysis and the procedure is simpler than RFLP

analysis as it does not require either restriction enzyme digestion or Southern blotting (Southern, 1975; Williams *et al*., 1990).

Amplification of DNA is based on the use of arbitrary primer DNA sequences available commercially. The amplification reaction depends on homology between the genomic DNA and these short oligonucleotide primers (10 bp). PCR products (DNA intercalated with ethidium bromide) are easily separated by standard electrophoretic techniques and visualised under ultraviolet (UV) light. Amplification products will vary in size. Distances vary between individuals, resulting in polymorphisms. Disadvantages of RAPD markers include the production of complex banding patterns with most primers, making comparisons among populations or laboratories difficult. The low annealing temperature at which the primers are used can result in bands of the same apparent size, representing different DNA regions. Furthermore, the degree of reproducibility among different DNA extraction preparations and different researchers is a problem (Burr, 1994).

RAPD analysis was successfully employed for the detection of genetic diversity in for example a French olive collection (Khadari *et al*., 2003), hybrid poplar cultivars (Rajora & Rahman, 2003), Korean tea populations (Kaundum & Park, 2002) and spring wheat cultivars (Sun *et al*., 2003).

1.3.2.1.1 Random amplified polymorphic DNA analysis in *Clivia*

RAPD markers can be used in the same way as RFLP markers except that the former is a dominant marker while RFLP is a codominant marker (Thottappilly *et al*., 2000). Ran *et al.* (2001b) extracted DNA from fresh root tips of *Clivia* and conducted RAPD analysis. The level of intraspecific polymorphism was variable for different taxonomic units (Ran *et al.*, 2001b). Populations of *C. miniata* showed the greatest variation with *C. nobilis* displaying the least variation with high levels of DNA polymorphisms between different species. Ran and his colleagues found that partitioning of genetic variance revealed that most of the total variance could be attributed to variation among species. This indicated that there were distinct genetic differences between species of *Clivia*. RAPD analysis revealed that *C. miniata* and *C. gardenii* were genetically close. *Clivia nobilis* was more distantly related to these species whereas *C. caulescens* occupied an intermediate position. These results supported their previous findings using karyotype analysis (Ran *et al*., 2001a).

Statistically, the variation found in populations was low, resulting in not all individual plants being uniquely distinguished. However, the major population groups in each species could be identified. *Clivia miniata* plants showed significantly greater variation between populations than among plants in the same population. Ran and his colleagues suggested that it should be highly beneficial to use plants from different populations as parents for hybrid combinations in any breeding programme for the improvement of cultivated *Clivia* (Ran *et al*., 2001b).

1.3.2.2 Microsatellites and Amplified fragment length polymorphisms

Plant breeding in its conventional form applied to crops is based on phenotypic selection of superior genotypes within segregating progenies obtained from crosses. Phenotyping procedures of crops generated in this manner are often expensive, time consuming or sometimes unreliable (Mohan *et al*., 1997; Francia *et al*., 2005). Knowledge regarding genetic diversity and relationships among diverse germplasm is of utmost importance to plant breeders. It supports decisions on the selection of parents for crossing and is helpful to widen the genetic basis for breeding programmes. Difficulties in manipulating traits are derived from genetic complexity, number of genes involved and interactions between genes (epistasis) and environment-dependent expression of genes (Dale & von Schantz, 2002; Francia *et al*., 2005).

The use of DNA markers is a very effective way of obtaining essential information on the genomic region around a given gene and ultimately isolating the gene of interest (Agrama *et al*., 2002). The capacity of a molecular marker to reveal polymorphisms implies its usefulness. Amplified fragment length polymorphisms (AFLPs) (Vos *et al*., 1995), microsatellites or simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs) reveal high levels of polymorphisms (Mohan *et al*., 1997; Pejic *et al*., 1998; Beyene *et al*., 2005; Francia *et al*., 2005).

SSR loci provide a high level of polymorphism as already mentioned. One school of thought is that SSR analysis presents the potential advantages of reliability, reproducibility, discrimination and standardisation over RFLP analysis. It has been reported that SSR analysis using high quality agarose gels can conveniently assess the

genetic diversity in inbred maize lines (Enoki *et al*., 2002). Since SSRs are codominant, distinguishing between homo- and heterozygotes is possible.

AFLP analysis (Vos *et al*., 1995) is a molecular technique for fingerprinting DNA of any origin and complexity. AFLPs can be used to monitor inheritance of agronomic traits in plant and animal breeding, pedigree analysis, parentage analysis and screening of DNA markers linked to genetic traits (Blears *et al*., 1998). AFLPs have the capacity to inspect the entire genome for polymorphisms being a multilocus marker technique (Pejic *et al*., 1998) while being highly reproducible. AFLPs detect the highest number of polymorphisms in a single assay compared to RFLPs, RAPDs and SSRs. The high assay efficiency index is a reflection of the efficiency of AFLPs to simultaneously analyse a large number of fragments rather than the levels of polymorphism detected at each locus. The high multiplex ratio of AFLPs offers a distinctive advantage when genome coverage is a major issue due to the presence of linkage disequilibrium, such as in inbred lines and breeding material (Pejic *et al*., 1998). The number of amplified DNA fragments can be controlled by choosing a different base number and composition of nucleotides in adapters. Genetic polymorphisms are identified by the presence or absence of DNA fragments following restriction and amplification of genomic DNA. AFLPs are not dependent on prior sequence knowledge (Blears *et al*., 1998), are inherited as Mendelian markers (Ajmone-Marsan *et al*., 1998) and are widely used to develop polymorphic markers (Mohan *et al*., 1997).

The genetic variation present at microsatellite and AFLP loci was assessed in seven Italian populations of wild cordoon *Cynara cardunculus* L. var. *sylvestris* (Lamk) Fiori, a non-domesticated robust perennial plant collected from Sicily and Sardinia (Portis *et al*., 2005). Thirty individuals, randomly sampled from each population, were genotyped at five SSR loci and fingerprinted using seven AFLP primer combinations. Genetic distance estimates both within and between populations were consistent between the two marker systems. As a result of geographical isolation, the Sardinian and Sicilian populations were clearly differentiated and formed two distinct gene pools. Most of the genetic variation was partitioned within rather than between populations (Portis *et al*., 2005). In a study done by Fargette *et al*. (2005), AFLP markers proved useful to analyse inter- and intraspecific genetic diversity of various organisms such as plants, insects, fishes etc. Different levels of discrimination and analysis were achieved such as identification of interspecific hybrids, analysis of patterns of genetic differentiation within an insect species complex, phylogeny of rapidly evolving clades or discrimination between closely related species (Fargette *et al*., 2005).

Unlike interspecific markers, AFLPs are specifically useful for investigating intraspecific variations and relatedness between closely related entities (Cai *et al*. 2005). Preliminary assessment of the genetic relationship between *Erianthus rokii* and a wild relative of sugar cane '*Saccharum* complex' revealed that RAPD, AFLP and SSR analysis resulted in sufficient resolution to detect differences between the genetic profiles of various strains of the same species (Cai *et al*., 2005). AFLP markers detected the highest number of polymorphisms in a single assay, with high resolution and good reproducibility. The use of AFLPs was technically much more complex than

the use of SSR markers, requiring numerous experimental steps at higher cost per informative marker. Despite those limitations, Cai *et al.* (2005) suggested that the AFLP technique has great value for use in genetic mapping and evolutionary studies. This could be attributed to the large number of loci distributed randomly throughout a genome.

Unlike microsatellites, AFLP markers were not highly variable, providing a less biased estimate of population variability than SSRs (Cai *et al*., 2005). SSR analysis revealed the highest genetic variability in the microbial population studied, also achieving the highest discriminatory power. SSRs proved to be the most efficient method with the highest number of effective alleles per assay. AFLP analysis has been used to study genetic relationships of a wide range of species, including ornamentals such as *Aglaonema* Schott., *Alocasia* G. Don., *Dieffenbachia* Schott., *Caladium* Venten., *Hemerocallis* L., *Philodendron* Schott and the popular ornamental Calatheas (Chao *et al*., 2005). AFLPs were proven to be extremely sensitive for distinguishing closely related cultivars (Xu *et al*., 1999; Barbarosa *et al*., 2003; Chao *et al*., 2005). To the present, AFLP analysis has not been applied to the study of genetic diversity in *Clivia*.

1.3.2.2.1 Microsatellites used in *Clivia*

Swanevelder (2003) developed microsatellites for *C. miniata* using template DNA from populations shown to be genetically different. Plants used were from the Oribi Gorge, Kentani area, Mzamba River, Port St Johns, Umtamvuna River, Donkeni and Broedershoek farm in South Africa. Primer sets designed for *C. miniata*, including designed product length and primer sequences, are presented in Chapter 2 (Swanevelder, 2003).

Swanevelder (2003) found that two primer sets, CLV2 and CLV4 showed polymorphisms between samples from different localities. The other two marker sets showed no polymorphisms between different *C. miniata* localities sampled. He proposed that these might still be useful in studies of other *Clivia* species (Swanevelder, 2003).

1.4 Aims of the study

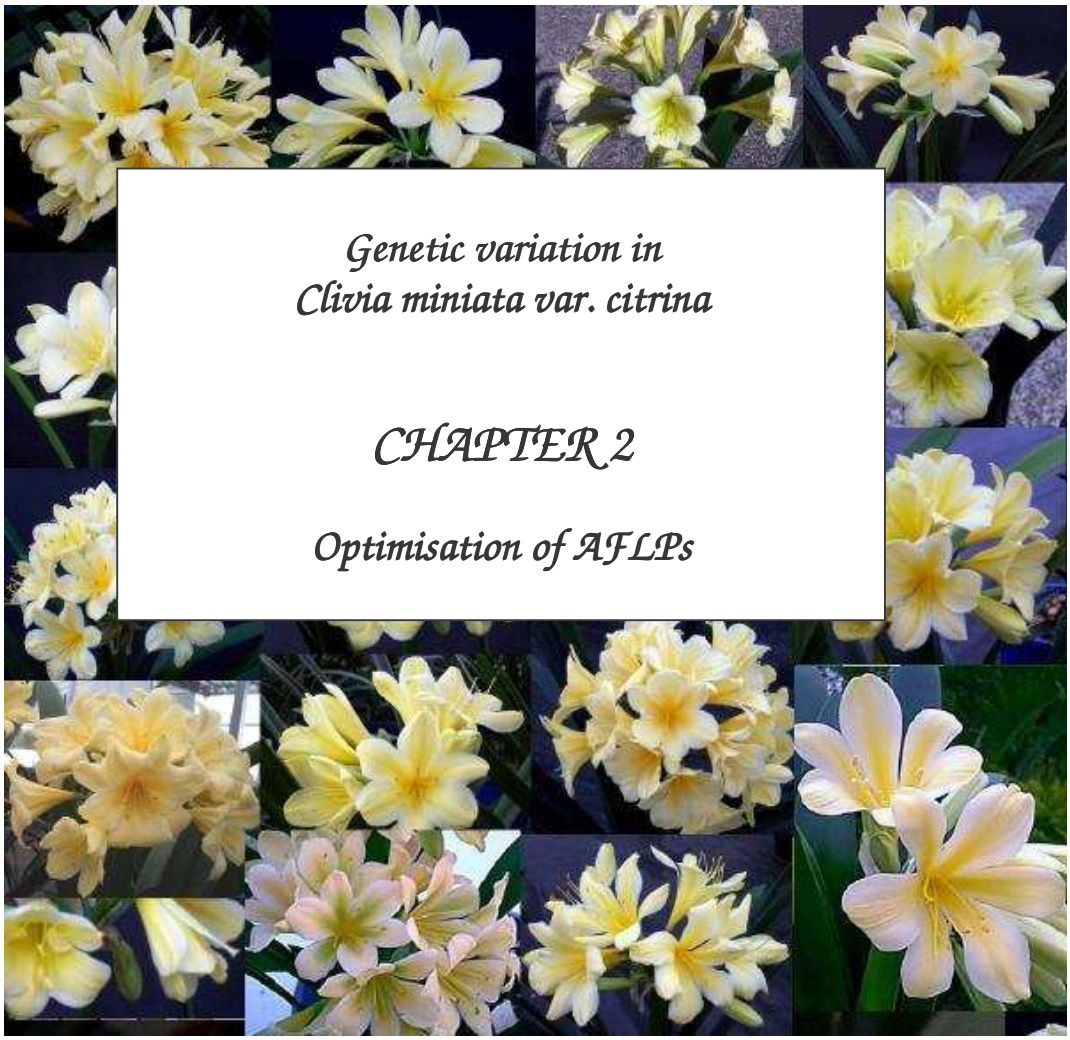
According to Koopowitz (2002) yellow *Clivia* are mutations of the orange-red standard forms that have appeared spontaneously in both wild and garden populations. Yellow Clivia plants are rare and desirable and were described as *Clivia miniata* var. *citrina* (Koopowitz, 2002; Van Niekerk, 2005). Hobbyists from around the world trade in these ornamental plants initiating entire enterprises. Although the yellow form occurs naturally, many yellow clones have arisen through cultivation. Clones passed on from breeder to breeder have acquired different names. For directed breeding purposes in a thriving industry it is important to identify genetically similar plants.

The aims of this study were to:

1. Evaluate microsatellites developed by Swanevelder (2003) for *Clivia miniata* on *C. miniata* var. *citrina*.

2. Determine if AFLP analysis can distinguish among individual plants within the genus *Clivia.*

3. Determine genetic relatedness between different plants of 'Vico', 'Giddy' and 'Natal Yellow' cultivars.



Genetic variation in
Clivia miniata var. citrina

CHAPTER 2

Optimisation of AFLPs

2.1 Introduction

DNA marker technologies have become increasingly important as effective tools in crop breeding programmes. However, application to ornamental crop species is lagging behind. The choice of marker system and technique to be used is critical when considering using DNA fingerprinting or marker-assisted selection. The most commonly used systems with related techniques employ PCR. PCR-based techniques are generally quick and straightforward to perform and require small amounts of DNA. An effective marker system should yield the maximum number of polymorphisms for the particular germplasm sampled in terms of fragments amplified per assay, frequency (%) of polymorphic fragments per assay unit and number of unique profiles generated (McGregor *et al*., 2000; Swanevelder, 2003).

Previous studies done on *Clivia* include RAPD analysis conducted by Ran *et al*. (2001b) and SSR analysis developed by Swanevelder (2003) for *Clivia*. Ran *et al*. (2001b) found that the level of intraspecific polymorphism was variable for different taxonomic units. Populations of *C. miniata* showed the greatest variation with *C. nobilis* showing the least variation. Swanevelder (2003) indicated that the developed SSRs may prove useful in studies of other *Clivia* species. No prior account of *Clivia* subjected to AFLP analysis could be found, neither could another account of SSR analysis applied to *Clivia* as described and developed by Swanevelder (2003) be found. The work done on *Clivia* in this study presents a first report of AFLP and SSR fingerprint analyses on *Clivia miniata* var. *citrina*.

Step one in a genetic variation study is the selection of a suitable marker system and optimisation of the selected system on the crop involved. The first aim was to evaluate

SSRs developed by Swanevelder (2003). Secondly, to incorporate the use of AFLPs to elucidate genetic similarities between members of the genus *Clivia* and within species of *Clivia.*

2.2 Materials and Methods

2.2.1 Plant material

Plant material was obtained from breeders in South Africa. Material used during optimisation is given in Table 2.1. Six samples were randomly selected for optimisation.

Table 2.1 Plant material used for optimisation

Name	Species
Nakamura Yellow	*C. miniata*
Giddy	*C. miniata*
Dwesa Yellow	*C. miniata*
Floradale Yellow	*C. miniata*
Tarrs Picotee	*C. miniata*
Nurenberger	*C. miniata*

2.2.2 DNA isolation using CTAB method

Total genomic DNA was isolated using a modified hexadecyltrimethylammonium bromide (CTAB) method based on Saghai-Maroof *et al*. (1984). Preferably young *Clivia* leaves were freeze-dried (using the FreezeMobile II apparatus) and ground to a fine powder in the presence of silica gel using a mortar and pestle. A volume of 750 µl CTAB buffer [100 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.0; 20 mM ethylenediaminetetraacetate (EDTA), pH 8.0; 1.4 M NaCl; 2% (w/v) CTAB and 0.2% (v/v) β-mercaptho-ethanol] was added to approximately 250 µl fine leaf powder in a 1.5 ml microfuge tube and incubated at 65°C for one hour. A volume of 500 µl chloroform: isoamylalcohol [24:1 (v/v)] was added to the suspension. Phases were separated by centrifugation at 12 000 g for 3 minutes. DNA was precipitated from the aqueous phase with 0.66 volumes 2-isopropanol at room temperature for 20 minutes. Centrifugation followed at 12 000 g for 10 minutes. The supernatant was discarded and tubes drained upside down. The precipitate was washed at room temperature with 500 µl 70% (v/v) ethanol for 20 minutes followed by centrifugation at 12 000 g for 10 minutes. The pellet was air-dried for one hour and resuspended in TE buffer (10 mM Tris.Cl, pH 8.0; 1 mM EDTA, pH 8.0). Overnight incubation followed at 4°C. DNase-free RNase (0.1µg/µl) was added to samples and incubation at 37°C followed for 2 hours. Ammonium acetate (0.75 M) and an equal volume of chloroform: isoamylalcohol [24:1 (v/v)] were added to the samples. DNA was precipitated from the aqueous layer with two volumes of ice-cold absolute ethanol followed by overnight incubation at -20ºC. DNA was recovered by centrifugation at 12 000 g for 15 minutes and washed twice with cold 70% (v/v) ethanol by centrifugation of 10 minutes each time. The pellet was air-dried and resuspended in TE buffer. DNA quantity and quality were estimated using an UV spectrophotometer by measuring

absorbencies at A_{260} and A_{280}. DNA samples were diluted, depending on the concentration, to 200 ng/µl.

2.2.3 SSR analysis

SSR analyses were performed in 20 µl reaction mixtures containing 20 ng/µl genomic DNA, 1x Promega *Taq* polymerase buffer (10 mM Tris.Cl, pH 9.0; 50 mM KCl; 0.1% (v/v) Triton X-100), 2 mM $MgCl_2$, 200 µM of each dNTP, 50 ng of each primer and 1 U *Taq* DNA polymerase (Promega, Madison, WI, USA). A total of four previously developed primers were tested on six *Clivia* plants. Primer sets designed for *C. miniata*, including the designed product length, primer sequences and annealing temperatures are presented in Table 2.2 (Swanevelder, 2003). Reactions were performed using a Touchdown PCR programme of 5 minutes denaturation at 94°C, followed by 6 cycles of 30 seconds at 94°C, 30 seconds at 55°C, decreasing with 1°C every cycle and 1 minute at 72°C. This was followed by 25 cycles of 30 seconds at 94°C, 30 seconds at primer set annealing temperature (Table 2.2), 1 minute at 72°C and a final extension time of 7 minutes at 72°C.

2.2.3.1 Gel electrophoresis

Prior to loading, PCR products were mixed with 10 µl formamide dye [98% de-ionized formamide; 10 mM EDTA, pH 8.0; 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol] and denatured by incubation for 5 minutes at 95°C. Mixtures were immediately placed on ice. PCR products (2.5 µl) were separated on 5% (w/v) denaturing polyacrylamide gels [19:1 acrylamide: bis-acrylamide; 7 M urea; 1X TBE buffer (0.89 M Tris.HCl; 0.89 M Boric acid; 2.0 mM EDTA)]. Electrophoresis was performed at constant power of 80 W for approximately 1 hour.

Table 2.2 **Primer sets designed for *Clivia miniata*, including the designed product length, primer sequences and primer annealing temperatures (Swanevelder, 2003).**

Primer Code	Primer sequence (5' – 3')	Microsatellite targeted	Designed product length	Annealing temperature (^{o}C)
CLV1F	CAATAATGTGGCTAATGGGTTG	T_4AT_6	**± 200 bp**	**53**
CLV1R	CTCAAGCTATGCATCCAACG			
CLV2F	CTTGTTGTAGCTTGTAATAGC	$(GT)_9$	**± 225 bp**	**51**
CLV2R	CTGAACGGCAGAGGAGTTG			
CLV3F	ACAACTCCTCTGCCGTTCAG	A_{11}	**± 246 bp**	**51**
CLV3R	GGGTGCAGTGCACTAGTGC			
CLV4F	GCATCCCTTGCTCCTCTAC	$(CCT)_2TCT(CCT)_2CGT$	**± 210 bp**	**55**
CLV4R	CTCAAGCTATGCATCCAACG			

2.2.3.2 Silver staining for DNA visualisation

The silver staining (Silver Sequence™ DNA Sequencing System of Promega) process of acrylamide gels included fixing the gel in 10% (v/v) acetic acid for 30 minutes, rinsing three times in de-ionised water (5 minutes per rinse) and staining for 30 minutes in a solution containing 0.1% (w/v) silver nitrate and 0.056% (v/v) formaldehyde. All steps were performed with slow agitation on a shaker. The stained gel was rinsed with de-ionised water for no longer than 5-10 seconds and immersed in cold (4-10°C) developing solution containing 3% (w/v) sodium carbonate, 0.056% (v/v) formaldehyde and 0.002 mg/ml sodium thiosulphate. Manual agitation followed until DNA fragments became visible. The development process was stopped by adding 10% acetic acid directly to the developing solution and shaking continued for 2-3 minutes. The gel was rinsed with de-ionised water and left upright to dry overnight. It was photographed by exposing photographic paper (Kodak Polymax II RC) directly under the gel to dim light for approximately 20 seconds.

2.2.4 AFLP analysis

AFLP primers used for optimisation of AFLP analysis on *Clivia* are presented in Table 2.3. Primers were named E and M, for *Eco*RI and *M*seI, respectively. Selective nucleotides at the 3'-end are indicated in Table 2.3, following E or M. Primers and adapters were synthesised by Integrated DNA Technologies, Inc. and oligonucleotides used for adapters were polyacrylamide gel electrophoresis (PAGE) purified. Adapters were prepared by adding equimolar amounts of both strands, heating for 10 minutes to 65°C in a water bath and then leaving the mixture to cool to room temperature. AFLP analysis was performed as described by Vos *et al.* (1995) and modified by Herselman (2003).

2.2.4.1 Restriction enzyme digestion and ligation reactions

Genomic DNA (1.0 µg) was digested for 5 hours at 37°C using 4 U of *Mse*I and Ix *Mse*I-buffer [50 mM NaCl; 10 mM Tris.Cl, pH 7.9; 10 mM $MgCl_2$ and 0.1 mM dithiothreitol (DTT)] in a final volume of 50 µl, followed by further digestion overnight at 37°C with 5 U *Eco*RI and NaCl to a final concentration of 100 mM. Adapter ligation was achieved by adding a solution containing 50 pmol *Mse*I-adapter, 5 pmol *Eco*RI-adapter, 1 U T4 DNA Ligase, 0.4 mM ATP and 1x T4 DNA Ligase buffer (66 mM Tris.Cl, pH 7.6; 6.6 mM $MgCl_2$; 10 mM DTT; 66 µM ATP) followed by overnight incubation at 16°C.

2.2.4.2 Preamplification reactions

Template DNA (5 µl of the restriction/ligation mixture) in a 50 µl reaction mixture for the preamplification reactions was added to 30 ng of each preamplification primer (*Eco*RI- and *Mse*I-primer+1), 1x Promega *Taq* polymerase buffer, 2 mM $MgCl_2$, 200 µM of each dNTP and 1 U *Taq* DNA polymerase (Promega, Madison, WI, USA). Reactions were performed using the following cycling programme: 5 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 60 seconds at 56°C and 60 seconds at 72°C and final elongation of 10 minutes at 72°C. Quality and quantity of preamplification reactions were determined by electrophoresis in 1.5% (w/v) agarose gels. Preamplification reactions were diluted accordingly (1:5; 1:10 or 1:15 times) prior to selective amplifications.

2.2.4.3 Selective amplification reactions

Selective amplifications were performed in a total of 20 µl reaction volumes containing 5 µl preamplification product, 1x Promega *Taq* polymerase buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 100 µg/ml bovine serum albumin, 30 ng *Mse*I-primer+3 or *Mse*I-primer+4, 30 ng *Eco*RI-primer+3 or *Eco*RI-primer+4 and 0.75 U Promega *Taq* DNA polymerase. The cycling programme for selective amplification was: one cycle of denaturation at 94°C for 5 minutes followed by one cycle of 30 seconds at 94°C, 30 seconds at 65°C and 60 seconds at 72°C. The annealing temperature was lowered by 1°C per cycle during the next eight cycles after which 25 cycles were performed at 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 60 seconds followed by a final elongation step of 10 minutes at 72°C. Primer combinations are listed in Table 2.3. Repeatability of selective amplifications was tested through three independent amplification reactions of each specific primer combination. AFLP products were separated in denaturing polyacrylamide gels and DNA fragments visualised using silver staining. Gel electrophoresis was done as described in section 2.2.3.1, except that PCR products were mixed with 20 µl formamide dye and electrophoresis was done for 2 hours. Silver staining was performed as described in section 2.2.3.2.

2.2.5 Data analysis

Primer combinations were evaluated for use in *Clivia* fingerprinting based on the following criteria: Number of generated fragments, ability to score generated fragments, repeatability, ability to detect polymorphism and level of polymorphic fragments (Subudhi *et al*., 1998; Aggarwal *et al*., 1999; Potokina *et al*., 2002).

Table 2.3 ***Eco*RI and *Mse*I adapter, primer+1, primer+3 and primer+4 sequences used in AFLP analysis**

Enzyme	Type	Sequence (5'-3')
*Eco*RI	Adapter-F	CTCGTAGACTGCGTACC
	Adapter-R	AATTGGTACGCAGTCTAC
*Mse*I	Adapter-F	GACGATGAGTCCTGAG
	Adapter-R	TACTCAGGACTCAT
*Eco*RI	Primer+1	GACTGCGTACCAATTCA
	Primer+3	GACTGCGTACCAATTCANN ANN=ACC, ACA
	Primer+4	GACTGCGTACCAATTCANNN ANNN= ACCT, AGCG
*Mse*I	Primer+1	GATGAGTCCTGAGTAAC
	Primer+3	GATGAGTCCTGAGTAACNN CNN=CAA, CAC, CAG, CAT, CTA, CTC, CTG, CTT
	Primer+4	GATGAGTCCTGAGTAACNNN CNNN=CATC, CTGG

2.3 Results

2.3.1 SSR analysis

The four SSR primers developed by Swanevelder (2003) known as CLV1-CLV4 were screened for their ability to be used in *Clivia* fingerprinting analysis. Using reaction conditions as described by Swanevelder (2003) CLV1 showed no amplification, CLV2 and CLV3 showed a smear and CLV4 amplified fragments that were too big (>1 000 bp). Even after varying $MgCl_2$ concentrations (1.5 mM, 2.0 mM and 2.5 mM), annealing temperatures and cycling programmes, no amplification of the expected sizes could be obtained.

2.3.2 AFLP analysis

To optimise AFLP analysis on *Clivia*, a total of 28 AFLP primer combinations were screened on six *Clivia* plants (Table 2.4). Amplified fragment lengths varied between 200 and 1 100 bp. E+3 primers in combination with M+3 primers amplified an average of 88 fragments. Results indicated that due to the high number of amplified fragments, scoring would be too complex. Subsequently, E+4 and M+4 primers were screened in combination with each other as well as M+3 and E+3 primers respectively. E+4 in combination with M+3 primers amplified an average of 32 fragments while E+3 in combination with M+4 primers amplified an average of 50 fragments. E+4 in combination with M+4 primers amplified an average of 24 fragments. Results indicated that E+4 primers in combination with either M+3 or M+4 primers did not amplify enough fragments. E+3 primers in combination with M+4 primers amplified enough loci to be useful in fingerprinting studies on *Clivia*. Fragments generated with this E+3-M+4 primer combinations were easy to score,

repeatable and detected polymorphisms within the six randomly selected *Clivia* plants screened. Primer combination E-ACC with M-CATC amplified 40 fragments and detected 80% polymorphic fragments, primer combination E-AGC with M-CTGG amplified 50 fragments and detected 92% polymorphic fragments. Primer combination E-AGC with M-CATC amplified 58 fragments and detected 95% polymorphic fragments. These three primer combinations were selected for further studies on *Clivia*.

Table 2.4 Different primer combinations tested to fingerprint six *Clivia* plants

***Eco*RI primer**		**Tested with *Mse*I primer**	
+3:	E-AAC	+3:	M-CAA; M-CAC; M-CAG; M-CAT; M-CTA; M-CTC; M-CTG; M-CTT
	E-ACA		M-CAA; M-CAC; M-CAG; M-CAT; M-CTA; M-CTC; M-CTG; M-CTT
+3:	**E-ACC**☺	+4:	**M-CATC**☺; M-CTGG
	E-AGC☺		**M-CATC**☺; **CTGG**☺
+4:	E-ACCT	+3:	M-CAT; M-CTG
	E-AGCG		M-CAT; M-CTG
+4:	E-ACCT	+4:	M-CATC; M-CTGG
	E-AGCG		M-CATC; M-CTGG

☺=indicates primer combinations that were successful

2.4 Discussion

SSR markers are based on tandem repeats of short (2-6 bp) DNA fragments scattered throughout the genome that lie between conserved sequences (Litt & Luty, 1989; Weber & May, 1989). Database searches demonstrated that both dinucleotide and trinucleotide repeats are frequent in the plant genome, with at least one repeat greater than 20 bp in length every 30 kb throughout the genome (Taramino & Tingey, 1996). In plant species, repeats containing $(AT)_n$ were found to be the most frequent dinucleotide repeat. In contrast to the human genome, $(AC)_n$ repeats were found to be much less abundant in plants (Akkaya *et al*., 1992). The frequency of each class of SSR appears to be different between plant species. Variation in the number of tandem repeats results in different PCR product lengths. These repeats are highly polymorphic, even among closely related cultivars. This is due to mutations causing variation in the number of repeating units. Different alleles can be detected at a locus by PCR using conserved DNA sequences that flank SSR as primers (Kochert, 1994).

Six randomly selected *Clivia* plants were used for SSR optimisation purposes. SSR analysis based on SSRs developed by Swanevelder (2003) was not found useful in detecting polymorphisms in *Clivia*. Since AT is the most common sequence in plants followed by AG or TC (Powell *et al*., 1996), it would be expected that CLV1 had the best theoretical odds of detecting polymorphism, being constructed to target T_4AT_6.

Amplification using CLV1 as primer set resulted in no amplification result even after changing reaction conditions as specified by Swanevelder (2003). Further optimisation of this primer set might be necessary. The absence of any amplification

product might be ascribed to the absence of these primer sequences in the tested *Clivia* plants compared to the presence of such sequences in the plants Swanevelder (2003) tested. This might also be the case for the other primer sets CLV2, CLV3 and CLV4.

Although developmental costs are high and development of new SSR primers is time-consuming, the availability of SSR primers for *Clivia* genotyping would be useful. SSR markers are codominant, allowing discrimination between homozygotes and heterozygotes (Subudhi *et al*., 1998; Aggarwal *et al*., 1999). SSRs have the advantage of being technically less demanding than AFLP analysis and more applicable for screening large populations, either for genetic diversity analysis or Marker-assisted selection (MAS). When SSRs have been developed and proven to detect polymorphisms, running costs are low. However, further SSR development is necessary for *Clivia*.

Most eukaryotic DNAs are AT-rich, as a result, *Mse*I (recognising sequence TTAA) will generally produce smaller restriction fragments than other enzymes. *Eco*RI is a reliable six-cutter enzyme of relatively low-cost. The use of *Mse*I and *Eco*RI limits incomplete restriction of DNA. During AFLP analysis fragments cut by both enzymes are preferentially amplified (Blears *et al*., 1998; Han *et al*., 1999; Heckenberger *et al.,* 2003). Most AFLP fragments correspond to unique positions in the genome and can be exploited as landmarks in genetic and physical maps. Each fragment is characterised by its size and the primers required for amplification (Vos *et al*., 1995). The number of amplified fragments may be controlled by the nature of selective bases

(Subudhi *et al*., 1998; Aggarwal *et al*., 1999; Han *et al*., 1999; Potokina *et al*., 2002). As the number of selective nucleotides is increased, the complexity of the DNA fingerprint decreases (Han *et al*., 1999). Furthermore, selective extensions with rare di- or trinucleotides will result in reduction of amplified fragments. The complexity of the DNA fingerprint can further be decreased by using eight-cutter rare cutter enzymes (e.g. *Sse*I) or methylation sensitive enzymes (e.g. *Pst*I).

AFLP optimisation was based on six randomly selected plants used for SSR optimisation. Of the 28 primer pair combinations tested, only three were considered successful. Based on ease of scorability, repeatability and ability to detect polymorphisms, E+3 (rare cutter) with M+4 (frequent cutter) proved most suitable. E+3 in combination with M+3 resulted in complex fingerprints. The complexity was reduced by the use of an additional selective base, using primers E+4 and M+4. Results indicated that E+4 in combination with M+3 were too specific, as were E+4 in combination with M+4, yielding not enough fragments. Vos *et al*. (1995) demonstrated a loss of selectivity when 4-base extensions were used; however, results for *Clivia* were contradictory to this. The +4-base extensions aided to reduce complexity of the fingerprint pattern, but were still selective enough to be used to distinguish between plants.

The use of AFLPs to evaluate genetic variation within the genus *Clivia* proved highly valuable. All six randomly selected plants could be clearly distinguished. Future research should involve evaluating other primer pair combinations, perhaps incorporating an eight-base cutter in the combination. Other enzyme combinations e.g. *Pst*1, *Sse*1 or *Mlu*1 could be used.

Genetic variation in
Clivia miniata var. citrina

CHAPTER 3

Genetic variation in Clivia
as revealed by AFLP analysis

3.1 Introduction

Today intense breeding activity for specific orange, yellow and other *Clivia* flower colours have become the trend (Koopowitz, 2002). Selection of specific pod and pollen parents has become important and deciding what breeding stock to use is critical for any current or prospective *Clivia* breeder.

Flower colour is often more important than form and people will often grow and treasure flowers with exceptionally poor form as long as the flowers have unusual colours or patterns (Koopowitz, 2002). This differs widely from the Japanese view where *Clivia* is treasured for its evergreen foliage with beautiful flowers being a bonus (Koopowitz, 2002; Swanevelder, 2003).

Yellow *Clivia* are mutations of the orange-red standard forms that have appeared spontaneously in wild and garden populations (Koopowitz, 2002). Yellow *Clivia* is rare and desirable and ranks among very special plants in the world (Koopowitz, 2002; Van Niekerk, 2005). As with standard *Clivia*, the past decade has seen a drive toward breeding and propagating yellow forms. Increased availability of yellow *Clivia* has lowered the price per plant substantially. Predictions are that orange and yellow forms of *C. miniata* will sell at the same price in the near future, where at the Longwood Gardens Rare Plant Auction in 2000, a yellow form fetched the highest price of US$ 2200 (Koopowitz, 2002; Swanevelder, 2003).

DNA fingerprinting offers the capacity to identify individual plants based on their genetic composition. Amplified fragment length polymorphism (AFLP) is a PCR-based assay that can be used for plant DNA fingerprinting. The specificity of

restriction analysis combined with PCR amplification may be used for DNA of any origin or complexity. Sequence variation detected is similar to RFLP analysis but the number of polymorphisms detected per assay is higher (Vos *et al*., 1995). AFLP analysis is relatively easy to perform, requires minimal amounts of DNA and generates a large number of fragments in a comparatively short time, covering nearly the entire genome. AFLPs are reliable and reproducible compared to RAPD markers because stringent reaction conditions are used (Vos *et al*., 1995; McGregor *et al*., 2000).

The aim of this study was to determine if AFLP analysis can distinguish among individual plants between and within the genus *Clivia*, with special reference to particular plant material obtained from established *Clivia* breeders (the reputedly different 'Vico Yellow', 'Natal Yellow' and 'Giddy' plants).

3.2 Materials and Methods

3.2.1 Plant material

Seventy-two plants obtained from South African *Clivia* breeders were assessed for genetic diversity using AFLP analysis (Table 3.1). These lines include 47 plants of *C. miniata* var. *citrina*, 18 plants of *C. miniata*, two *C. caulescens* plants and one plant each of *C. gardenii* var. *citrina, C. gardenii*, *C. nobilis* and *C. mirabilis* and an interspecific *C. caulescens* x *C. mirabilis* hybrid.

3.2.2 DNA isolation

Total genomic DNA was isolated using the CTAB method (Sahgai-Maroof *et al*., 1984) as described in Section 2.2.2.

3.2.3 AFLP analysis

DNA fingerprints were generated based on the optimised conditions described in section 2.2.4. Primers were synthesised by Integrated DNA Technologies, Inc. Genomic DNA digests and ligation of adapters were performed as described in section 2.2.4.1. Preamplification DNA was diluted 1:15 prior to selective amplification. Selective reactions were performed as described in section 2.2.4.3.

Successful primer pair combinations used in selective amplification reactions to fingerprint 72 *Clivia* plants are given in Table 3.2. The cycling programme was used as described in section 2.2.4.3. AFLP products were separated in denaturing polyacrylamide gels and DNA fragments visualised by Silver Staining (section 2.2.3.2).

In order to ensure and test the repeatability of the generated AFLP data, plant material from the four *Clivia* plants used to standardise the AFLP technique (Chapter 2) was again subjected to DNA extraction, digestion, ligation and preamplification. Both DNA samples of each of these *Clivia* plants were included during selective amplification reactions. Furthermore, since each primer combination's amplification reactions of all the samples had to be run on at least two separate gels, six samples were included as controls on each of the two gels per primer combination. This was done to align fragments for scoring purposes.

Table 3.1 **Names of *Clivia* species, perceived colour (if known) and name of breeder plants were collected from, natural occurring populations (N), collection localities in South Africa (indicated if known) and Group numbers (if known) used in this study**

Species	Colour	Plant	Natural occurring populations (N), Locality	Breeder / Collector
C. nobilis	Orange	*C. nobilis*	N, Unknown	J. Spies
C. gardenii	Orange	*C. gardenii*	N, Unknown	J. Spies
C. gardenii var. *citrina*	Yellow	Ngome Yellow	N, Unknown	J. Spies
C. caulescens	Orange	*C. caulescens*	N, Unknown	M. Dower
	Orange	*C. caulescens*	N, Unknown	J. Spies
C. mirabilis	Orange	*C. mirabilis*	N, Unknown	
Interspecific	Hybrid	*C. caulescens* x *C. mirabilis*		J. Winter
C. miniata var. *miniata*		Andrew Gibson	N, Unknown	F. van Niekerk
	Apricot	Apricot		L. van der Merwe
	Apricot	Floradale Apricot		M. Dower
	Blush	Peacevale Blush	N, Unknown	F. van Niekerk
	Blush	Greendale Blush Yellow	N, Unknown	F. van Niekerk
	Blush	Q2 Apple Blossom	N, Unknown	M. Dower
	Unknown	Floradale Apricot x Umtamwuna 32C		M. Dower
	Yellow	Nakamura Big Powerful Yellow x Vico → Pinstripe Yellow		M. Dower
	Yellow	Yellow Offspring		M. Dower
	Peach	Peach Offspring		M. Dower
	Peach	Chubb's Peach (PG)	N, Unknown	P. Gore
	Peach	Gill Hornby Peach	N, Unknown	F. van Niekerk
	Peach	Chubb's Peach (FvN)	N, Unknown	F. van Niekerk

Table 3.1 (continued)

Species	Colour	Plant	Natural occurring populations (N), Locality	Breeder / Collector
C. miniata var. *miniata*	Peach	Gail's Peach	N, Unknown	F. van Niekerk
	Peach	Bonnie Peach	N, Unknown	F. van Niekerk
	Peach	Mvuma Peach	N, Upper Tongaat, KZN	F. van Niekerk
	Peach	De Villiers Variegated Peach		M. Dower
	Picotee	Tarrs Picotee		F. van Niekerk
C. miniata var. *citrina*	Yellow	Watkins Yellow Grobler		A. Grobler
	Yellow	Nakamura Yellow		Unknown
	Yellow	Holl Frick [Group 2]		M. Dower
	Yellow	New Dawn		J. Holmes
	Yellow	Kirstenbosch Yellow		Kirstenbosch Botanical Gardens
	Yellow	Floradale Yellow (MD) [Group 2]		M. Dower
	Yellow	Yellow Hybrid		D. Visser
	Yellow	Karkloof [Group 1]	N, north of Howick, KZN	I. Vermaak
	Yellow	Natal Yellow (FvN) [Group 2]	N, Unknown	F. van Niekerk
	Yellow	Giddy [Group 2]		M. Dower
	Yellow	Wittig Yellow	N, Unknown	A. Grobler
	Yellow	Giddy's Best [Group 2]		R. Lotter
	Yellow	Natal Yellow [Group 2]	N, Unknown	F. van Niekerk

Table 3.1 (continued)

Species	Colour	Plant	Natural occurring populations (N), Locality	Breeder / Collector
C. miniata var. *citrina*	Yellow	Floradale Yellow (FvN) [Group 2]		F. van Niekerk
	Yellow	Cynthia's Best [Group 2]		F. van Niekerk
	Yellow	Pretoria Yellow		Unknown
	Yellow	Vico Gold Nakamura		M. Dower
	Yellow	Vico Gold Smithers		M. Dower
	Yellow	Vico Yellow Nakamura		M. Dower
	Yellow	Nakamura Vico Meristem		M. Dower
	Yellow	Umtamwuna 32C	N, Unknown	M. Dower
	Yellow	Vico Meristem 2		M. Dower
	Yellow	Barbara's Yellow		Unknown
	Yellow	Potterrill	N, Unknown	Unknown
	Yellow	Nogqaza	N, Unknown	Clivia Plantation
	Yellow	Howick Yellow [Group 1]	N, near Howick, KZN	F. van Niekerk
	Yellow	Karkloof Yellow [Group 1]	N, north of Howick, KZN	F. van Niekerk
	Yellow	Blinkwater Yellow [Group 1]	N, Albert Falls Dam, east of Howick, KZN	F. van Niekerk
	Yellow	Dwesa Yellow [Group 2]	N,Transkei, EC	F. van Niekerk
	Yellow	Cobb Inn Yellow	N, Unknown	F. van Niekerk
	Yellow	Smith's Yellow [Group 2]	N, Dwesa, SEC	F. van Niekerk
	Yellow	Eric Dodd / Bashee Yellow [Group 2]	N, Bashee River, SEC	F. van Niekerk
	Yellow	Port St John Yellow / Neville Wyllie [Group 2]	N, east of Umtata, EC	F. van Niekerk

Table 3.1 (continued)

Species	Colour	Plant	Natural occurring populations (N), Locality	Breeder / Collector
C. miniata var. *citrina*	Yellow	Port St John / Rod Ellis [Group 2]	N, east of Umtata, EC	F. van Niekerk
	Yellow	Celtis Kloof	N, Unknown	F. van Niekerk
	Yellow	King Hamelin [Group 1]	N, Darnell area, KZN	F. van Niekerk
	Yellow	Byrne Valley Yellow	N, near Richmond, EC	F. van Niekerk
	Yellow	Mpumulo Yellow [Group 1]	N, Mpumulo, KZN	F. van Niekerk
	Yellow	Alpha Ndwedwe	N, between Stanger and Durban, KZN	F. van Niekerk
	Yellow	Echo Ndwedwe	N, between Stanger and Durban, KZN	F. van Niekerk
	Yellow	Zulu Ndwedwe	N, between Stanger and Durban, KZN	F. van Niekerk
	Yellow	Qora	N, close to coast, SEC	F. van Niekerk
	Yellow	Eshowe [Group 1]	N, near Eshowe, KZN	F. van Niekerk
	Yellow	Nurenberger	N, Unknown	F. van Niekerk
	Yellow	Mvuma Yellow	N, upper Tongaat, KZN	F. van Niekerk
	Yellow	Oribi Yellow	N, near Oribi Gorge, KZN	F. van Niekerk
	Yellow	Dwesa [Group 2]	N, south of Port St John, EC	M. Dower

Localities: EC= Eastern Cape, SEC=South Eastern Cape, KZN=KwaZulu-Natal
FvN=F. van Niekerk, MD=M. Dower, PG=P. Gore

3.3 Data analysis

A binary matrix recording specific AFLP fragments as present (1) or absent (0) was generated for each of the 72 *Clivia* plants. Only reliable (between 200 and 500 bp) and repeatable (at least three replications) fragments were considered. Pairwise genetic distances were expressed as Dice coefficient (Dice, 1945) and cluster analysis was performed using UPGMA (unweighted pairgroup method using arithmetic averages; Sokal & Michener, 1958). Statistical analyses were performed using NTSYS-pc version 2.02i (Rohlf, 1998; Exeter Software, NY, USA) software. Dendrograms were created using the SAHN programme and goodness of fit of clustering to data matrices was calculated using the COPH programme of NTSYS.

Table 3.2 Successful primer pair combinations used to fingerprint all 72 *Clivia* plants

***Eco*RI+3 primer used with *Mse*I+4 primer**	
E-ACC	M-CATC
E-AGC	M-CTGG
E-AGC	M-CATC

3.4 Results

3.4.1 Genetic diversity of all 72 *Clivia* plants

AFLP analysis produced highly reproducible bands showing reliable fragments between 200 and 500 bp. Relatively high levels of polymorphism were detected

among the 72 *Clivia* plants. Reliable results were obtained with the three *Eco*RI/*Mse*I primer combinations selected based on optimisation results (Chapter 2). Primers amplified a total of 148 fragments of which 90% were polymorphic. All cultivars could be individually distinguished.

The number of polymorphic fragments per primer combination ranged from 32 to 55 with an average of 44 polymorphic fragments. Among the three primer combinations tested (E-ACC with M-CATC, E-AGC with M-CATC and E-AGC with M-CTGG), E-AGC in combination with M-CTGG detected the highest number of polymorphic fragments and the highest number of total fragments. (An example of an AFLP profile generated using primer combination E-AGC with M-CATC is given in Figure 3.1.). Each primer combination was evaluated based on the number of polymorphic fragments and total number of amplified fragments. Values are given in Table 3.3.

Pair-wise genetic similarity values (using Dice coefficient) were produced (see Appendix A) to determine the genetic diversity among plants. The co-phenetic correlation coefficient was calculated to test the association between the Dice coefficient matrix and the symmetrical matrix produced from the UPGMA based dendrogram. The co-phenetic correlation can be used as a measure of goodness of fit for cluster analysis. The co-phenetic correlation coefficient was 0.83, indicating a good fit [r (co-phenetic correlation coefficient) >0.9 indicates a very good fit, r=0.9-0.8 indicates a good fit and r<0.8 indicates a poor fit].

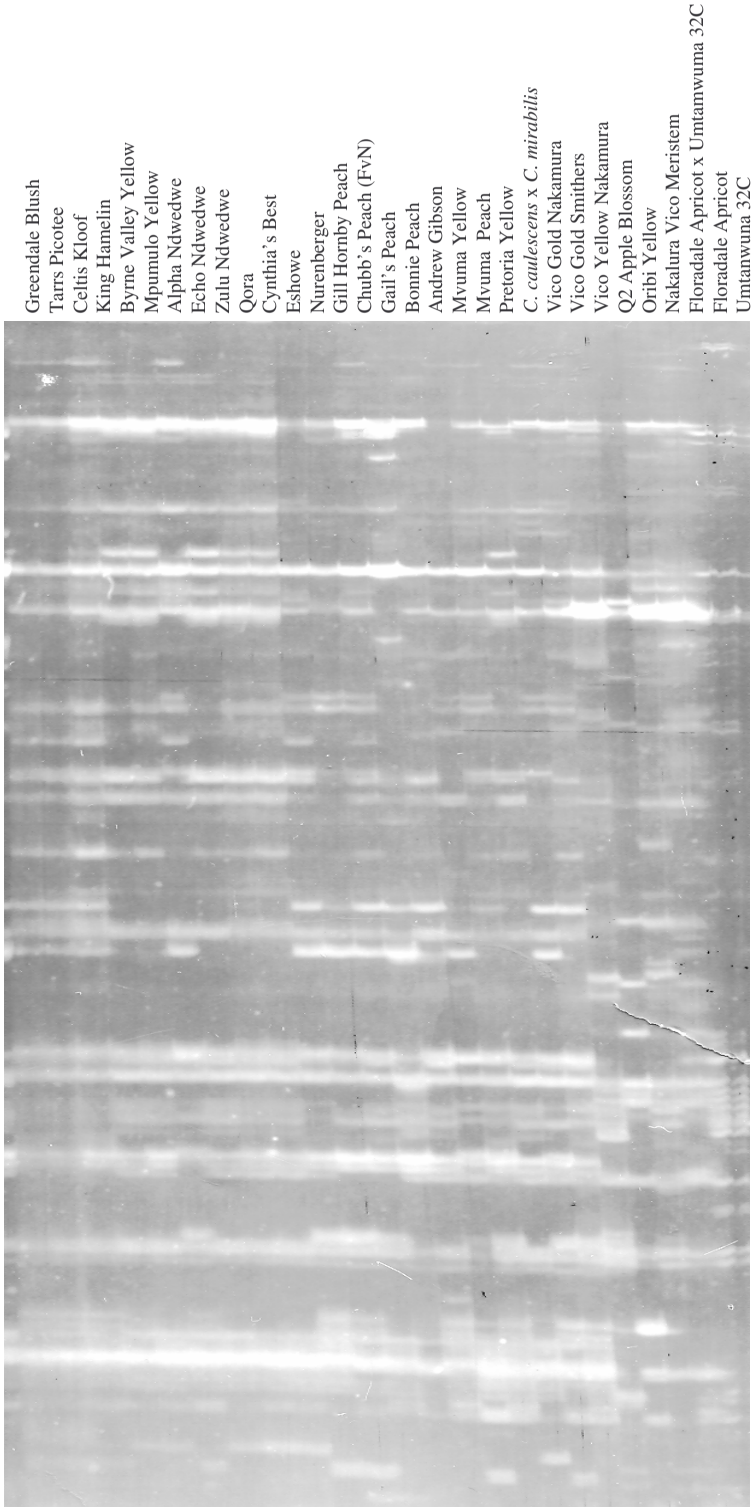


Figure 3.1 **An example of an AFLP profile generated using the primer combination E-AGC with M-CATC. AFLP PCR amplification products were separated on a 5% (w/v) denaturing polyacrylamide gel. The figure represents 31 of the 72 *Clivia* plants tested**

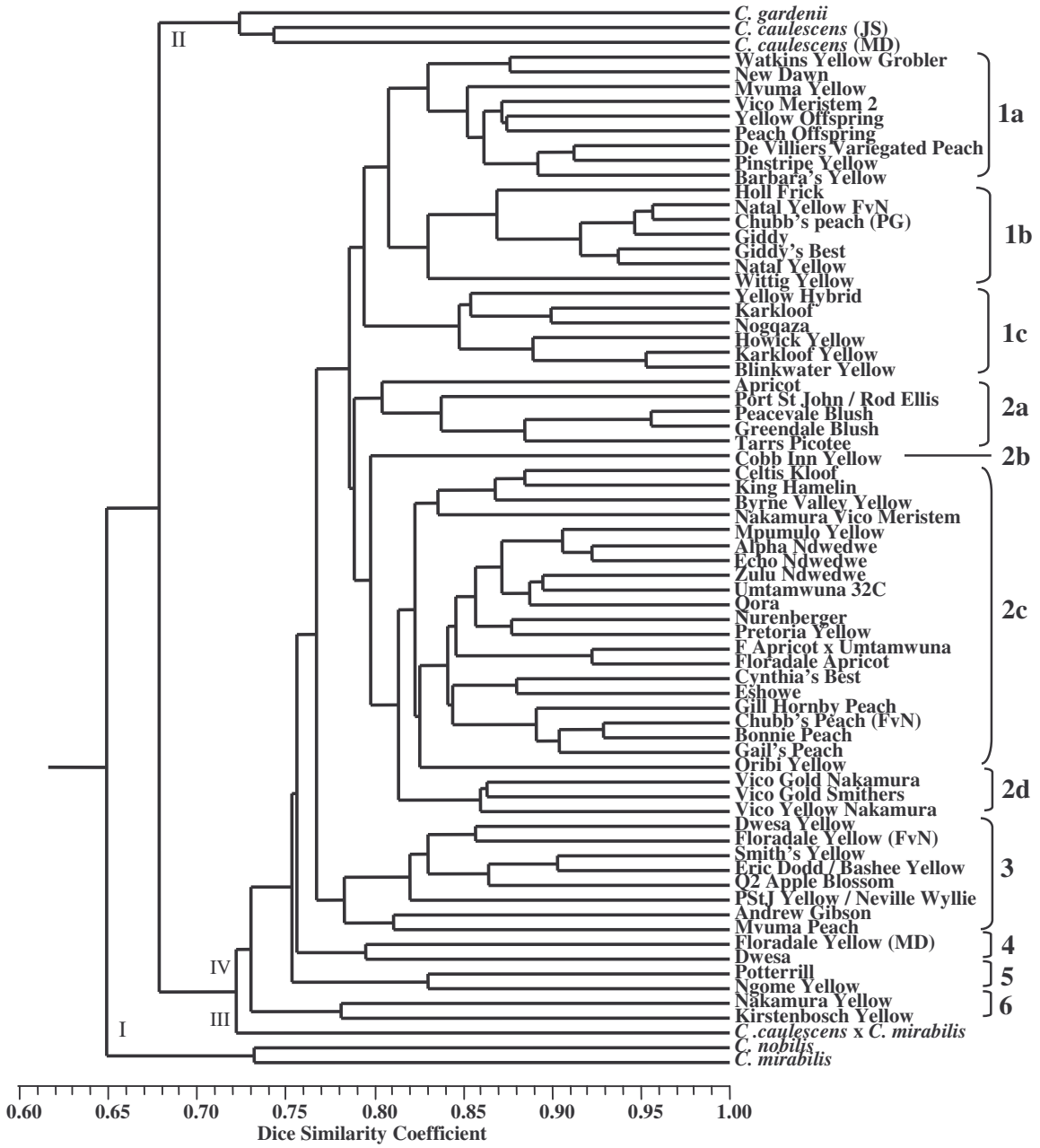
Pair-wise genetic similarity coefficients were calculated using 148 fragments generated by three primer combinations. Pair-wise genetic similarity coefficients varied from 0.520 to 0.957 with an average genetic similarity (GS) of 0.768. Plants Port St John / Neville Wyllie and *C. mirabilis* were the most dissimilar. Within the *C. miniata* var. *citrina* plants, the most dissimilar plants were Cob Inn Yellow and Kirstenbosch Yellow with a GS of 0.611. The two most similar plants were Natal Yellow (FvN) and Chubb's Peach (PG) with a genetic similarity of 0.957.

Table 3.3 Primer combinations, total number of fragments, number of polymorphic fragments and percentage (%) polymorphic fragments used to fingerprint 72 *Clivia* plants

Primer combination	Total number of fragments	Number of polymorphic fragments	% polymorphism
E-ACC; M-CATC	40	32	80
E-AGC; M-CATC	58	55	95
E-AGC; M-CTGG	50	46	92

3.4.1.1 Dendrogram of 72 *Clivia* plants

A dendrogram constructed using Dice's coefficient of similarity and the UPGMA clustering method is given in Figure 3.2. AFLP results correlated well with known pedigree and species data.



F Apricot x Umtamwuna = Floradale Apricot x Umtamwuna 32C; PStJ Yellow / Neville Wyllie = Port St John Yellow / Neville Wyllie

Figure 3.2 Dendrogram of 72 *Clivia* plants generated using three AFLP primer combinations, Dice similarity coefficient and UPGMA cluster analysis with the aid of NTSYS-pc version 2.02i computer package

At a genetic similarity (GS) of 0.72, the 72 *Clivia* plants were divided into four clusters (I-IV). Cluster I contained two species, *C. nobilis* and *C. mirabilis*, cluster II also contained two species, *C. gardenii* and *C. caulescens*, cluster III contained the *C. aulescens* x *C. mirabilis* interspecific hybrid and cluster IV contained the *C. miniata* plants (henceforth referred to as the Miniata cluster).

The Miniata cluster could be further divided into six subclusters. The first subcluster could be subdivided into three subclusters (1a, 1b and 1c). Subcluster 1a contained nine plants. Subcluster 1b consisted of seven plants. The two most similar plants, Natal Yellow (FvN) and Chubb's Peach (PG) (GS of 0.957) were present in this subcluster. Subcluster 1c contained a total of six plants including the third most similar plants, Karkloof Yellow and Blinkwater Yellow (GS of 0.953).

The second subcluster within the Miniata cluster could be subdivided into four subclusters (2a, 2b, 2c and 2d). Subcluster 2a contained the two second most similar plants, Peacevale Blush and Greendale Blush (GS of 0.955). Subcluster 2b contained a single plant, Cobb Inn Yellow. Subcluster 2c was the biggest subcluster containing 21 plants. This subcluster contained the plants Nakamura Vico Meristem, the Ndwedwe cultivars (Alpha, Echo and Zulu), Floradale Apricot and Floradale Apricot x Umtamwuna 32C. Subcluster 2d contained the three Vico plants. The third subgroup contained eight plants. Subgroup 4, 5 and 6 each contained two plants. Subgroup 6 contained the two plants (Nakamura Yellow and Kirstenbosch Yellow) most dissimilar to the rest of the *C. miniata* var. *citrina* plants.

Different flower colours were included in the dendrogram (Apricot, Blush, Peach, Picotee, Orange and Yellow). Two plants of unknown colour, one interspecific hybrid namely Caulescens x Mirabilis and an intraspecific hybrid namely Floradale Apricot x Umtamwuna 32C, were also included. The largest number of *Clivia* plants, namely 50 was known to bare yellow colours (47 *C. miniata* var. *citrina* plants, one *C. gardenii* var. *citrina* and two hybrid plants namely Yellow Offspring and Nakamura Big Powerful Yellow x Vico → Pinstripe Yellow). Two Apricot, three Blush, eight Peach (including the hybrid plant Peach Offspring), six Orange (which include the species *C. nobilis*, *C. gardenii* and, *C. caulescens*) and one Picotee plant were included.

Clusters I and II contained only orange flowered plants whereas cluster III contained the interspecific hybrid *C. caulescens* x *C. mirabilis* of unknown colour. Cluster IV contained the colour forms Apricot, Blush, Peach, Picotee and Yellow. Subcluster 1a contained nine plants with two Peach plants (Peach Offspring and De Villiers Variegated Peach) and the rest (7 of 9 plants) being Yellow. Subcluster 1b contained seven plants with one Peach plant, Chubb's Peach (PG) and the remaining six Yellow plants. Subcluster 1c contained only Yellow plants. Subcluster 2a contained one Apricot, two Blush, one Picotee and one Yellow plant, respectively. Subcluster 2b consisted of a Yellow plant, Cobb Inn Yellow. Fifteen of the 21 plants in subcluster 2c were Yellow, one plant of unknown colour (the hybrid Floradale Apricot x Umtamwuna 32C), one Apricot plant (Floradale Apricot) and four Peach plants (Gill Hornby Peach, Chubb's Peach, Bonnie Peach and Gail's Peach). Three plants in subcluster 2d were Yellow.

The third subgroup contained five Yellow plants, one Blush plant (Q2 Apple Blossom) and a Peach plant (Mvuma Peach). Subgroups 4, 5 and 6 contained only Yellow plants.

3.4.1.2 Yellow 'Group' allocation in 72 *Clivia* plants

Subcluster 1a contained three Group 2 Yellows (Holl Frick, Giddy (MD) and Giddy's Best) out of seven Yellow plants. Out of the six Yellow plants in subcluster 1c, three were Group 1 Yellows (Howick Yellow, Karkloof Yellow and Blinkwater Yellow). Port St John / Rod Ellis was the only Group 2 Yellow in subcluster 2a. Subcluster 2c contained King Hamelin, Mpumulo Yellow and Eshowe as Group 1 Yellows and one Group 2 Yellow (Cynthia's Best).

Subgroup 3 had the largest number of Group 2 Yellows clustering together (Dwesa Yellow, Floradale Yellow (FvN), Smith's Yellow, Eric Dodd / Bashee Yellow and Port St John Yellow / Neville Wyllie). Subgroup 4 contained 2 plants which were both Group 2 Yellows (Floradale Yellow and Dwesa).

3.4.2 Genetic diversity of four *Clivia* species

A species dendrogram constructed using Dice's coefficient of similarity (Dice, 1945) and the UPGMA clustering method is presented in Figure 3.3. Only the four *Clivia* species are indicated together with *C. gardenii* var. *citrina.* AFLP results correlated well with known species data for *C. gardenii*, *C. caulescens* (JS), *C. caulescens* (MD), *C. nobilis* and *C. mirabilis*.

At a GS of 0.660, the six *Clivia* plants divided into two clusters. One cluster contained *C. gardenii* and *C. gardenii* var. *citrina* (at GS 0.680). The second cluster contained *C. nobilis* and *C. mirabilis* (at GS 0.730). The two *C. caulescens* plants formed a subcluster (at GS 0.745) inside the *C. gardenii*-*C. gardenii* var. *citrina* cluster.

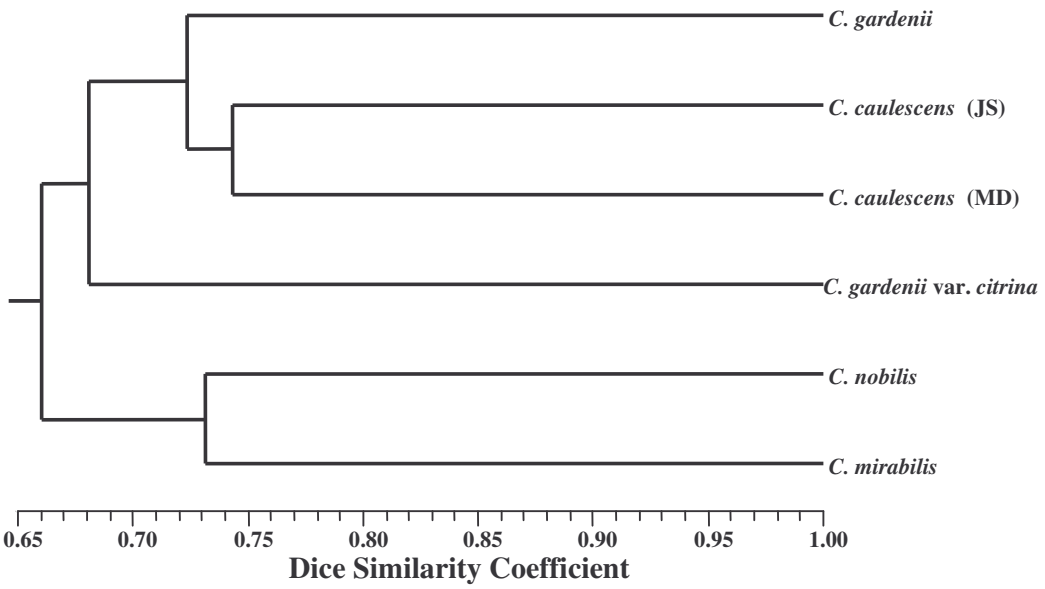


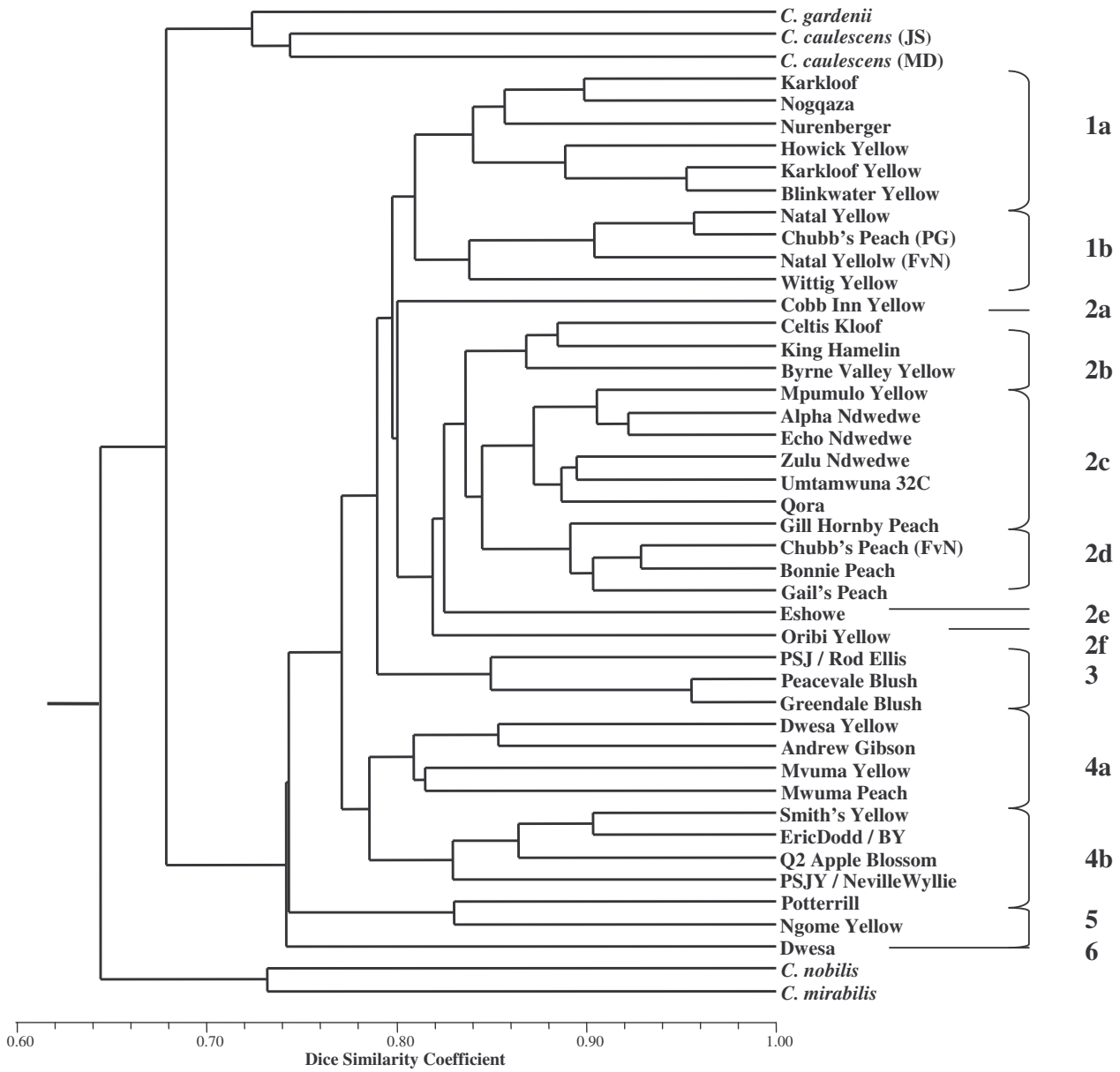
Figure 3.3 Dendrogram of four *Clivia* species and *C. gardenii* var. *citrina* plants indicating their genetic similarity (GS)

3.4.3 Genetic diversity of *Clivia* plants obtained from natural populations

A dendrogram was constructed using a total of 45 *Clivia* plants were collected from natural populations (presented in Figure 3.4). At a GS of 0.680 the *Clivia* plants were divided into three clusters. One cluster contained the species *C. gardenii*, *C. caulescens* (JS) and *C. caulescens* (MD), the second cluster contained the two species

C. nobilis and *C. mirabilis* and the third cluster contained plants obtained from natural populations of *Clivia* (henceforth known as the Natural cluster).

The Natural cluster could be further divided into six subclusters. Subcluster 1 could be subdivided into two subclusters (1a and 1b). Subcluster 1a contained Karkloof, Nogqaza, Nurenberger, Howick Yellow, Karkloof Yellow and Blinkwater Yellow. Karkloof Yellow and Blinkwater Yellow were the third most similar plants (GS of 0.942). Subcluster 1b contained Natal Yellow, Chubb's Peach (PG), Natal Yellow (FvN) and Wittig Yellow. Chubb's Peach (PG) and Natal Yellow were the second most similar plants (GS of 0.948). Subcluster 2 could be divided into six subclusters (2a to 2f). Subcluster 2a contained Cobb Inn Yellow and subcluster 2b Celtis Kloof, King Hamelin and Byrne Valley. Subcluster 2c contained Mpumulo Yellow and the Ndwedwes (Alpha Ndwedwe, Echo Ndwedwe and Zulu Ndwedwe) together with Umtamwuna 32C and Qora. Subcluster 2d contained Gill Hornby Peach, Chubb's Peach (FvN), Bonnie Peach and Gail's Peach. Subcluster 2e contained Eshowe and subcluster 2f Oribi Yellow. Subcluster 3 contained the plants Port St John / Rod Ellis, Peacevale Blush and Greendale Blush. Peacevale Blush and Greendale Blush were the most similar plants (GS of 0.955). Subcluster 4 could be subdivided into two subclusters (4a and 4b). Subcluster 4a contained the plants Dwesa Yellow, Andrew Gibson, Mvuma Yellow and Mvuma Peach. Subcluster 4b contained the plants Smith's Yellow, Eric Dodd / Bashee Yellow, Q2 Apple Blossom and Port St John / Neville Wyllie. Subgroup 5 contained two plants (Potterrill and Ngome Yellow) and subgroup 6 one plant (Dwesa).



PSJY / Neville Wyllie = Port St John / Neville Wylliw; PSJ / Rod Ellis= Port St John / Rod Ellis; Eric Dodd / BY = Eric Dodd / Bashee Yellow

Figure 3.4 Dendrogram of 45 *Clivia* plants obtained from natural populations

3.4.4 Genetic diversity of *Clivia* obtained from cultivation

A dendrogram was constructed using twenty-seven plants from the cultivated group (Figure 3.5). At a GS of 0.778 the *Clivia* plants divided into five clusters. Cluster 1 could be subdivided into subclusters 1a to 1e. Subcluster 1a contained Watkins Yellow and New Dawn. Subcluster 1b contained Vico Meristem 2, Yellow Offspring, Peach Offspring, De Villiers Variegated Peach, Pinstripe Yellow and Barbara's Yellow. Subcluster 1c contained Holl Frick, Giddy (MD) and Giddy's Best (RL). Subcluster 1d contained only Nakamura Yellow, whereas subcluster 1e contained Apricot and Tarrs icotee. Only Floradale Yellow (MD) occupied subcluster 2a and subcluster 2b contained Yellow Hybrid, Cynthia's Best, Pretoria Yellow, the hybrid Floradale Apricot x Umtamwuna and Floradale Apricot. Subcluster 2c contained Vico plants (Vico Gold Nakamura, Vico Gold Smithers and Vico Yellow Nakamura). Nakamura Yellow was the only plant in subcluster 2d. Clusters 3, 4 and 5 contained one plant each, Floradale Yellow (FvN), *C. caulescens* x *C. mirabilis* and Kirstenbosch, respectively.

The most dissimilar plants were Kirstenbosch Yellow and *C. caulescens* x *C. mirabilis* (at a GS of 0.600). Most similar plants were Holl Frick and Giddy's Best at a GS of 0.923.

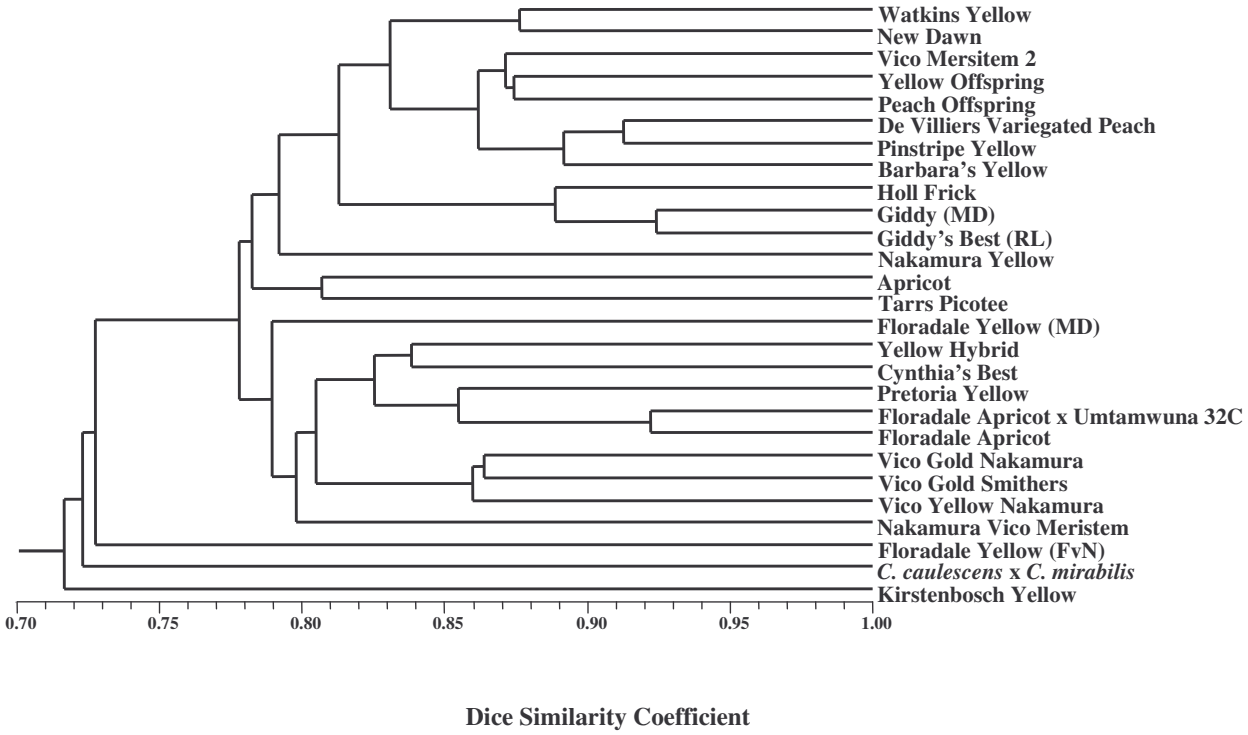


Figure 3.5 Dendrogram of 27 *Clivia* plants obtained from cultivation

3.4.5 Genetic diversity of the Giddy plants

A dendrogram of the Giddy plants was constructed using Dice's coefficient of similarity and UPGMA clustering (Figure 3.6). AFLP results correlated well with known pedigree data.

The dendrogram divided into two main clusters at GS 0.832. Cluster 1 subclustered into 1a and 1b. Subcluster 1a contained the plant Holl Frick. Subcluster 1b contained the most similar plants Giddy (MD) and Giddy's Best (RL) (GS of 0.924). Cluster 2 contained Cynthia's Best.

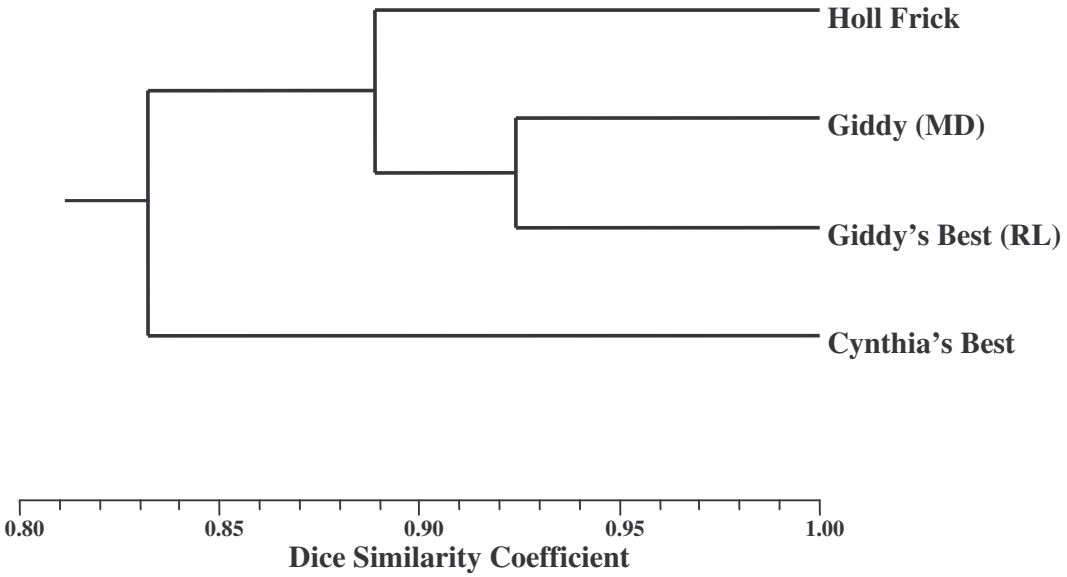


Figure 3.6 Dendrogram of four different Giddy plants showing their genetic similarity (GS)

3.4.6 Genetic diversity of the Vico plants

A dendrogram of the Vico plants was constructed using Dice's coefficient of similarity and UPGMA clustering (Figure 3.7). AFLP results correlated well with known pedigree data.

The dendrogram divided into two main clusters. Cluster 1 divided into subclusters 1a and 1b. Subcluster 1a contained the third most similar plants, Vico Gold Nakamura and Vico Yellow Nakamura (GS of 0.863). Subcluster 1b contained the second most similar plants Vico Gold Smithers and Umtamwuna (GS of 0.869) and the most similar plants Floradale Apricot x Umtamwuna 32C and Floradale Apricot (GS of 0.922). Cluster 2 contained only a single plant, Nakamura Vico Meristem. Floradale Apricot x Umtamwuna 32C is a hybrid obtained from the two plants Umtamwuna 32C and Floradale Apricot. Results indicated that the hybrid was closer related to one parent (Floradale Apricot) (GS of 0.922) than to the other parent (Umtamwuna 32C) (GS of 0.843).

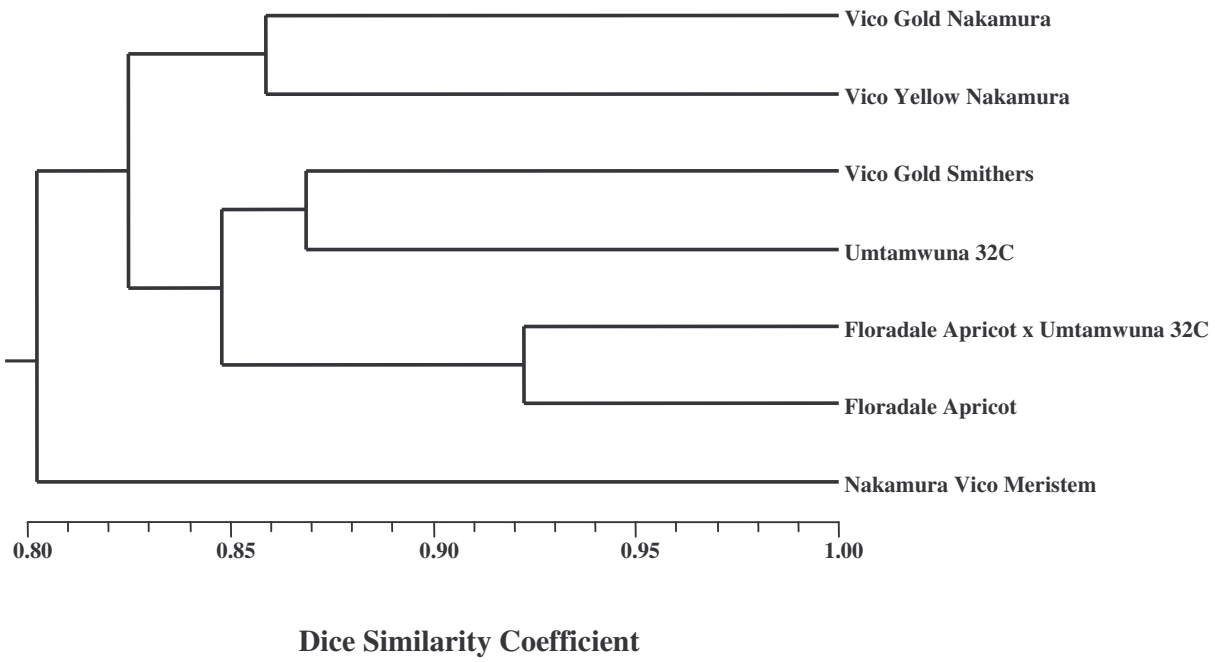


Figure 3.7 Dendrogram containing four different *Clivia* Vico plants: a reputed Vico plant (Nakamura Vico Meristem), Floradale Apricot, Umtamwuna 32C and a hybrid Floradale Apricot x Umtamwuna 32C

3. 5 Discussion

AFLP analysis was successful in detecting genetic diversity and determining genetic relationships within closely related cultivated *Clivia* plants. Relative levels of genetic diversity (35%), as expected from known pedigree and species data, existed among *Clivia* plants. Genetic diversity within *C. miniata* and *C. miniata* var. *citrina* plants was also relatively high at 27%. Levels of polymorphism are high in comparison to cultivated crops like groundnut *Arachis hypogaea* L. (2.78%) (Herselman, 2003) and coffee *Coffea arabica* L. (30.4%) (Bekele, 2005). Previous findings using RAPD

analysis by Ran *et al*. (2001b) to distinguish *Clivia* plants support findings as far as differentiating between species is concerned. The level of AFLP polymorphic fragments discovered (average of 90% between three primer combinations tested) represents inherent variability among *Clivia* plants at DNA level. Similar results were obtained by Ran *et al.* (2001b) using RAPD analysis where 94% of the detected RAPD fragments were polymorphic. All 72 *Clivia* plants, some very closely related, could be uniquely differentiated.

Morphologically most cultivated *C. miniata* var. *citrina* plants cannot be distinguished between by *Clivia* enthusiasts. Cultivars bearing names misrepresentative of their true origin and pedigree often left breeders to base entire breeding programmes on colour alone. Flower colour varies in shade and intensity is strongly influenced by environmental factors, such as exposure to sun or light. Based on colour alone, distinguishing between different cultivars remained a challenge, until AFLP analysis opened up the possibility to distinguish between different cultivars at DNA level.

This is the first report on using AFLP analysis to distinguish between closely related *Clivia* plants, intraspecifically. Previous studies using karyotyping, DNA sequencing and RAPD analysis (Ran *et al*., 1999, 2001a, b; Conrad & Reeves, 2002, Booysen, 2003, Swanevelder, 2003) focussed on interspecies relationships.

3.5.1 Genetic diversity of all 72 *Clivia* plants

One of the four main clusters (at a GS of 0.72) in the AFLP dendrogram constructed using all 72 *Clivia* plants contained a single plant namely *C. caulescens* x *C. mirabilis*. This plant was developed from an interspecific cross between *C. caulescens* and *C. mirabilis*. *Clivia caulescens* x *C. mirabilis* clustered between *C. caulescens* and *C. mirabilis* in the dendrogram and not between or with the other *C. miniata* or *C. miniata* var. *citrina* plants. The *C. caulescens* x *C. mirabilis* hybrid was more closely related to *C. caulescens* (GS of 0.680) than *C. mirabilis* (GS of 0.650), but even more closely related to the *C. miniata* var. *citrina* plants (GS of 0.720). Furthermore, clustering based on AFLP genetic data confirmed that *C. gardenii*, *C. caulescens*, *C. nobilis* and *C. mirabilis* are clearly distinct from all colour forms of *C. miniata* (including *C. miniata* var. *citrina*). Subcluster 5 contained Ngome Yellow. This is said to be the yellow form of *C. gardenii*. However, this cultivar does not group closely to the *C. gardenii* species.

Subcluster 2a and 2b (Figure 3.2) contained members of the reputed Vico cultivars. Subcluster 1a contained Vico Meristem 2, a reputed member of the Vico cultivars. The separate grouping could be due to wrong name allocation by breeders in the process of cultivation.

Giddy and Giddy's Best clustered together in subcluster 1b. Pair-wise Dice genetic similarity correlations confirmed the close relationship with a GS of 0.924. This confirms known pedigree data that Giddy and Giddy's Best are closely related.

The plants Natal Yellow and Natal Yellow (FvN) clustered together in subcluster 1b in the dendrogram confirming their close relationship. The name 'Natal Yellow' is esteemed as one of the few yellow *Clivia*, producing yellow flowers of high quality. This line has been under cultivation for approximately 20 years and due to its popularity, this name has been applied to various plants.

Based on pedigree data, Floradale Apricot was crossed with Umtamwuna 32C to produce the hybrid Floradale Apricot x Umtamwuna 32C. The hybrid clustered closer to Floradale Apricot (GS of 0.922) than to Umtamwuna 32C (GS of 0.843) suggesting a closer genetic similarity to Floradale Apricot compared to Umtamwuna 32C. Many *Clivia* enthusiasts hold the opinion that offspring are closer related to the pod parent than the pollen parent. Floradale Apricot was the pod parent whereas Umtamwuna 32C was the pollen parent.

Another paternity test was included in the sample group of 72 *Clivia* plants. According to known pedigree data, when De Villiers Variegated Peach as a pod parent is crossed to a Group 1 Yellow (Group 1 and Group 2 Yellows will be discussed in section 3.5.2), Peach flower coloured offspring will result. When De Villiers Variegated Peach as a pollen parent is crossed with a Group 1 Yellow, yellow flower coloured offspring will result. Offspring of De Villiers Variegated Peach were included in the sample group of 72 *Clivia* plants, namely Yellow Offspring and Peach Offspring. In the dendrogram De Villiers Variegated Peach clustered with Yellow Offspring and Peach Offspring in subcluster 1a. A GS of 0.838 for De Villiers Variegated Peach and Peach Offspring was observed and a GS of 0.901 was observed

for De Villiers Variegated Peach and Yellow Offspring (Spies, personal communication)

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The Miniata cluster contained various colour forms of Apricot, Blush, Peach, Picotee, Orange and Yellow. As would be expected, from the number of plants included in the dendrogram, Yellow was the predominant colour represented, followed by Peach. With regard to the Blush flower coloured *Clivia* plants, Peacevale Blush clustered with Greendale Blush in subcluster 2a whereas Q2 Apple Blossom was relatively removed being in subgroup 3. Peach Offspring and De Villiers Variegated Peach clustered in subgroup 1a whereas Chubb's Peach and Mvuma Peach clustered in subcluster 1b and subgroup 3 respectively. The other Peach plants Gill Hornby Peach, Chubb's Peach (FvN), Bonnie Peach and Gail's Peach clustered together in subcluster 2c making this the largest colour cluster other than Yellow.

3.5.1.1 Known 'Group' number allocations to *C. miniata* var. *citrina* plants and geographical distribution

Clivia plants obtained from natural populations (localities of plants are presented in Table 3.1) in the natural geographic distribution areas (areas as presented in Table 1.1) were included in AFLP analysis. Only subclusters 1c, 2c and 3 in Figure 3.1 contained plants that were collected from similar geographical areas. In subcluster 1c Karkloof, Blinkwater, Howick Yellow and Karkloof Yellow were all obtained from areas in and around the town of Howick in KwaZulu-Natal. Seven of the 21 plants from subcluster 2c were from areas in KwaZulu-Natal (Darnell, Richmond, Eshowe and Ndwedwe) representing a broader distribution range than subcluster 1c. Within subcluster 3, Dwesa Yellow and Port St John / Neville Wyllie were collected in the

Eastern Cape, Smith's Yellow and Eric Dodd / Bashee Yellow from the South Eastern Cape and Mvuma Peach from KwaZulu-Natal. This again represents a sizeable distribution range within a cluster.

Since plants from the same geographical areas were distributed throughout the dendrogram with only a few clustering together, it is an indication that there exists wide genetic diversity within geographical populations. This is important for Biodiversity conservation programmes and conserving genetic diversity within *C. miniata*.

Known Group 1 Yellow and Group 2 Yellow plants were present throughout the entire dendrogram. The majority of known Group 1 Yellow plants grouped together in subcluster 1a having been collected from areas in Kwazulu Natal. Group 2 Yellow plants were grouped together more towards the lower half of the dendrogram, all having been collected from the Eastern Cape.

In subcluster 1b, five of the seven plants were known to be Group 2 Yellow plants. Four of the six plants contained in subcluster 1c were Group 1 Yellows and five out of eight plants in subcluster 3 were Group 2 plants. All plants in subcluster 4 were Group 2 Yellow plants.

It would seem that dendrogram information generated from this study can not offer clear clues to exact geographical distribution of plants obtained from natural populations. More plants from natural populations would have to be included. (Chapter 4 examines the possible phylogeny of plants from natural populations and

Group number allocation that can be applied to plants from known areas with unknown Group numbers). However, destruction of natural *Clivia* habitat is severe and populations of naturally occurring *Clivia* plants are rapidly disappearing (F. van Niekerk, personal communication; Williams, 2005). Further research is required to establish exact geographical distribution patterns in relation to GS between plants from natural populations.

3.5.2 Genetic diversity of four *Clivia* species

From the Species dendrogram (Figure 3.2) different species of *Clivia* were genetically dissimilar enough to be detected as separate plants and species. The two plants of *C. caulescens* clustered together, confirming results of Ran *et al*. (2001b).

Ran *et al*. (2001b) used RAPD analysis to distinguish between plants of *C. nobilis*, *C. miniata*, *C. gardenii*, *C. caulescens* and *C. robusta*. Results indicated that *C. nobilis* was the most dissimilar species to *C. miniata*. This result was confirmed during the present study, since *C. nobilis* and *C. mirabilis* (not included in Ran *et al*.'s (2001b) study) were genetically most distantly related to *C. miniata*. In Ran *et al.*'s study (2001b) *C. miniata* and *C. gardenii* clustered together with *C. caulescens* clustering in between *C. nobilis* and *C. miniata* / *C. gardenii*. Results indicated that *C. miniata* was closely related to *C. gardenii*. The preset study revealed that *C. miniata* was closely related to both *C. gardenii* and *C. caulescens* and the most distintly related to *C. nobilis* and *C. mirabilis*.

Ngome Yellow, the *C. gardenii* var. *citrina* plant, clustered together with the *C. miniata* plants, being more similar to *C. miniata* compared to *C. caulescens* or *C.*

gardenii. According to information from breeders, Ngome Yellow is taxonomically classified as *C. gardenii* var. *citrina*, a yellow form of *C. gardenii*. Genetic fingerprinting results from this study did not confirm this classification.

When the position of plants belonging to *C. miniata* var. *citrina* (being regarded as yellow forms of *C. miniata*) are considered, these plants fell well within the Miniata cluster containing both *C. miniata* and *C. miniata* var. *citrina* plants which furthermore includes different colours (Apricot, Peach, Picotee, Blush and Yellow). Evaluation at subgenomic level using AFLP analysis did not separate *C. miniata* var. *miniata* from *C. miniata* var. *citrina.* No exclusive clustering together of only *C. miniata* var. *citrina* was observed, therefore no clear taxonomic distinction is suggested between *C. miniata* var. *miniata* and *C. miniata* var. *citrina*.

3.5.3 Genetic diversity of the Vico plants

Plants bearing the 'Vico' name are reported to be of superior flower quality, possessing a depth of yellow unlike most other yellow *Clivia*. Vico is believed to have Eshowe Yellow ancestors from a wild population of *Clivia* near Eshowe and was sent to Europe and from there to Dr Hirao, a respected breeder of ornamentals, in Japan. Hirao named it Smither's Yellow after the man who sent him the plant. The 'Vico' designation refers to Vico Morcote in Switzerland (Dixon, 2005). Plants produced from meristem tissue culture were sold under the name 'Vico'. The plant bearing the name Nakamura Vico Meristem did not cluster together with the other Vico plants (Vico Gold Nakamura, Vico Yellow Nakamura or Vico Gold Smithers). Vico Meristem 2 is also removed from the Vico cluster. Could the plants Nakamura Vico Meristem and Vico Meristem have originated from meristem culture? Variation might

be due to factors such as choice of plant part to culture as well as spontaneous mutations occurring during the tissue culture process.

3.5.4 Genetic diversity of the Giddy plants

Cynthia Giddy was a formidable *Clivia* enthusiast. Plants bearing the 'Giddy' name are sought-after and expensive. Based on AFLP data and known pedigree information, plants bearing the 'Giddy' name available for this study were true Giddy. Giddy, Giddy's Best and Cynthia's best all clustered together. Results confirmed that the name Giddy was correctly applied to these plants.

3.6 Conclusion

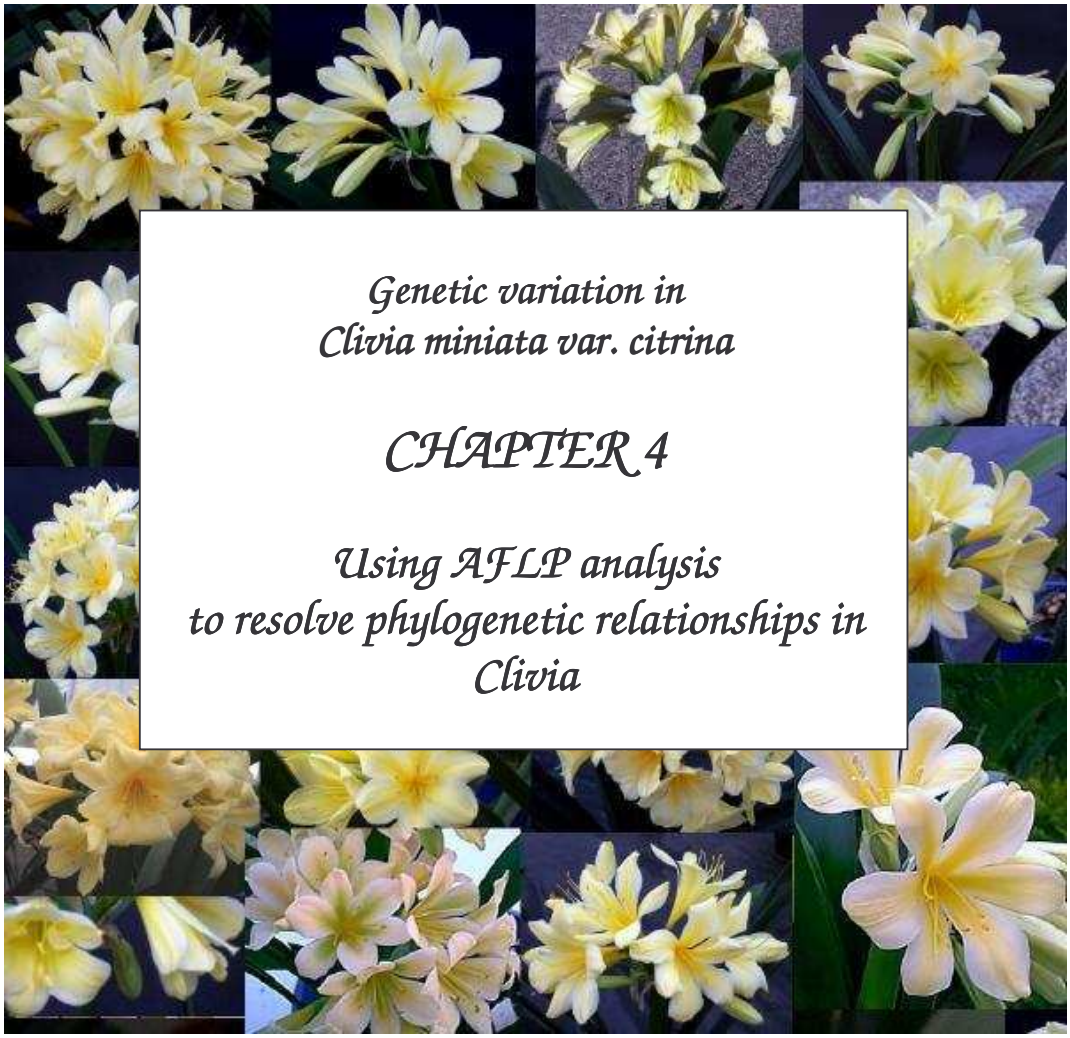
Using AFLP analysis we succeeded in distinguishing between different *Clivia* plants. The plants available for scrutiny were all genetically distinct enough. However, based on known pedigree data, names allocated to plants might not be truly representative of the true origin of plants (e.g. Vico Meristem plants). Material obtained from different breeders (Natal Yellow, Vico and Giddy) could be distinguished on DNA level.

Clivia breeders interested in a particular plant as basis for a new breeding programme can now select distantly related plants, based on data generated from AFLP data. Better directed breeding practices can be applied and misnomers can be eliminated from the commercial *Clivia* industry.

Knowing how closely related a breeder's breeding stock is, is an ideal. Furthermore, knowing how closely related different breeder's breeding stock is, can offer insights into dormant areas of the commercial industry.

Registration of new commercial cultivars accompanied by DNA fingerprint evidence (similar to what was done in this study to distinguish between plants) can now be done. Legal implications for this type of research conducting plant fingerprinting is that fraudulent claims against breeders selling plants dissimilar to the allocated name the plant was sold under can now be exposed.

Generation of genetic linkage maps can speed progress toward unravelling the colour heritability aspect. As the ever-changing market trend toward preference for Apricot, Peach or Blush, the knowledge generated from such linkage maps can speed up production of quality plants before demand surpasses supply. Considering the present market trend in the *Clivia* industry, it seems that a study evaluating these colour aspects might be just timely for the next colour preference demand.



Genetic variation in
Clivia miniata var. citrina

CHAPTER 4

Using AFLP analysis to resolve phylogenetic relationships in Clivia

4.1 Introduction

Inferring phylogenetic relationships among closely related plant species is often difficult due to lack of molecular markers exhibiting enough nucleotide variability at this taxonomic level (Despres *et al*., 2003). Booysen (2003) investigated phylogenetic relationships among members of the genus *Clivia* using chloroplast DNA (*trnL-F* region) and the gene *matK*. The *trnL-F* region and *matK* gene provided enough variation to partially resolve the phylogeny of the genus *Clivia*. The genus *Clivia* was found to be monophyletic when placed into phylogenetic relationship within the family Amaryllidaceae (Booysen, 2003). Interspecies relationships between *C. miniata*, *C. gardenii* and *C. caulescens* could not be resolved using *matK* (Booysen, 2003).

Evolutionary relationships between members of the genus *Clivia* have been elusive. Determining the phylogeny of species is important in order to indicate the evolutionary path of the organism and the relationship that exists between organisms by combining molecular and statistical techniques (Li, 1997; Qui *et al*., 1999). However, the low level of nucleotide variability detected at intragenus level for both *trnL-F* and *matK* necessitated the use of an additional molecular technique. The aim of this study was to determine if AFLP data could be used to establish phylogenetic relationships in the genus *Clivia* by evaluating plants obtained from natural populations of *Clivia*.

4.2 Materials and methods

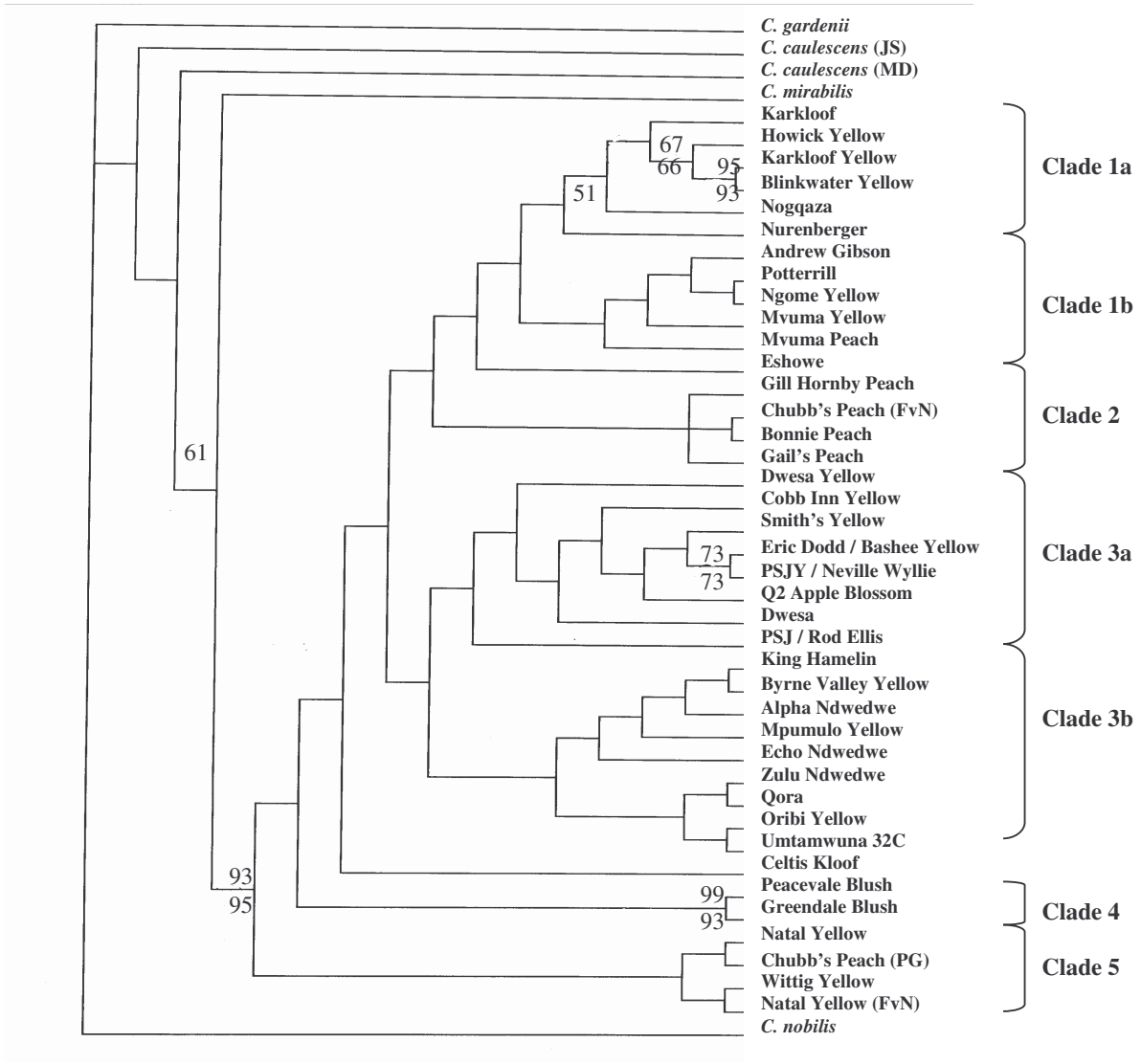
4.2.1. AFLP analysis

AFLP analysis (as described in Chapter 3) was conducted on 72 plants of the genus *Clivia* incorporating the species *C. nobilis, C. miniata*, *C. miniata* var. *citrina, C. gardenii*, *C. gardenii* var. *citrina*, *C. caulescens* and *C. mirabilis.*

Parsimonious cladograms were generated for 45 *Clivia* plants obtained from natural populations using a heuristic search and Tree bisection and reconnection (TBR) on the AFLP data using the software package PAUP* version 4.01b (Swofford, 2002). The species *C. nobilis* (one plant), *C. miniata* var. *miniata* (10), *C. miniata* var. *citrina* (29 plants), *C. gardenii* (one plant), *C. gardenii* var. *citrina* (one plant), *C. caulescens* (two plants) and *C. mirabilis* (one plant) completed the 45 *Clivia* plants.

4.3 Results

Two equally parsimonious trees were obtained from the total data set, with a tree length of 786 and a Consistency Index (CI) of 0.995. A strict consensus tree was computed and is given in Figure 4.1. In order to determine the evolutionary relationships between *Clivia* plants obtained from natural populations, Group 1 and Group 2 Yellow plants were plotted onto a map of South Africa (Figure 4.2).



PSJY / Neville Wyllie= Port St John Yellow / Neville Wyllie; Eric Dodd / Bashee Ye=Eric Dodd / Bashee Yellow; PSJ / Rod Ellis = Port St John / Rod Ellis

Figure 4.1 **Cladogram for *Clivia* plants obtained from natural populations. A strict consensus cladogram was generated from AFLP data containing 45 of the 72 plants analysed. Only species and plants originating from natural populations were included to attempt to establish evolutionary relationships within natural populations (Bootstrap values indicated above branch, Jacknife values indicated below branch).**

Pretoria
Soshanguve
Nelspruit
Matsulu
Witbank
Barberton
Maputo
Mmabatho
Krugersdorp
Tembisa
Itoseng
Soweto
Johannesburg
Mbabane
Potchefstroom
Vereeniging
Manzini
Sharpeville
Klerksdorp
SWAZILAND
Volksrust
Kroonstad
Kutloanong
Welkom
Osizweni
Mondlo
Dundee
Harrismith
Postmasburg
Phuthaditjhaba
Upington
Kimberley
Ladysmith
Bloemfontein
Teyateyaneng
Stanger
Botshabelo
Maseru
Pietermaritzburg
SOUTH AFRICA
LESOTHO
Durban
30°
Park Ryne
De Aar
Aliwal North
Ibhayi
Umtata
Cradock
Queenstown
Rini
INDIAN OCEAN
Graaff-Reinet
Sada
Gcuwa
Fort Beaufort
Somerset East
Bisho
East London
Mdantsane
Grahamstown
Oudtshoorn
Uitenhage
George
Knysna
Port Elizabeth
30°
35°
25°
South Africa, Lesotho, and Swaziland
0 100 200 300 400
Kilometers
© World Sites Atlas (sitesatlas.com)
Area 2 (Group1 Yellow plants)
Unknown Group numbers
Area 1 (Group 2 Yellow plants)

Figure 4.2 **Map of South Africa indicating geographic localities of *Clivia* plants obtained from natural populations. Group 1 Yellow plants were found to be from Area 2 (KwaZulu-Natal) whereas Group 2 Yellow plants were found to be from Area 1 (Eastern Cape). Plants found between Areas 1 and 2 had Unknown Group numbers.**

4.4 Discussion

To determine phylogenetic relationships only plants not subjected to recent human attempts at trait improvement were considered. Cultivated plants were consequently excluded from this study (the hybridisation process would have introduced convergence rather than divergence in the cladogram resulting in reticulate evolution).

The cladogram revealed a number of well defined clades. Clade 1a consisted of the two Karkloof specimens (Karkloof and Karkloof Yellow), Blinkwater Yellow and Howick Yellow. Monophyly of plants in this clade were well supported by Bootstrap and Jackknife values (Figure 4.1). The most interesting aspect of this clade was that the two Karkloof specimens differed significantly. This may be the result of possible improvement of the plant obtained from a particular breeder, whereas the other plant was collected from a natural population. The notion that Karkloof and Blinkwater represent the same taxonomic entity (Fred van Niekerk, personal communication) was strongly supported by high Bootstrap and Jackknife values (Figure 4.1).

The four plants in clade 1a all represent Group 1 Yellow plants. These plants were all collected in area 2 (Figure 4.2). Nogqaza and Nurenberger appeared as basal taxa to this clade. Classification of these two plants (Group 1 or Group 2 Yellow) and their localities are unknown. However, their grouping in the cladogram suggested that they should probably be Group 1 Yellows from the same geographic area.

Clade 1b contained the plants Andrew Gibson, Potterrill, Ngome Yellow, Mvuma Yellow, Mvuma Peach and Eshowe. Ngome Yellow was collected in the Ngome

Forest, north of region 2 (Figure 4.2). This plant is classified as *C. gardenii* var. *citrina* taxonomically but seems to be closer related to *C. miniata* plants represented in clade 1b than to the species *C. gardenii.* Mvuma Peach, Mvuma Yellow and Eshowe had known geographic localities, in the Upper Tongaat area and near Eshowe. This suggested that Mvuma Peach, Mvuma Yellow and Eshowe were obtained from area 2, making them probable Group 1 Yellow plants.

Plants in clade 2 were all peach flower coloured. Chubb's Peach (FvN) originally came from an unknown geographic locality as did the other peach flowered plants. However, clade 3a contained the plants Dwesa Yellow, Smith's Yellow, Eric Dodd / Bashee Yellow, Port St John Yellow / Neville Wyllie, Dwesa and Port St John / Rod Ellis. These plants were known Group 2 Yellow plants, all with known localities in the Eastern Cape (area 1 in Figure 4.2). Eric Dodd / Bashee Yellow and Port St John Yellow / Neville Wyllie were well supported with bootstrap and jacknife values (as indicated in Figure 4.1). Within clade 3a, only Q2 Apple Blossom and Cobb's Inn were of unknown locality and group. Based on the grouping within clade 3a, Q2 Apple Blossom and Cobb Inn are most likely from area 1, belonging to Group 2 Yellow plants.

Clade 3b contained plants from different Group numbers. Group 1 Yellow plants in this clade were King Hamelin and Mpumulo Yellow. Both plants had known geographic localities (falling within area 2 in Figure 4.2). Byrne Valley Yellow, Qora, Oribi Yellow and the Ndwedwe plants (Alpha, Echo and Zulu) had known geographic localities but unknown Group numbers, whereas Umtamwuna 32C had both unknown geographic locality and Group number. Based on geographic localities, Byrne Valley

Yellow, Qora and Oribi Yellow fell within area 2 (Figure 4.2), indicating the probability that these plants are Group 2 Yellow. Umtamwuna 32C was a sister clade to Oribi Yellow and Qora, suggesting that it might also be a Group 2 Yellow. Geographically, the Ndwedwe plants occurred between areas 1 and 2. Currently, the Ndwedwe plants have a separate group allocation, that of Alpha Group.

Celtis Kloof forms a sister clade to clade 3b. Geographically, it falls near area 2 (Figure 4.2). This plant is the only known Group 3 Yellow. Clade 4 was well supported (Figure 4.1) and contained the plants Peacevale Blush and Greendale Blush. Clade 5 contained Chubb's Peach, Wittig Yellow and the two Natal Yellow plants [Natal Yellow and Natal Yellow (FvN)].

From the name these plants have in common, it would be expected that both Chubb's Peach (PG) and Chubb's Peach (FvN) would group together with the other peach flowered plants. Somehow, plant material could have gotten mixed up and the wrong material could have been supplied for DNA extraction. Human error in lab and field might be responsible for this grouping.

The majority of clades observed correspond with either genetic (grouping into a specific yellow group) or geographical data (Figure 4.2). Since bootstrap and jackknife support do not confirm all lineages beyond doubt, more taxa should be included to confirm the monophyletic origin of these groups. The inclusion of plants with orange flowers should also be investigated as this may strengthen the support for certain branches. Although certain colour mutations such as peach and blush mainly occur in a specific clade, deviations from this pattern, for example Mvuma Peach,

indicate that these colour mutations are not always linked to a divergence in the tree and similar mutations may occur more than once in the phylogenetic development. The two plants totally deviating from this pattern, Ngome Yellow and Chubb's Peach (PG) should be collected and studied again to determine if any human error caused these deviations or if these plants were wrongly named.

Group 1 Yellows are much more frequent in cultivation than Group 2 Yellows. An explanation for this phenomenon may be the cultivation history of *Clivia*. *Clivia* export started in the mid-nineteenth century when the first plants were sent to England and Belgium and later to Japan and other countries. Although the first plants were said to be *C. nobilis*, it was *C. miniata* that captured the World's eye. Gardeners of noblemen, noblemen and members of the general public discovered the ease with which *Clivia* could be propagated.

Plants available to the first collectors travelling on expedition in South Africa would have been *Clivia miniata*, mainly from KwaZulu-Natal. The greater number of Group 1 Yellows available in breeder's collections today could be ascribed to the geographical origins of these yellow forms and the fact that many Group 1 Yellows are fertile when self-pollinated. Greater accessibility to area 2 in KwaZulu-Natal in the mid-nineteenth century meant more material of Group 1 Yellows were probably collected from natural *Clivia* populations in those surrounding areas. These plants were probably self-pollinated and produced numerous seeds for the propagation of true breeding yellows.

Group 2 plants possibly originated in the Eastern Cape or areas of KwaZulu Natal and neighbouring KwaZulu Natal. This area was less accessible. Added to this, Group 2 Yellows were limited in being self-incompatible. It is therefore logical that more Group 1 Yellows would be produced than Group 2 Yellows.

It appears as if the mutation for Group 2 Yellows occurred first.This was followed by a mutation to form Group 1 Yellows. Apparently mutations to form blush and peach are restricted to certain clades with some exceptions. To study the evolution of the colour mutations more samples, especially from orange flowering individuals, should be included in the study. Only clade 3b appears to contain a mixture of Group 1 and 2 Yellows, as well as the different Ndwedwe groups.

4.5 Conclusion

The phylogenetic relationships of natural populations of *Clivia miniata* indicated that all *C. miniata* plants shared a common ancestor. *Clivia miniata* from the same geographical area grouped together in the cladogram. More data would be required to prove these observations for all *Clivia.*

The taxonomic status of *Clivia miniata* var. *citrina* would depend on the monophyly of the yellow *Clivia* specimens. Orange flowered forms should be included to determine the validity of the current taxonomic status of these groups.

Genetic variation in
Clivia miniata var. citrina

CHAPTER 5

Conclusions and Recommendations

5.1 Conclusions and Recommendations

Previous studies of DNA sequencing using chloroplast DNA and the gene *matK* did not adequately resolve questions surrounding the phylogeny of the genus *Clivia* (Booysen, 2003). Vorster (1994) suggested that the similarity in morphology, particularly for vegetative characters, made it difficult to justify the separation of *Clivia* into different species. Karyotype analysis done by Ran *et al*. (1999) showed that *Clivia* species (at that time only four species, *C. nobilis*, *C. miniata*, *C. gardenii* and *C. caulescens*) shared similar genomes. Sharing occurred mostly of the *miniata* and *gardenii* genomes. Booysen (2003) suggested that *Clivia* was monophyletic.

The tremendous morphological diversification of flower colour gave rise to the taxa designation 'var. *citrina*'. Olmstead and Palmer (1994) found that an increase in the number of taxa used in a phylogenetic study usually increased the resolution of unrelated taxa but decreased the resolution of closely related taxa. Could this have taken place within the classification system used for *Clivia*? What was happening to the *Clivia* genome?

Phylogenetic inference at low taxonomic levels is often limited in plants by lack of a suitable molecular marker system such as mtDNA in animals. This is especially true when considering species complexes where hybridisation takes place more or less regularly, either by means of cultivation or natural hybridisation (Despres *et al.*, 2003).

Availability of microsatellites developed for *Clivia* by Swanevelder (2003) offered a new means of scrutiny at DNA level. Testing these microsatellites during the present

study was not successful. However, future studies should focus on the development of microsatellites that can be successfully applied to molecular marker studies on *Clivia*, including DNA fingerprinting and marker-assisted breeding. In such a context the AFLP technique which provided information on the whole genome variability provided a tool suitable for analysis of genetic differentiation in relation to morphological diversification. The advantage of evaluating the entire genome to obtain many polymorphic markers without prior sequence knowledge served as one of the motivations for use of AFLP analysis.

From AFLP analysis we concluded that there existed high levels of polymorphic fragments (average of 90% for the three primer combinations used) throughout the *Clivia* genome. Such tremendous genetic variation implies that the genus is modern and still evolving at a rapid pace. The total genetic variation between 72 *Clivia* plants tested, including plants from five different species, was also relatively high (35%) compared to other cultivated crops. Furthermore, variation within *C. miniata* plants obtained from natural populations (GS of 0.720) was similar to variation within *C. miniata* plants obtained through cultivation (GS of 0.715). Added to the natural rate of evolution, *Clivia* under cultivation develop into avenues of human preference (as with flower colour). Where that will lead to is dependent on human whims.

As to the taxonomic classification that *Clivia* is subjected to, reconsideration of species status is needed. From AFLP data it is suggested that *C. miniata* species only exist as a single species with many morphological expressions and that the 'var. *citrina*' classification should be reconsidered. Use of one molecular technique might not offer a complete solution to taxonomic classification questions that still need to be addressed.

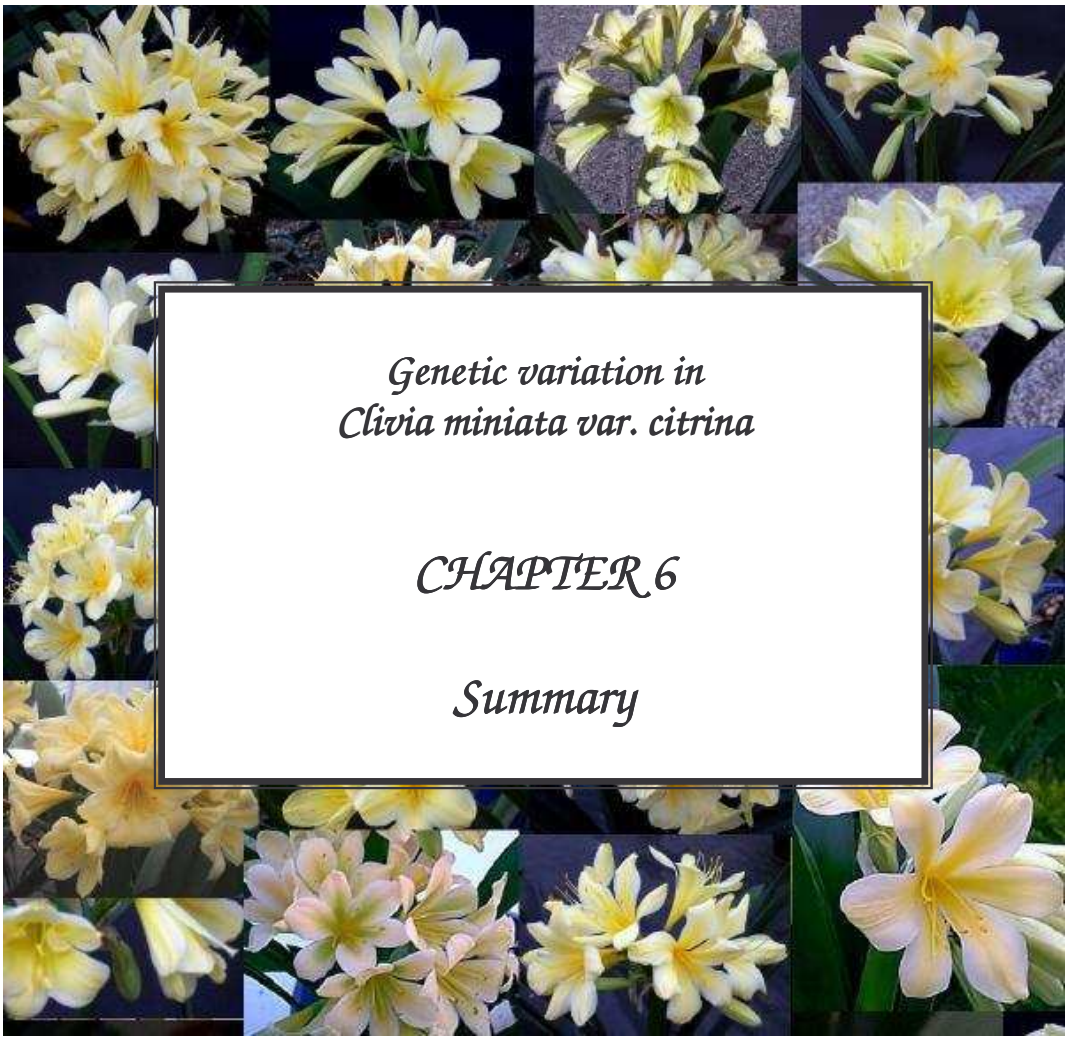
The phylogenetic relationships of natural populations of *C. miniata* indicated that all *C. miniata* plants shared a common ancestor.

For directed breeding purposes it is important to know relative genetic similarity between plants to incorporate as much genetic variation for breeding programmes as possible. Results obtained from this study can aid *Clivia* breeders in decision making processes with regard to the selection of suitable breeding parents.

Identification of plants become important as breeding programmes select parents for new phenotypes to be developed that must breed true for a particular trait. Selling plants bearing wrong names can be ruled out with the AFLP DNA fingerprinting technique.

Clivia as an ornamental crop provides infinite scope for further development of cultivars and lines that can be commercially produced. Application of marker-assisted selection might be the next step for the *Clivia* industry.

Results from this study elucidated some of the important questions regarding genetic diversity between different *Clivia* species and genetic variation within *C. miniata* and *C. miniata* var. *citrina*. Results indicated that AFLP analysis can be applied to elucidate questions regarding both genetic diversity and phylogeny in *Clivia*. Data from this study strongly suggest that the current taxonomic classification of *Clivia* should undergo fresh scrutiny. Future studies focussing on the taxonomy of the genus may proceed from the present study.



Genetic variation in
Clivia miniata var. citrina

CHAPTER 6

Summary

KEYWORDS: *Clivia;* AFLP; fingerprinting; *Clivia miniata* var. *citrina*; Phylogeny

The genus *Clivia* is from the African tribe Haemanthaceae and a member of the family Amaryllidaceae. *Clivia* is endemic to southern Africa. Yellow *Clivia* are mutations of the orange-red standard forms that have appeared spontaneously in both wild and garden populations. Yellow *Clivia* plants are rare and desirable and were described as *Clivia miniata* var. *citrina*. Hobbyists from around the world trade in these ornamental plants initiating entire enterprises. Although the yellow form occurs naturally, many yellow clones have arisen through cultivation. Clones passed on from breeder to breeder have acquired different names. For directed breeding purposes in a thriving industry it is important to identify genetically similar plants. The aims of this study were to evaluate existing microsatellites for *Clivia miniata* var. *citrina*, to determine if AFLP analysis can distinguish among different plants within the genus *Clivia* and to determine genetic relatedness between different plants of 'Vico', 'Giddy' and 'Natal Yellow' cultivars.

Previous studies done on *Clivia* include RAPD analysis and SSR analysis for *Clivia.* Work done in this study presents a first report of AFLP and SSR fingerprint analyses on *C. miniata* var. *citrina*. SSR fingerprint analysis revealed that the existing four SSR primer combinations were not applicable for studies on *C. miniata* var. *citrina*. AFLP analysis was optimised using a total of 28 *Eco*RI / *Mse*I primer combinations. Primer combinations were evaluated using six randomly selected *Clivia* plants based on number of generated fragments, ability to score generated fragments, ability to detect polymorphism and level of polymorphic fragments. Fragments generated using *Eco*RI+3 primers in combination with *Mse*+4 primer combinations conformed to the

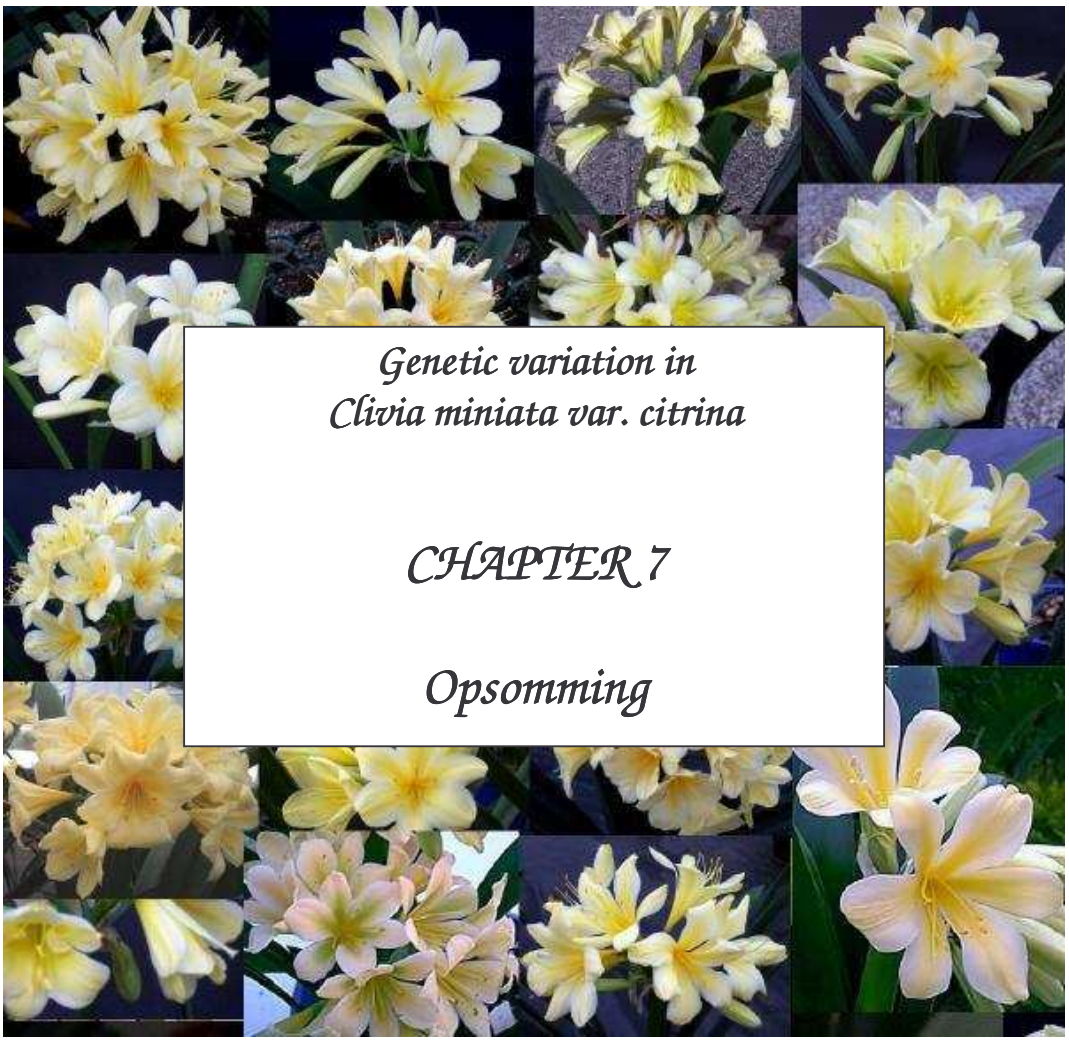
chosen criteria. Primer combinations E-ACC with M-CATC, E-AGC with M-CATC and E-AGC with M-CTGG were selected for further studies on *Clivia*.

AFLP analysis using three preselected primer combinations on 72 *Clivia* plants was successful in detecting genetic diversity and determining genetic relationships within closely related cultivated *Clivia* plants. Relatively high levels of genetic diversity (35%), as expected from known pedigree and species data, existed among *Clivia* plants. Genetic diversity within *C. miniata* and *C. miniata* var. *citrina* plants was high at 27%. Plants available for scrutiny were all genetically distinct. However, based on known pedigree data, names allocated to plants might not be truly representative of the true origin of the plants (e.g. Vico Meristem plants). Material obtained from different breeders could be distinguished at DNA level (e.g. 'Giddy' and 'Natal Yellow' cultivars).

AFLP analysis revealed that different flower coloured plants (Apricot, Blush, Peach, Orange and Yellow) as well as plants from the same geographic areas were distributed together throughout the dendrogram with only a few of a certain colour grouping together. Known Group 1 Yellow and Group 2 Yellow were also present throughout the entire dendrogram, although the majority of known Group 1 Yellow plants grouped together.

Clustering of the different species of the genus *Clivia* agreed with known pedigree data and hybrids included with their parents clustered according to known pedigree data.

The phylogenetic relationships of natural populations of *C. miniata* indicated that all *C. miniata* plants shared a common ancestor. *Clivia miniata* from the same geographical area grouped together in the cladogram. More data would be required to prove these observations for all *Clivia*. Taxonomic status of the *C. miniata* var. *citrina* would depend on the monophyly of yellow *Clivia* plants. Orange flowered forms should be included to determine the validity of the current taxonomic status of these groups.



Genetic variation in
Clivia miniata var. citrina

CHAPTER 7

Opsomming

SLEUTELWOORDE: *Clivia*; AFLP; vingerafdruk-analises; *Clivia miniata* var. *citrina*; filogenie

Die genus *Clivia* is uit die tribes Haemanthaceae, deel van die familie Amaryllidaceae, en is endemies tot suider-Afrika. Geel *Clivia* blomme is ‘n kleurmutasie van die wilde oranje-rooi blomtipe wat spontaan binne beide natuurlike en gekultiveerde *Clivia* populasies verskyn. Die geel blomkleur is skaars en gesog en word as *Clivia miniata* var. *citrina* beskryf. Geel *Clivia* is ‘n gesogte rariteit wat deur geesdriftiges in ‘n handelsbedryf omskep is. Alhoewel die geel kleur in die natuur voorkom, is baie geelkleurige klone deur plantveredeling geskep en naamsverwarring het ontstaan aangesien elke teler ‘n plant na ‘n bekende persoon vernoem. Om spesifiek-gedrewe teling toe te pas is dit noodsaaklik om geneties identiese plante te identifiseer. Hierdie studie het ten doel gehad om bestaande mikrosatelliete op *C. miniata* var. *citrina* te toets, te bepaal of AFLP-analises tussen verskillende plante binne die genus *Clivia* kan onderskei en om te bepaal wat die genetiese verwantskappe tussen die verskillende ‘Vico’, ‘Giddy’ en ‘Natal Yellow’ kultivars is.

Vorige werk wat op *Clivia* uitgevoer is, sluit RAPD- en SSR-analises in. Die huidige studie verteenwoordig die eerste verslag rakende die gebruik van AFLP- en SSR-analises op *Clivia miniata* var. *citrina*. SSR-vingerafdrukke het getoon dat die bestaande vier SSR-inleier kombinasies nie op *C. miniata* var. *citrina* werk nie. AFLP-analise is gestandaardiseer deur ‘n total van 28 *Eco*RI / *Mse*I-voorvoerder kombinasies te gebruik. Die inleier kombinasies is op ses willekeurig geselekteerde *Clivia* plante getoets en is geëvalueer op grond van die aantal gegenereerde

fragmente, vermoë om fragmente te dokumenteer, potensiaal om polimorfismes te erken asook aantal polimorfismes. Fragmente verkry deur van *Eco*RI+3 inleier in kombinasie met *Mse*+4 inleier gebruik te maak het bogenoemde kriteria die beste gepas. Inleier kombinasies E-ACC met M-CATC, E-AGC met M-CATC en E-AGC met M-CTGG is gekies vir verdere studies op *Clivia*.

AFLP-analise met behulp van die bogenoemde inleier kombinasies is suksesvol op die hele steekproef van 72 *Clivia* plante toegepas en is suksesvol aangewend vir die bepaling van genetiese diversiteit asook genetiese verwantskappe tussen naverwante veredelde *Clivia* plante. Relatief hoë vlakke van genetiese diversiteit (35%), soos verwag gebaseer op bekende stamboom en spesiedata, is tussen lede van *C. miniata* en die ander *Clivia* species en tussen *C. miniata* en *C. miniata* var. *citrina* (27%) waargeneem. Al die plante binne die steekproef kon geneties van mekaar onderskei word. Gebaseer op bekende stamboomdata is gevind dat alle naam toevoegings nie in lyn met beskikbare stamboom inligting was nie, byvoorbeeld die Vico Meristeem plante. Materiaal wat van verskillende telers ontvang is kon op DNA-vlak onderskei word (bv. die 'Giddy' en 'Natal Yellow' kultivars).

AFLP-analises het aangetoon dat verskillende blomkleur plante (Appelkoos, Ligpienk, Perskekleurig, Oranje en Geel) asook plante afkomstig van dieselfde geografiese area nie afsonderlik binne die dendrogramme gegroepeer het nie. Slegs 'n paar plante het volgens kleur gegroepeer. Bekende Groep 1 Geel en Groep 2 Geel plante was deurgaans binne die dendrogram versprei, alhoewel die meeste Groep 1 Geel plante saam gegroepeer het.

Groepering van die verskillende spesies in die genus *Clivia* het met beskikbare stamboomdata ooreengestem en basters met hul ouers het volgens stamboomdata saamgegroepeer.

Die filogenetiese verwantskappe tussen natuurlike populasies van *C. miniata* het getoon dat alle *C. miniata* plante van ‘n gemeenskaplike voorouer afkomstig is. *Clivia miniata* plante afkomstig van dieselfde geografiese areas het saam gegroepeer in die cladogram. Meer inligting word benodig om hierdie bevinding toepaslik tot alle *Clivia* te kan maak. Die taksonomiese stand van *C. miniata* var. *citrina* berus op die monofelie van geel blomkleurige *Clivia* plante. Plante met oranje blomme behoort in toekomstige studies by geelkleuriges ingesluit te word om die taksonomiese stand van *C. miniata* var. *citrina* beter te verklaar.

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APPENDIX 1

Dice similarity coefficient matrix

APPENDIX A: DICE PAIRWISE SIMILARITY COEFFICIENTS

	C. gardenii	*C. caul* (JS)	*C. nobilis*	*C. caul* (MD)	*C. mirabilis*	Watkins Y G	Naka Y	Holl Frick
C. gardenii	1.000							
***C. caul* (JS)**	0.743	1.000						
C. nobilis	0.694	0.701	1.000					
***C. caul* (MD)**	0.705	0.744	0.658	1.000				
C. mirabilis	0.612	0.646	0.732	0.616	1.000			
Watkins Y G	0.716	0.667	0.634	0.635	0.647	1.000		
Naka Y	0.691	0.687	0.627	0.623	0.614	0.814	1.000	
Holl Frick	0.654	0.712	0.651	0.636	0.680	0.767	0.772	1.000
New Dawn	0.667	0.710	0.629	0.671	0.658	0.876	0.842	0.854
Kirstenbosch Y	0.587	0.702	0.586	0.582	0.600	0.725	0.781	0.753
Apricot	0.659	0.710	0.598	0.658	0.599	0.802	0.768	0.793
Floradale Y	0.667	0.686	0.663	0.614	0.683	0.717	0.706	0.741
Y Hybrid	0.682	0.689	0.667	0.624	0.663	0.804	0.731	0.754
Karkloof	0.675	0.647	0.625	0.650	0.654	0.805	0.776	0.715
Nogqaza	0.697	0.693	0.659	0.659	0.630	0.811	0.819	0.772
Natal Y (FvN)	0.746	0.764	0.707	0.687	0.707	0.802	0.786	0.867
Chubb' s P (PG)	0.731	0.750	0.703	0.695	0.715	0.778	0.772	0.854
Giddy (MD)	0.727	0.746	0.689	0.691	0.699	0.817	0.767	0.895
Wittig Y	0.650	0.683	0.575	0.600	0.587	0.777	0.769	0.782
Giddy's Best (RL)	0.724	0.754	0.707	0.675	0.707	0.815	0.777	0.882
Natal Y (FvN)	0.683	0.702	0.655	0.650	0.688	0.780	0.736	0.847
Howick Y	0.674	0.693	0.670	0.643	0.607	0.809	0.770	0.747
Karkloof Y	0.663	0.671	0.625	0.642	0.591	0.827	0.788	0.739
Blinkwater Y	0.683	0.655	0.655	0.624	0.612	0.802	0.773	0.749
Dwesa Y	0.685	0.670	0.649	0.647	0.631	0.787	0.736	0.759
Floradale Y	0.603	0.650	0.593	0.667	0.552	0.679	0.711	0.715
Cobb Inn Y	0.694	0.656	0.652	0.667	0.633	0.672	0.713	0.712
Smith's Y	0.687	0.695	0.612	0.680	0.588	0.740	0.767	0.755
Eric Dodd/BasheeY	0.646	0.667	0.594	0.653	0.564	0.702	0.727	0.753
PSJY/Nev	0.667	0.638	0.554	0.640	0.520	0.698	0.697	0.697

PSJ / Rod Ellis	0.713	0.674	0.663	0.712	0.622	0.761	0.788	0.777
Peacevale Blush	0.683	0.716	0.631	0.720	0.609	0.737	0.739	0.777
Greendale Blush Y	0.691	0.675	0.604	0.702	0.618	0.756	0.734	0.785
Tarrs Picotee	0.713	0.697	0.652	0.667	0.634	0.783	0.753	0.753
Celtis Kloof	0.671	0.667	0.667	0.663	0.638	0.744	0.759	0.783
	C. gardenii	***C. caul* (JS)**	***C. nobilis***	***C. caul* (MD)**	***C. mirabilis***	**Watkins Y G**	**Naka Y**	**Holl Frick**
King Hamelin	0.646	0.630	0.619	0.623	0.596	0.737	0.764	0.764
Byrne Valley Y	0.688	0.638	0.655	0.662	0.617	0.757	0.756	0.756
Mpumulo Y	0.709	0.671	0.718	0.675	0.663	0.736	0.738	0.762
Alpha Ndwedwe	0.697	0.682	0.692	0.671	0.630	0.789	0.749	0.795
Echo Ndwedwe	0.702	0.663	0.685	0.671	0.634	0.751	0.743	0.767
Zulu Ndwedwe	0.708	0.693	0.714	0.719	0.619	0.745	0.747	0.759
Qora	0.719	0.670	0.692	0.671	0.655	0.766	0.747	0.747
Cynthia's Best	0.714	0.731	0.708	0.717	0.674	0.759	0.751	0.785
Eshowe	0.709	0.728	0.659	0.625	0.654	0.736	0.750	0.762
Nurenberger	0.705	0.667	0.678	0.646	0.639	0.785	0.744	0.744
Gill Hornby P	0.689	0.697	0.674	0.594	0.643	0.759	0.751	0.751
Chubb' s P (FvN)	0.714	0.709	0.674	0.654	0.609	0.774	0.743	0.802
Gail's P	0.715	0.722	0.677	0.671	0.604	0.773	0.766	0.766
Bonnie P	0.746	0.715	0.696	0.663	0.651	0.796	0.736	0.763
Pretoria Y	0.731	0.685	0.736	0.678	0.659	0.806	0.780	0.747
Caul x Mirabilis	0.663	0.647	0.682	0.710	0.692	0.682	0.642	0.728
Vico Gold Naka	0.706	0.678	0.667	0.688	0.638	0.767	0.783	0.747
Vico Gold Smither's	0.674	0.717	0.682	0.696	0.630	0.747	0.750	0.726
Vico Y Naka	0.675	0.709	0.688	0.603	0.621	0.773	0.763	0.763
Q2 Apple Blossom	0.667	0.703	0.659	0.675	0.615	0.724	0.761	0.745
Oribi Y	0.671	0.671	0.667	0.610	0.654	0.728	0.704	0.745
Naka Vico Mer	0.658	0.671	0.641	0.613	0.586	0.756	0.745	0.750
FlAprxUmtam	0.720	0.663	0.678	0.645	0.680	0.791	0.721	0.745
Floradale Apricot	0.743	0.678	0.696	0.679	0.667	0.776	0.709	0.740
Umtamwuna	0.726	0.711	0.682	0.684	0.637	0.777	0.744	0.752
Andrew Gibson	0.648	0.707	0.645	0.619	0.662	0.780	0.745	0.772
Mvuma Y	0.745	0.734	0.667	0.662	0.684	0.831	0.790	0.805

Mwuma P	0.644	0.662	0.607	0.638	0.593	0.780	0.759	0.748
Vico Meristem 2	0.657	0.731	0.628	0.602	0.667	0.809	0.800	0.794
Dwesa	0.649	0.732	0.662	0.634	0.640	0.745	0.748	0.725
Y Offspring	0.671	0.712	0.687	0.645	0.662	0.842	0.764	0.810
P Offspring	0.737	0.769	0.688	0.648	0.680	0.836	0.782	0.803
DV Variegated P	0.697	0.705	0.675	0.635	0.693	0.812	0.795	0.803
Pinstripe Y	0.693	0.701	0.667	0.639	0.685	0.822	0.792	0.800
Barbara's Y	0.662	0.671	0.630	0.613	0.658	0.871	0.808	0.777
Potterrill	0.658	0.676	0.607	0.686	0.648	0.805	0.786	0.803
Ngome Y	0.654	0.708	0.675	0.680	0.684	0.793	0.748	0.764

	New Dawn	**Kirstenbosch Y**	**Apricot**	**Floradale Y**	**Y Hybrid**	**Karkloof**	**Nogqaza**	**Natal Yellox (FvN)**
New Dawn	1.000							
Kirstenbosch Y	0.790	1.000						
Apricot	0.847	0.750	1.000					
Floradale Y	0.727	0.671	0.761	1.000				
Y Hybrid	0.796	0.699	0.756	0.791	1.000			
Karkloof	0.807	0.706	0.795	0.791	0.846	1.000		
Nogqaza	0.859	0.730	0.802	0.776	0.862	0.899	1.000	
Natal Yellox (FvN)	0.838	0.745	0.805	0.778	0.811	0.811	0.828	1.000
Chubb' s P (PG)	0.825	0.730	0.802	0.776	0.819	0.809	0.837	0.957
Giddy (MD)	0.854	0.725	0.820	0.804	0.847	0.816	0.843	0.941
Wittig Y	0.827	0.792	0.765	0.679	0.809	0.785	0.805	0.830
Giddy' s Best (RL)	0.841	0.747	0.807	0.780	0.813	0.814	0.842	0.941
Natal Yellolw (FvN)	0.805	0.715	0.769	0.743	0.811	0.788	0.807	0.899
Howick Y	0.789	0.679	0.789	0.753	0.880	0.840	0.877	0.825
Karkloof Y	0.830	0.693	0.784	0.712	0.824	0.826	0.876	0.800
Blinkwater Y	0.805	0.689	0.750	0.709	0.833	0.812	0.864	0.798
Dwesa Y	0.800	0.679	0.760	0.796	0.827	0.774	0.845	0.794
Floradale Y	0.713	0.657	0.701	0.773	0.762	0.709	0.764	0.719
Cobb Inn Y	0.733	0.611	0.705	0.768	0.773	0.744	0.787	0.781
Smith's Y	0.788	0.721	0.739	0.772	0.761	0.759	0.791	0.793
Eric Dodd/BasheeY	0.750	0.704	0.763	0.783	0.737	0.770	0.778	0.757

PSJY/Nev	0.733	0.671	0.745	0.778	0.721	0.728	0.726	0.718
PSJ / Rod Ellis	0.807	0.722	0.784	0.758	0.738	0.780	0.820	0.822
Peacevale Blush	0.785	0.731	0.810	0.734	0.736	0.768	0.788	0.802
Greendale Blush Y	0.793	0.712	0.817	0.729	0.743	0.788	0.807	0.809
Tarrs Picotee	0.784	0.658	0.807	0.769	0.770	0.780	0.809	0.800
Celtis Kloof	0.779	0.662	0.779	0.775	0.798	0.786	0.827	0.840
King Hamelin	0.773	0.676	0.749	0.746	0.793	0.756	0.800	0.826
Byrne Valley Y	0.753	0.685	0.741	0.771	0.784	0.803	0.828	0.840
Mpumulo Y	0.747	0.680	0.736	0.800	0.796	0.766	0.802	0.853
Alpha Ndwedwe	0.791	0.679	0.780	0.798	0.819	0.787	0.859	0.839
Echo Ndwedwe	0.786	0.684	0.798	0.793	0.794	0.793	0.833	0.835
Zulu Ndwedwe	0.778	0.691	0.778	0.785	0.764	0.785	0.845	0.836
Qora	0.778	0.679	0.767	0.796	0.744	0.807	0.824	0.836
Cynthia's Best	0.813	0.710	0.749	0.798	0.838	0.809	0.835	0.867
Eshowe	0.770	0.705	0.751	0.778	0.843	0.789	0.818	0.853
Nurenberger	0.787	0.663	0.764	0.772	0.815	0.838	0.876	0.845
	New Dawn	**Kirstenbosch Y**	**Apricot**	**Floradale Y**	**Y Hybrid**	**Karkloof**	**Nogqaza**	**Natal Yellox (FvN)**
Gill Hornby P	0.760	0.646	0.764	0.768	0.811	0.778	0.807	0.840
Chubb' s P (FvN)	0.798	0.671	0.775	0.771	0.804	0.759	0.811	0.879
Gail's P	0.785	0.687	0.807	0.813	0.802	0.758	0.819	0.821
Bonnie P	0.764	0.638	0.775	0.804	0.832	0.771	0.839	0.840
Pretoria Y	0.819	0.671	0.798	0.804	0.834	0.836	0.872	0.833
Caul x Mirabilis	0.702	0.600	0.702	0.724	0.693	0.686	0.731	0.723
Vico Gold Naka	0.802	0.675	0.756	0.775	0.743	0.798	0.827	0.807
Vico Gold Smither's	0.805	0.718	0.805	0.767	0.800	0.789	0.829	0.787
Vico Y Naka	0.785	0.709	0.752	0.805	0.786	0.760	0.822	0.807
Q2 Apple Blossom	0.767	0.676	0.739	0.814	0.798	0.778	0.832	0.789
Oribi Y	0.767	0.680	0.771	0.821	0.807	0.795	0.814	0.800
Naka Vico Mer	0.747	0.737	0.770	0.748	0.767	0.755	0.777	0.823
FlAprxUmtam	0.767	0.676	0.776	0.821	0.802	0.790	0.811	0.807
Floradale Apricot	0.771	0.663	0.768	0.794	0.819	0.805	0.817	0.830
Umtamwuna	0.784	0.697	0.781	0.780	0.811	0.807	0.843	0.833
Andrew Gibson	0.829	0.721	0.770	0.750	0.805	0.733	0.826	0.764

Mvuma Y	0.823	0.736	0.803	0.708	0.798	0.734	0.785	0.812
Mwuma P	0.795	0.686	0.782	0.753	0.808	0.768	0.808	0.772
Vico Meristem 2	0.836	0.790	0.797	0.763	0.772	0.730	0.763	0.820
Dwesa	0.732	0.676	0.732	0.795	0.724	0.706	0.747	0.788
Y Offspring	0.834	0.698	0.753	0.747	0.786	0.736	0.798	0.828
P Offspring	0.828	0.747	0.777	0.738	0.802	0.726	0.790	0.847
DV Variegated P	0.828	0.775	0.777	0.725	0.790	0.726	0.803	0.785
Pinstripe Y	0.813	0.743	0.748	0.747	0.800	0.748	0.825	0.808
Barbara's Y	0.840	0.762	0.778	0.739	0.802	0.778	0.814	0.821
Potterrill	0.821	0.686	0.782	0.727	0.721	0.755	0.782	0.785
Ngome Y	0.783	0.667	0.733	0.727	0.779	0.721	0.747	0.786

	Chubb' s P (PG)	**Giddy (MD)**	**Wittig Y**	**Giddy' s Best (RL)**	**Natal Yellolw (FvN)**	**Howick Y**	**Karkloof Y**	**Blinkwater Y**
Chubb' s P (PG)	1.000							
Giddy (MD)	0.951	1.000						
Wittig Y	0.840	0.835	1.000					
Giddy' s Best (RL)	0.929	0.924	0.845	1.000				
Natal Yellolw (FvN)	0.909	0.893	0.845	0.937	1.000			
Howick Y	0.845	0.840	0.802	0.828	0.827	1.000		
Karkloof Y	0.820	0.793	0.810	0.802	0.812	0.895	1.000	
Blinkwater Y	0.818	0.791	0.795	0.789	0.798	0.883	0.953	1.000
Dwesa Y	0.824	0.819	0.744	0.807	0.793	0.821	0.796	0.793

	Chubb' s P (PG)	**Giddy (MD)**	**Wittig Y**	**Giddy' s Best (RL)**	**Natal Yellolw (FvN)**	**Howick Y**	**Karkloof Y**	**Blinkwater Y**
Floradale Y	0.727	0.739	0.653	0.695	0.688	0.714	0.742	0.764
Cobb Inn Y	0.794	0.797	0.695	0.724	0.740	0.779	0.738	0.750
Smith's Y	0.791	0.775	0.764	0.772	0.756	0.743	0.783	0.781
Eric Dodd/BasheeY	0.755	0.762	0.737	0.747	0.730	0.706	0.745	0.742
PSJY/Nev	0.702	0.698	0.693	0.719	0.675	0.690	0.691	0.688
PSJ / Rod Ellis	0.831	0.804	0.750	0.813	0.800	0.774	0.768	0.754
Peacevale Blush	0.824	0.819	0.761	0.805	0.778	0.763	0.781	0.741
Greendale Blush Y	0.819	0.826	0.756	0.812	0.773	0.782	0.800	0.761
Tarrs Picotee	0.798	0.804	0.726	0.813	0.777	0.807	0.791	0.743

Celtis Kloof	0.838	0.833	0.744	0.843	0.807	0.791	0.786	0.749
King Hamelin	0.824	0.807	0.787	0.840	0.815	0.775	0.805	0.778
Byrne Valley Y	0.814	0.833	0.771	0.838	0.818	0.772	0.785	0.783
Mpumulo Y	0.873	0.857	0.759	0.856	0.832	0.789	0.761	0.747
Alpha Ndwedwe	0.837	0.854	0.757	0.863	0.841	0.813	0.809	0.784
Echo Ndwedwe	0.833	0.851	0.776	0.827	0.802	0.776	0.770	0.756
Zulu Ndwedwe	0.824	0.830	0.721	0.828	0.793	0.800	0.796	0.771
Qora	0.824	0.809	0.756	0.828	0.771	0.768	0.785	0.771
Cynthia's Best	0.866	0.872	0.760	0.839	0.807	0.802	0.777	0.753
Eshowe	0.829	0.835	0.795	0.811	0.775	0.794	0.789	0.786
Nurenberger	0.832	0.839	0.777	0.815	0.757	0.840	0.816	0.814
Gill Hornby P	0.807	0.813	0.714	0.811	0.775	0.762	0.756	0.742
Chubb' s P (FvN)	0.856	0.873	0.752	0.860	0.837	0.765	0.793	0.756
Gail's P	0.809	0.836	0.728	0.813	0.800	0.775	0.769	0.733
Bonnie P	0.845	0.862	0.721	0.839	0.816	0.811	0.793	0.780
Pretoria Y	0.831	0.837	0.744	0.825	0.802	0.828	0.815	0.802
Caul x Mirabilis	0.743	0.761	0.675	0.736	0.743	0.708	0.675	0.683
Vico Gold Naka	0.816	0.800	0.744	0.787	0.772	0.791	0.809	0.819
Vico Gold Smither's	0.829	0.802	0.771	0.811	0.786	0.794	0.846	0.832
Vico Y Naka	0.807	0.807	0.732	0.798	0.780	0.781	0.813	0.823
Q2 Apple Blossom	0.789	0.784	0.733	0.763	0.767	0.757	0.755	0.752
Oribi Y	0.793	0.800	0.725	0.767	0.759	0.750	0.767	0.756
Naka Vico Mer	0.798	0.785	0.759	0.800	0.760	0.758	0.750	0.747
FlAprxUmtam	0.800	0.800	0.721	0.821	0.790	0.764	0.769	0.762
Floradale Apricot	0.828	0.813	0.737	0.832	0.809	0.787	0.782	0.775
Umtamwuna	0.832	0.827	0.781	0.848	0.824	0.800	0.795	0.800
Andrew Gibson	0.769	0.782	0.712	0.779	0.771	0.783	0.745	0.760
Mvuma Y	0.819	0.807	0.753	0.810	0.808	0.757	0.763	0.760

	Chubb' s P (PG)	**Giddy (MD)**	**Wittig Y**	**Giddy' s Best (RL)**	**Natal Yellolw (FvN)**	**Howick Y**	**Karkloof Y**	**Blinkwater Y**
Mwuma P	0.767	0.793	0.735	0.744	0.753	0.778	0.784	0.768
Vico Meristem 2	0.829	0.809	0.779	0.818	0.809	0.764	0.803	0.785
Dwesa	0.795	0.783	0.738	0.760	0.744	0.732	0.710	0.706
Y Offspring	0.835	0.847	0.805	0.826	0.800	0.786	0.788	0.773

P Offspring	0.854	0.842	0.784	0.832	0.825	0.774	0.767	0.764
DV Variegated P	0.793	0.793	0.745	0.783	0.775	0.762	0.780	0.764
Pinstripe Y	0.803	0.803	0.768	0.793	0.798	0.783	0.803	0.800
Barbara's Y	0.817	0.817	0.798	0.807	0.812	0.809	0.817	0.803
Potterrill	0.805	0.793	0.721	0.795	0.753	0.765	0.758	0.742
Ngome Y	0.793	0.781	0.713	0.771	0.756	0.763	0.761	0.758
	Dwesa Y	**Floradale Y**	**Cobb Inn Y**	**Smith's Y**	**Eric Dodd/BasheeY**	**PSJY/Nev**	**PSJ / Rod Ellis**	**Peacevale Blush**
Dwesa Y	1.000							
Floradale Y	0.857	1.000						
Cobb Inn Y	0.806	0.807	1.000					
Smith's Y	0.823	0.863	0.817	1.000				
Eric Dodd/BasheeY	0.800	0.824	0.757	0.903	1.000			
PSJY/Nev	0.819	0.792	0.702	0.833	0.861	1.000		
PSJ / Rod Ellis	0.828	0.805	0.781	0.866	0.819	0.790	1.000	
Peacevale Blush	0.740	0.729	0.730	0.798	0.784	0.727	0.864	1.000
Greendale Blush Y	0.747	0.711	0.718	0.767	0.766	0.736	0.835	0.955
Tarrs Picotee	0.763	0.695	0.760	0.737	0.735	0.731	0.813	0.864
Celtis Kloof	0.802	0.750	0.810	0.778	0.765	0.736	0.820	0.824
King Hamelin	0.786	0.755	0.793	0.785	0.745	0.727	0.781	0.744
Byrne Valley Y	0.807	0.792	0.807	0.782	0.782	0.711	0.778	0.753
Mpumulo Y	0.822	0.749	0.828	0.777	0.764	0.723	0.856	0.798
Alpha Ndwedwe	0.856	0.776	0.815	0.767	0.755	0.726	0.831	0.777
Echo Ndwedwe	0.809	0.745	0.828	0.786	0.761	0.707	0.827	0.795
Zulu Ndwedwe	0.821	0.786	0.824	0.811	0.765	0.725	0.850	0.809
Qora	0.800	0.750	0.785	0.823	0.765	0.760	0.871	0.809
Cynthia's Best	0.822	0.770	0.800	0.835	0.768	0.753	0.839	0.811
Eshowe	0.772	0.741	0.797	0.793	0.744	0.715	0.800	0.790
Nurenberger	0.777	0.723	0.794	0.763	0.726	0.686	0.804	0.795
Gill Hornby P	0.783	0.755	0.779	0.782	0.746	0.718	0.768	0.733
Chubb' s P (FvN)	0.798	0.758	0.823	0.762	0.749	0.707	0.793	0.783
Gail's P	0.806	0.757	0.788	0.796	0.772	0.744	0.824	0.793
Bonnie P	0.830	0.759	0.791	0.744	0.707	0.691	0.789	0.775
Pretoria Y	0.828	0.743	0.832	0.765	0.730	0.726	0.835	0.774

	Dwesa Y	Floradale Y	Cobb Inn Y	Smith's Y	Eric Dodd/BasheeY	PSJY/Nev	PSJ / Rod Ellis	Peacevale Blush
Caul x Mirabilis	0.719	0.692	0.738	0.712	0.709	0.642	0.770	0.721
Vico Gold Naka	0.780	0.750	0.846	0.802	0.765	0.712	0.820	0.776
Vico Gold Smither's	0.783	0.765	0.790	0.793	0.768	0.715	0.811	0.826
Vico Y Naka	0.810	0.795	0.793	0.821	0.800	0.733	0.781	0.737
Q2 Apple Blossom	0.834	0.837	0.793	0.882	0.846	0.795	0.828	0.778
Oribi Y	0.766	0.745	0.764	0.761	0.760	0.747	0.763	0.758
Naka Vico Mer	0.763	0.739	0.767	0.781	0.723	0.732	0.803	0.750
FlAprxUmtam	0.798	0.761	0.766	0.765	0.752	0.717	0.791	0.763
Floradale Apricot	0.830	0.759	0.758	0.775	0.738	0.757	0.826	0.772
Umtamwuna	0.811	0.785	0.778	0.824	0.788	0.765	0.830	0.785
Andrew Gibson	0.854	0.731	0.680	0.750	0.757	0.766	0.800	0.727
Mvuma Y	0.817	0.695	0.685	0.787	0.735	0.735	0.783	0.729
Mwuma P	0.803	0.746	0.781	0.811	0.800	0.743	0.792	0.750
Vico Meristem 2	0.797	0.756	0.659	0.794	0.756	0.778	0.830	0.806
Dwesa	0.793	0.735	0.699	0.772	0.747	0.718	0.782	0.671
Y Offspring	0.828	0.699	0.713	0.761	0.737	0.737	0.795	0.744
P Offspring	0.798	0.671	0.704	0.765	0.726	0.699	0.800	0.752
DV Variegated P	0.798	0.662	0.642	0.725	0.726	0.726	0.775	0.711
Pinstripe Y	0.832	0.725	0.722	0.776	0.764	0.778	0.772	0.708
Barbara's Y	0.798	0.676	0.691	0.727	0.715	0.715	0.764	0.727
Potterrill	0.739	0.612	0.680	0.685	0.700	0.686	0.753	0.778
Ngome Y	0.762	0.667	0.708	0.706	0.680	0.689	0.739	0.710

	Greendale Blush Y	Tarrs Picotee	Celtis Kloof	King Hamelin	Byrne Valley Y	Mpumulo Y	Alpha Ndwedwe	Echo Ndwedwe
Greendale Blush Y	1.000							
Tarrs Picotee	0.906	1.000						
Celtis Kloof	0.843	0.865	1.000					
King Hamelin	0.777	0.805	0.885	1.000				
Byrne Valley Y	0.774	0.778	0.859	0.877	1.000			
Mpumulo Y	0.781	0.785	0.848	0.833	0.836	1.000		
Alpha Ndwedwe	0.795	0.820	0.860	0.871	0.893	0.912	1.000	
Echo Ndwedwe	0.802	0.793	0.857	0.843	0.878	0.899	0.922	1.000

Zulu Ndwedwe	0.805	0.785	0.846	0.809	0.837	0.876	0.877	0.885
Qora	0.805	0.796	0.824	0.786	0.800	0.887	0.834	0.874
Cynthia's Best	0.818	0.798	0.836	0.778	0.802	0.854	0.845	0.853
Eshowe	0.774	0.778	0.818	0.778	0.788	0.854	0.818	0.836
Nurenberger	0.814	0.804	0.811	0.772	0.828	0.842	0.843	0.851
Gill Hornby P	0.751	0.811	0.818	0.791	0.805	0.804	0.828	0.813
	Greendale Blush Y	**Tarrs Picotee**	**Celtis Kloof**	**King Hamelin**	**Byrne Valley Y**	**Mpumulo Y**	**Alpha Ndwedwe**	**Echo Ndwedwe**
Chubb' s P (FvN)	0.802	0.838	0.869	0.843	0.847	0.854	0.889	0.841
Gail's P	0.800	0.866	0.853	0.816	0.826	0.839	0.883	0.880
Bonnie P	0.793	0.850	0.851	0.805	0.840	0.850	0.877	0.853
Pretoria Y	0.780	0.845	0.842	0.807	0.844	0.850	0.872	0.869
Caul x Mirabilis	0.704	0.736	0.729	0.683	0.717	0.763	0.754	0.760
Vico Gold Naka	0.759	0.775	0.782	0.776	0.817	0.814	0.805	0.823
Vico Gold Smither's	0.798	0.789	0.796	0.790	0.783	0.827	0.829	0.825
Vico Y Naka	0.729	0.749	0.793	0.800	0.797	0.800	0.822	0.783
Q2 Apple Blossom	0.753	0.770	0.817	0.795	0.790	0.793	0.805	0.807
Oribi Y	0.763	0.791	0.821	0.767	0.841	0.793	0.814	0.824
Naka Vico Mer	0.750	0.756	0.829	0.833	0.844	0.790	0.808	0.813
FlAprxUmtam	0.770	0.786	0.817	0.778	0.850	0.830	0.846	0.830
Floradale Apricot	0.779	0.815	0.811	0.786	0.817	0.843	0.860	0.835
Umtamwuna	0.793	0.818	0.849	0.824	0.832	0.865	0.876	0.874
Andrew Gibson	0.741	0.776	0.792	0.747	0.719	0.768	0.784	0.760
Mvuma Y	0.715	0.738	0.752	0.720	0.699	0.765	0.778	0.767
Mwuma P	0.750	0.771	0.800	0.755	0.766	0.792	0.826	0.842
Vico Meristem 2	0.794	0.788	0.779	0.768	0.711	0.782	0.779	0.752
Dwesa	0.658	0.684	0.724	0.731	0.723	0.748	0.752	0.766
Y Offspring	0.744	0.776	0.778	0.748	0.755	0.776	0.778	0.805
P Offspring	0.725	0.767	0.748	0.738	0.703	0.798	0.770	0.785
DV Variegated P	0.725	0.755	0.736	0.711	0.685	0.755	0.783	0.772
Pinstripe Y	0.735	0.764	0.771	0.735	0.755	0.767	0.805	0.808
Barbara's Y	0.740	0.768	0.738	0.727	0.733	0.756	0.795	0.785
Potterrill	0.806	0.811	0.787	0.699	0.681	0.719	0.736	0.737
Ngome Y	0.723	0.768	0.758	0.706	0.680	0.759	0.759	0.749

	Zulu Ndwedwe	Qora	Cynthia's Best	Eshowe	Nurenberger	Gill Hornby P	Chubb' s P (FvN)	Gail's P
Zulu Ndwedwe	1.000							
Qora	0.895	1.000						
Cynthia's Best	0.883	0.873	1.000					
Eshowe	0.826	0.848	0.880	1.000				
Nurenberger	0.851	0.872	0.851	0.846	1.000			
Gill Hornby P	0.825	0.836	0.857	0.853	0.856	1.000		
Chubb' s P (FvN)	0.842	0.809	0.842	0.814	0.829	0.890	1.000	
Gail's P	0.869	0.827	0.869	0.832	0.815	0.884	0.891	1.000
Bonnie P	0.847	0.809	0.851	0.831	0.845	0.900	0.929	0.916
Pretoria Y	0.869	0.849	0.829	0.802	0.878	0.822	0.859	0.864

	Zulu Ndwedwe	Qora	Cynthia's Best	Eshowe	Nurenberger	Gill Hornby P	Chubb' s P (FvN)	Gail's P
Caul x Mirabilis	0.775	0.764	0.714	0.709	0.716	0.701	0.702	0.760
Vico Gold Naka	0.846	0.868	0.794	0.784	0.833	0.785	0.777	0.787
Vico Gold Smither's	0.848	0.848	0.838	0.832	0.824	0.809	0.814	0.832
Vico Y Naka	0.826	0.812	0.811	0.810	0.788	0.836	0.825	0.845
Q2 Apple Blossom	0.834	0.796	0.842	0.795	0.782	0.818	0.800	0.832
Oribi Y	0.818	0.820	0.826	0.814	0.832	0.846	0.817	0.825
Naka Vico Mer	0.805	0.817	0.802	0.831	0.813	0.843	0.837	0.825
FlAprxUmtam	0.843	0.816	0.815	0.802	0.836	0.814	0.826	0.809
Floradale Apricot	0.847	0.857	0.851	0.820	0.845	0.834	0.831	0.825
Umtamwuna	0.895	0.879	0.866	0.830	0.856	0.860	0.834	0.862
Andrew Gibson	0.777	0.756	0.785	0.773	0.745	0.753	0.757	0.795
Mvuma Y	0.781	0.781	0.800	0.772	0.741	0.827	0.795	0.829
Mwuma P	0.841	0.777	0.822	0.808	0.813	0.774	0.779	0.854
Vico Meristem 2	0.797	0.788	0.792	0.773	0.754	0.800	0.821	0.814
Dwesa	0.755	0.755	0.764	0.745	0.726	0.752	0.729	0.767
Y Offspring	0.786	0.805	0.793	0.773	0.790	0.778	0.795	0.805
P Offspring	0.790	0.790	0.798	0.782	0.775	0.800	0.831	0.834
DV Variegated P	0.778	0.765	0.798	0.769	0.725	0.775	0.779	0.822
Pinstripe Y	0.788	0.788	0.807	0.779	0.772	0.785	0.776	0.820
Barbara's Y	0.767	0.790	0.798	0.733	0.788	0.776	0.780	0.798

Potterrill	0.739	0.752	0.749	0.702	0.761	0.710	0.752	0.739
Ngome Y	0.750	0.762	0.770	0.741	0.735	0.747	0.763	0.762

	Bonnie P	**Pretoria Y**	**Caul x Mirabilis**	**Vico Gold Naka**	**Vico Gold Smither's**	**Vico Y Naka**	**Q2 Apple Blossom**	**Oribi Y**
Bonnie P	1.000							
Pretoria Y	0.878	1.000						
Caul x Mirabilis	0.739	0.774	1.000					
Vico Gold Naka	0.789	0.832	0.777	1.000				
Vico Gold Smither's	0.815	0.823	0.733	0.864	1.000			
Vico Y Naka	0.819	0.770	0.713	0.859	0.861	1.000		
Q2 Apple Blossom	0.818	0.794	0.739	0.805	0.802	0.844	1.000	
Oribi Y	0.818	0.831	0.764	0.812	0.814	0.810	0.819	1.000
Naka Vico Mer	0.832	0.810	0.693	0.790	0.795	0.819	0.760	0.808
FlAprxUmtam	0.838	0.848	0.768	0.807	0.821	0.793	0.767	0.838
Floradale Apricot	0.862	0.862	0.766	0.818	0.842	0.798	0.784	0.834
Umtamwuna	0.844	0.856	0.778	0.832	0.869	0.827	0.828	0.852
Andrew Gibson	0.782	0.810	0.738	0.732	0.742	0.777	0.811	0.779
Mvuma Y	0.829	0.795	0.722	0.721	0.758	0.772	0.777	0.718

	Bonnie P	**Pretoria Y**	**Caul x Mirabilis**	**Vico Gold Naka**	**Vico Gold Smither's**	**Vico Y Naka**	**Q2 Apple Blossom**	**Oribi Y**
Mwuma P	0.780	0.817	0.715	0.753	0.766	0.809	0.827	0.773
Vico Meristem 2	0.803	0.781	0.711	0.746	0.824	0.810	0.800	0.765
Dwesa	0.763	0.747	0.706	0.744	0.744	0.794	0.797	0.719
Y Offspring	0.828	0.807	0.749	0.795	0.759	0.770	0.778	0.753
P Offspring	0.842	0.812	0.739	0.775	0.780	0.795	0.803	0.734
DV Variegated P	0.805	0.800	0.701	0.738	0.780	0.778	0.752	0.726
Pinstripe Y	0.813	0.810	0.731	0.785	0.764	0.780	0.805	0.753
Barbara's Y	0.802	0.823	0.704	0.788	0.793	0.770	0.745	0.745
Potterrill	0.752	0.781	0.715	0.766	0.753	0.719	0.720	0.707
Ngome Y	0.774	0.823	0.721	0.756	0.739	0.725	0.767	0.730

	Naka Vico Mer	**FlAprxUmtam**	**Floradale Apricot**	**Umtamwuna**	**Andrew Gibson**	**Mvuma Y**	**Mwuma P**	**Vico Meristem 2**
Naka Vico Mer	1.000							
FlAprxUmtam	0.805	1.000						

Floradale Apricot	0.795	0.922	1.000					
Umtamwuna	0.811	0.844	0.885	1.000				
Andrew Gibson	0.761	0.779	0.782	0.773	1.000			
Mvuma Y	0.757	0.771	0.788	0.792	0.805	1.000		
Mwuma P	0.750	0.792	0.782	0.813	0.811	0.815	1.000	
Vico Meristem 2	0.763	0.748	0.783	0.818	0.833	0.837	0.791	1.000
Dwesa	0.770	0.708	0.736	0.765	0.733	0.780	0.724	0.737
Y Offspring	0.762	0.747	0.788	0.780	0.805	0.828	0.778	0.873
P Offspring	0.771	0.758	0.795	0.813	0.831	0.864	0.782	0.870
DV Variegated P	0.723	0.753	0.808	0.787	0.818	0.877	0.782	0.857
Pinstripe Y	0.719	0.768	0.803	0.821	0.790	0.870	0.805	0.853
Barbara's Y	0.712	0.752	0.805	0.798	0.755	0.838	0.770	0.861
Potterrill	0.726	0.740	0.740	0.716	0.789	0.764	0.747	0.770
Ngome Y	0.740	0.752	0.781	0.772	0.821	0.807	0.755	0.775
	Dwesa	**Y Offspring**	**P Offspring**	**DV Variegated P**	**Pinstripe Y**	**Barbara's Y**	**Potterrill**	**Ngome Y**
Dwesa	1.000							
Y Offspring	0.793	1.000						
P Offspring	0.810	0.874	1.000					
DV Variegated P	0.760	0.838	0.901	1.000				
Pinstripe Y	0.769	0.897	0.850	0.913	1.000			
Barbara's Y	0.749	0.861	0.838	0.874	0.909	1.000		
Potterrill	0.737	0.815	0.813	0.800	0.784	0.838	1.000	
Ngome Y	0.745	0.807	0.829	0.817	0.810	0.805	0.830	1.000

Caul=Caulescens; DV Variegated P= De Villiers Variegated Peach; FvN=F van Niekerk; JS=J Spies; MD= M Dower; Mer=Mersitem; Naka=Nakamura; P= Peach PG= P Gore; PSJ/Rod Ellis=Port St John / Rod Ellis; PSJY/Nev=Port St John Yellow / Neville Willy; RL=R Lotter; Watkins Y G=Watkins Yellow Grobler; Y=Yellow