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Diversity and population structure of Clivia miniata Lindl. (Amaryllidaceae): Evidence from molecular genetics and ecology

By
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Opgedra aan
My Ouers, Suster & Vriende

Aan God die dank

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ABBREVIATIONS

A	Adenosine
ABI	Applied Biosystems Incorporated
AC	Albany Centre
AFLP	Amplified fragment length polymorphism
APS	Ammonium persulfate
AUS$	Australian dollars
B	Cytosine, Guanosine or Thymine
BC	Barberton Centre
BMM	Bearded Man Mountain
bp	Base pairs
BRU	Broedershoek farm
C	Cytosine
CFR	Cape Floristic Region
cpDNA	Chloroplast DNA
CR	Critically endangered
CTAB	Cetyltrimethylammonium bromide
D	Adenosine, Guanosine or Thymine
ddH_2O	Double-distilled water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides
DON	Donkeni
€	Euro
EDTA	Ethylenediaminetetraacetic acid
EN	Endangered
EtBr	Ethidium bromide
G	Guanosine
g/l	Grams per litre
H	Adenosine, Cytosine, or Thymine

HCl	Hydrochloric acid
HOW	Howick Falls
ICNCP	International Code of Nomenclature for Cultivated Plants
IPTG	Isopropylthio-β-D-galactoside
ISSR	Inter-simple sequence repeat
ITS	Internal transcribed spacer
IUCN	International Union for the Conservation of Nature
KAR	Karkloof
kb	Kilobases (=1 000 basepairs)
KCl	Potassium chloride
KEI	Kei River Mouth
KEN	Kentani Area
KOE	Koek Koek River
KZN	KwaZulu-Natal
LB	Luria-Bertani medium
LEB	Lebombo Mountains
M	Molar
m	Metre
m^2	Square metre
MBA	Mbashe
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
min	Minutes
ml	Millilitre
mM	Millimolar
mm	Millimetres
MPB	Mpumalanga Parks Board
MPR	Maputaland-Pondoland Region
MZA	Mzamba River
N	Any base
NaCl	Sodium chloride

NBI	National Botanical Institute
ng	Nanograms
ng/µl	Nanograms per microlitre
NGO	Ngoye Forest
NQO	Nqobara River
NTO	Ntomeni Forest
°	Degree
°C	Degree Celsius
ORI	Oribi Gorge
PAGE	Polyacrylamide gel electrophoresis
PC	Pondoland Centre
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction restriction fragment length polymorphism
pers. comm.	Personal communication
pmol	Picomole
pmol/µl	Picomole per microlitre
PRE	National Herbarium, Pretoria
PRECIS	Pretoria (PRE) Computerised Information System
PRU	H.G.W.J. Schweickerdt Herbarium
PSJ	Port St Johns
QOR	Qora
R	Rand
RAMs	Random amplified microsatellites
RAPDs	Random amplified polymorphic DNAs
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
s	Second
SAMPL	Selectively amplified microsatellite polymorphic locus
SC	Soutpansberg Centre
SDS	Sodium dodecyl sulphate
SSM	Slip-strand mispairing

SSRs	Simple sequence repeats
STMs	Sequence-tagged microsatellites
STRs	Short tandem repeats
T	Thymine
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TEMED	N,N,N',N'-tetramethylene-ethylenediamine
T_m	Melting temperature
U	Unit: One unit of *Taq* polymearse enzyme is defined as the amount of enzyme that will incorporate 10 nmoles of dNTPs into acid insoluble material per 30 minutes at 74°C under standard analytical conditions.
UCO	Unequal cross-over
µg/ml	Microgram per millilitre
µl	Microlitre
µM	Micromolar
UMT	Umtamvuna Nature Reserve
US$	United States of America dollars
UV	Ultraviolet
V	Degenerate primer sequences: Adenosine, Cytosine or Guanosine
V/cm	Volt per centimetre
v/v	Volume per volume
var.	Variety
VU	Vulnerable
w/v	Weight per volume
WC	Wolkberg Centre
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1

INTRODUCTION

Clivia Lindl. (1928), a genus endemic to southern Africa, is classified in the sub-Saharan African tribe Haemantheae (Pax) Hutch. (1934) of the family Amaryllidaceae J. St-Hil. (1805). At present the genus comprises five described species, namely *Clivia nobilis* Lindl. (1828), *Clivia miniata* (Lindl.) Regel (1854), *Clivia gardenii* Hook. (1856), *Clivia caulescens* R.A.Dyer (1943) and *Clivia mirabilis* Rourke (2002) (Chapter 3).

All the species, but notably *Clivia miniata*, are currently the subject of considerable horticultural attention (reviewed in Chapter 5). The genus is also extensively utilised by traditional healers (Chapter 4). Despite all this attention, essentially nothing is known regarding the genetic diversity within natural populations of *Clivia*. A principal objective of the present study was to investigate the genetic diversity and structure of *Clivia miniata* populations in nature. With no previous work having been done at population level on any member in the genus, the first step was to develop the necessary molecular tools needed for such a study.

Existing molecular studies on both *Clivia* and Amaryllidaceae focus mainly on clarifying the taxonomic structure (phylogeny) of the taxa (Chapter 3). No infraspecific studies, i.e. on the population level, could be traced. Hence the need for the present study to develop the necessary molecular tools for further population work within the group. Whilst involved in the molecular work, other gaps in our knowledge of *Clivia* were revealed. To address some of these questions, the present study also focuses on the taxonomy, ecology, phytogeography, conservation status and horticultural significance of the genus.

Chapters are presented as self-contained units. Initial chapters address questions regarding the genus as a whole, with increased focus being placed in later chapters on the development of molecular tools for *Clivia miniata*. It is believed that the research

presented here will contribute towards an improved understanding of the genus *Clivia* and would lay the foundation for more intensive future research into the genetic structure and diversity of *Clivia* species in nature, especially *Clivia miniata*.

General materials and methods employed during the study are set out in Chapter 2. Selected molecular techniques, buffers and reagents are described in some detail, with alterations to existing protocols indicated. The general section of this chapter also covers aspects of terminology and lists all voucher specimens used in the molecular part of the study. Methods described in this chapter are only briefly referred to in subsequent chapters.

The genus *Clivia* is introduced in Chapter 3. This chapter provides a historical background on the taxonomy of *Clivia*, including its current taxonomic status and proposed phylogenetic relationships. Diagnostic characteristics for the different infrageneric taxa and an identification key, down to the variety level, are presented for the first time. Included in the chapter is a new, as yet undescribed species, and a new variety. Interspecific hybridisation within the genus and the cultivar-group classification system for *Clivia* hybrids, are discussed.

Chapter 4 presents the results of a study on the geographical distribution of the various members of the genus *Clivia*. The geographical distribution of each species is mapped and described based on the analysis of numerous herbarium and other records, supplemented by extensive fieldwork. Notes on the ecology of different populations of the species, based on the literature, field observations and herbarium records are presented. To explain the current distribution of the various species, an hypothesis is offered, exploring ancient migratory routes induced by historical environmental changes.

The trade in and horticultural significance of *Clivia*, with the focus on *Clivia miniata*, is investigated in Chapter 5. Notes are provided on the history of the industry, including the aims of *Clivia miniata* breeders in different regions of the world. Estimates are presented

which highlight the considerable size and monetary value of *Clivia* markets worldwide, especially the horticultural significance of *Clivia miniata*.

Chapter 6 addresses the lack of knowledge regarding the diversity of natural *Clivia miniata* populations and describes the development and application of chloroplast PCR-RFLP molecular markers. Biogeographic structure within the distribution range of this species is indicated.

The development of microsatellite markers for *Clivia miniata* is described in Chapter 7. An overview of the technology is given and the isolation method is described in detail. The first ever microsatellite markers developed for any member of the genus *Clivia* are presented in this chapter.

Chapter 8 gives an overview of the work presented in this dissertation. Included are new questions resulting from this study, potential future applications utilising the developed molecular tools and general and specific conclusions reached as a result of this study.

Data used in the construction of biogeographic distribution maps for the different species (Chapter 4), are presented in Appendix I. A RFLP data matrix, the experimental results of Chapter 6, is included in Appendix II. Sequenced data, used in Chapter 7 for the development of microsatellite markers, are presented in Appendix III.

CHAPTER 2

MATERIALS & METHODS

2.1 Introduction

Chapters in this dissertation are presented as individual units, but some techniques are common to two or more of them. Techniques used are given here in more detail to prevent unnecessary duplication. The principal aim of this chapter is to give an account of the materials and methods tested and used during the course of this study. Methods, tried but not used, are summarised and the reasons for discarding them are given. The selected methods that were used during the course of this study are briefly described, with all alterations indicated.

This chapter is divided into two sections: a general section, covering all the chapters and a molecular section, covering specifically Chapters 6 and 7.

2.2 General techniques

2.2.1 Descriptors

In many scientific fields qualitative terms are used to express approximate values. These descriptors, being subjective, indicate different values depending on the authors' viewpoint. Descriptors used in this dissertation are based mainly on the objective guidelines proposed by Schmid (1982). Table 2.1 is a summary from Schmid (1982) and gives an indication of the approximated percentages used.

Table 2.1 Descriptors as expressed by approximate percentages.[1]

0%	Absent; completely absent; lacking; never; not at all; wanting	31–54%	Often; fairly commonly; frequently; in many cases; repeatedly
V<2%	Very[2] rarely; almost never; in a very few cases; not very commonly/ frequently/often; uniquely; very exceptionally/seldom/unusually	55–64%	Very often; very frequently; in very many cases
2–4%	Rarely; exceptionally; extraordinary; in a few cases; seldom; uncommonly; unusually	65–94%	Usually; as a rule; characteristically; chiefly; commonly; consistently; customarily; generally; in most cases mainly; mostly; normally;
5–10%	Very occasionally; very sporadically	95–99%	Nearly[3] always; almost always; very commonly
11–30%	Occasionally; at times; often; now and then; on occasion; sometimes; sporadically	100%	Always; absolutely; all; all the time; completely; entirely; universally; with no exception

[1] From Schmid (1982).

[2] Very = exceeding, exceptionally, extraordinary, extremely, highly, etc.

[3] Nearly = all but, almost, not quite, very nearly.

2.2.2 Voucher specimens

Vouchers are required for any serious questioning or re-examination of results and conclusions. They are essential to reassess the identity of a sample and, if necessary, to duplicate the result (Goldblatt *et al.* 1992).

Vouchers were made of all plant samples (*Clivia miniata*) used during this study with the exception of material obtained from Kirstenbosch National Botanical Garden[1] (Table 2.2). All material collected or donated during the course of this study was deposited in the H.G.W.J. Schweickerdt Herbarium, University of Pretoria (Table 2.2 & Appendix I). Voucher numbers are subdivided according to the number of samples collected/obtained for that specific locality, with different voucher numbers assigned for each collector/donor.

[1] National Botanical Garden, Kirstenbosch, Private Bag X7, Claremont 7735, South Africa

Table 2.2 Voucher specimens for *Clivia miniata* material collected or donated during the course of this study.

Collection and voucher/ accession numbers	Collector/donor	Number of samples	Locality
ZH5, PRU92693, 2096, PRU92682	Z.H. Swanevelder; J.T. Truter	5	Bearded Man Mountain
431/99	Kirstenbosch NBG	9	Broedershoek Farm
327/00	Kirstenbosch NBG	9	Donkeni
ZH3, PRU92687	M. Exelby	1	Howick Falls
ZH4, PRU92699, 3014, PRU92687	Z.H. Swanevelder; J.T. Truter	5	Karkloof
3107, PRU92688	J.T. Truter	1	Kei River Mouth
3108, PRU92684, 3284, PRU92685	J.T. Truter	2	Koek Koek River
3611, PRU92683	J.T. Truter	1	Kentani Area, Transkei
3213, PRU92689	J.T. Truter	1	Lebombo Mountains
524/98	Kirstenbosch NBG	14	Mbashe
520/98	Kirstenbosch NBG	10	Nqobara River
435/99	Kirstenbosch NBG	11	Ntomeni Forest
3619, PRU92686	J.T. Truter	1	Oribi Gorge
ZH1, PRU92697, PSJ729/96	Z.H. Swanevelder; J.T. Truter; Kirstenbosch NBG	31	Port St Johns
720/96	Kirstenbosch NBG	10	Qora
436/99	Kirstenbosch NBG	10	Ngoye Forest
PRU91194, 515/98	A. Hardinge; Kirstenbosch NBG	26	Umtamvuna Nature Reserve
PRU91195	A. Hardinge	6	Mzamba River

Plant collection was conducted in accordance with the rules and regulations of the particular provinces. The necessary permits were obtained from Ezemvelo KwaZulu-Natal Wildlife (permits 27110/2001, 30443/2002 & 966/2003), Mpumalanga Parks Board (permits MPB. 1039 & MPB. 1056) and Department of Economic Affairs, Environment and Tourism, Province of the Eastern Cape (General Permit 01/07/2001).

2.3 Molecular techniques

2.3.1 DNA isolation and purification

Meerow *et al.* (1999) proposed the CTAB (cetyltrimethylammonium bromide) method of Doyle & Doyle (1987) for DNA (deoxyribonucleic acid) extraction from *Clivia* leaves. Several subsequent publications described DNA extraction procedures for *Clivia*, including the CTAB method (Ran *et al.* 2001c; Conrad & Reeves 2002) and the Nucleon Phytopure kit of Amersham Pharmacia Biotech (used on root tips) (Ran *et al.* 2001a; Ran *et al.* 2001b).

Initially the CTAB method (Doyle & Doyle 1987) was attempted on *Clivia* leaves. This method was discarded even though DNA was obtained, largely due to poor PCR (Polymerase Chain Reaction) results using these samples as templates and the lengthiness of the protocol. The Plant DNAzol™ Reagent (GibcoBRL, LifeTechnologies™) for genomic DNA isolation from plants and the monocot DNA isolation method (Edwards *et al.* 1991) were also tested, but both isolated less DNA than the CTAB method.

The genomic DNA isolation method of Raeder & Broda (1985) for fungal mycelia was tried. Though this method isolated large quantities of genomic DNA, PCR results with these templates were still unreliable. The higher amounts of DNA isolated, however, compensated for DNA losses that occurred during additional purification steps. Together with alterations made to the extraction buffer, better DNA templates were isolated.

The altered DNA isolation method, based on that of Raeder & Broda (1985), consisted of 200 mM Tris-HCl pH 8, 150 mM NaCl, 25 mM ethylenediaminetetra-acetic acid (EDTA) pH 8, 0.5% (w/v) SDS (sodium dodecyl sulphate) and 1% (v/v) 2-mercaptoethanol. Fresh leaf disks are homogenized in the extraction buffer (700 µl), as dried leaves produced poorer DNA templates.

Homogenisation was either done in a Bio 101 FastPrep machine at setting 2 for one minute or by hand in an eppendorf tube till material was fully macerated. Phenol-chloroform (3:5) purifications were performed till no interface was visible (centrifugal

steps performed at 10 000 rpm, 4°C). Chloroform-phenol in a 1:1 ratio or chloroform-isoamyl alcohol (24:1) can be used as substitutes in the purification steps. DNA in the aqueous layer was precipitated with two volumes of ice-cold absolute ethanol, after a final chloroform purification step. The addition of 0.1 volume 3M sodium acetate (pH 5) to the two volumes of absolute ethanol, further promotes the precipitation of the DNA. DNA forms a small, whitish pellet after centrifuging (11 000 rpm) at 4°C. Salts were removed from the DNA pellets by washing them with 70% (v/v) ethanol. DNA pellets were dried under vacuum, before re-suspending them in sterile, double-distilled water (ddH_2O).

2.3.2 Visualising and separating DNA

Agarose gels

DNA was separated in 1–3% (w/v) agarose gels and run in 1× (v/v) TAE buffer (50× TAE buffer: 2 M Tris-acetate and 0.05 M EDTA, pH 8) at 5 V/cm for 60 min (Sambrook *et al.* 1989).

Staining

DNA fragments were visualised by the addition of ethidium bromide (EtBr) at 0.5 µg/ml to the melted gel. The DNA, with the chelated EtBr, was viewed under UV light and photographed (Sambrook *et al.* 1989).

Loading buffers

As standard protocol, DNA samples were loaded with a 6× loading buffer (15% w/v ficoll and 0.25% w/v bromophenol blue indicator dye, ddH_2O). Bromophenol blue migrates through agarose gels at approximately the same rate as linear double-stranded DNA of 300 bp (base pair) in length (Sambrook *et al.* 1989).

Commercial blue/orange 6× loading dye (10% w/v ficoll, 0.25 % w/v xylene cyanol FF, migrating at ± 4 kb, 0.25% w/v bromophenol blue and 0.25% w/v orange G, migrating at 50 bp, in water) was used in conjunction with a 100 bp ladder (Promega Corporation, Madison, WI) (Sambrook *et al.* 1989).

Polyacrylamide gel electrophoresis (PAGE)

As standard, 6% polyacrylamide gels were used for the testing of microsatellite markers. Gels (75 ml) contained 2 % (v/v) Long Ranger® gel solution (BioWhittaker Molecular Applications, Rockland, ME), 1× (v/v) TBE buffer (10× TBE buffer: 0.9 M Tris-borate and 0.02 M EDTA, pH 8), 10% (w/v) APS (ammonium persulfate) and 0.004% (v/v) TEMED (N,N,N',N'-tetramethylethylene-diamine) (Sambrook *et al.* 1989). APS and TEMED are added last because they activate polymerisation.

Gel solutions were evacuated for 5 min before pouring commenced. A 30 min setting time preceded a pre-run of 7 V/cm for 30 min. All PAGE were run 1× (v/v) TBE buffer. Wells were washed with 1× TBE buffer before samples were loaded.

Samples (10 µl) were loaded into the wells with a mixture of 2 µl SYBR Green I, diluted 1:500 (Roche Diagnostics GmbH, Mannheim, Germany) and 2 µl commercial 6× loading buffer (10% w/v ficoll, 0.25 % w/v xylene cyanol FF, 0.25% w/v bromophenol blue and 0.25% w/v orange G) (Promega Corporation, Madison, WI). Because SYBR Green I dye is light sensitive, the four-hour runs were done in darkness at a constant 7 V/cm. Gels were visualised under UV light and photographed.

2.3.3 Molecular markers

DNA Molecular weight marker III

λ DNA (250 ng/µl) was restricted for three hours with *Eco*RI (24 U) and *Hind*III (20 U) (Promega Corporation, Madison, WI) in 10% (v/v) buffer B (Promega Corporation, Madison, WI), resulting in fragment sizes of 21 226, 5 148, 4 973, 4 268, 3 530, 2 027, 1 904, 1 584, 1 375, 947, 831 and 564 base pairs. Restricted λ DNA was denatured at 60°C for 10 min and 6× (v/v) loading buffer (previous section) was added to obtain a final 1× (v/v) concentration. The resulting DNA molecular weight marker III (50 ng/µl) is stored at –20°C till needed. Five microlitres of this marker is used as size standard in agarose gels.

100 bp ladder

The 100 bp ladders were obtained commercially (Promega Corporation, Madison, WI) and used in conjunction with 2% (and higher) agarose gels and PAGE.

2.3.4 Polymerase chain reaction (PCR)

PCRs were either performed on Applied Biosystems GeneAmp 2700 or the 9700. Reactions work best with Super-Therm DNA polymerase, magnesium chloride (25 mM $MgCl_2$ stock) and 10× PCR reaction buffer (composition unknown), all supplied by Southern Cross Biotechnology (Pty) Ltd. (Cape Town, South Africa). The $MgCl_2$ concentrations varied between the different reactions. Primers stocks (100 pmol/µl) were diluted to a working stock of 10 pmol/µl and deoxynucleotide triphosphates (dNTPs, 100 mM) to a working stock of 50 µM each. Template DNA was diluted to a final concentration of 30 ng/µl.

Problems with amplifications were addressed by changing the reaction conditions, proposed by Innis & Gelfand (1990). Additional additives such as 2-pirrolidinone (usually at 480 mM) and cosolvents like butaine (0.5–2.0 M), formamide (1–5%, v/v) and DMSO (1–5%, v/v), were sometimes added (in different concentrations) in an attempt to facilitate a successful reaction.

Reactions were started with a warm block (hotstart) to prevent any artefacts from being produced. Touchdown PCRs were performed during the optimisation steps, in accordance to the protocol of Don *et al.* (1991). Detailed reaction conditions for PCRs are presented in Chapter 6 and 7.

2.3.5 Cloning

Vector

The pGEM®-T Easy vector (Promega Corporation, Madison, WI, USA) was used for cloning. This vector contains single 3'-thymine (T)-overhangs that greatly increases the cloning efficiency of PCR products with an adenosine (A)-overhang. The Super-Therm DNA polymerase (Southern Cross Biotechnology (Pty) Ltd., Cape Town, South Africa) used has no proofreading capability and therefore adds the A-overhang needed for successful ligation (Promega Corporation, Madison, WI, USA).

Li & Guy (1996) reported that by increasing the final extension time (60–120 min) of the PCR, greater cloning efficiency of PCR products could be obtained. The extension time of 7 min that was used gave sufficient colonies with the fragment of interest.

The pGEM®-T Easy vector contained T7 and SP6 primer sites, one on each side of the insert. This allows for amplification of fragments out of the plasmid (colony PCRs) without going though lengthy plasmid extractions. The insertion site is located within the enzyme β-galactosidase. Inserted fragments that inactivate this enzyme allow for direct colour screening of recombinant clones. The vector also contains an ampicillin resistance gene that only allows transformed colonies to grow on the selective medium (Technical manual: pGEM®-T and pGEM®-T Easy Vector Systems, Promega Corporation, Madison, WI, USA).

Ligation and transformation

The ligation and transformation protocol of Promega (Technical manual: pGEM®-T and pGEM®-T Easy Vector Systems, Promega Corporation, Madison, WI, USA) were used with the following alterations: total reaction volumes were lowered to 10 µl, with 2× Rapid Ligation Buffer and PCR products lowered in accordance, 50 ng pGEM®-T Easy Vector and 3 units T4 DNA ligase were used (pGEM®-T and pGEM®-T Easy Vector Systems, Promega Corporation, Madison, WI, USA). Reactions were done overnight.

The JM109 high efficiency competent cells provided with the pGEM®-T Easy Vector Systems (Promega Corporation, Madison, WI, USA) were used in transformations. The standard transformation protocol supplied with the cell line JM109 was used (Promega Corporation, Madison, WI, USA). A brief summary of the protocol is as follows: 100 µl competent *E. coli* cells (JM109) were heat shocked (42°C), together with 50 ng vector (containing the PCR insert) for a period of 45–50 s in a water bath, after an ice treatment of 10 min; heat shock was followed with a 2 min ice treatment and SOC medium (900 µl, 4°C) was added before incubating at 37°C (60 min); the cells were plated out onto LB/ampicillin/ IPTG/X-Gal plates and grown overnight.

The clones with inserted PCR products generally produce white colonies on the LB/ampicillin/IPTG/X-Gal plates, but fragments cloned in-frame may produce blue colonies. The white colonies were first selected for further analysis and only if fragments were absent, were the blue colonies randomly chosen and tested for those fragments.

2.3.6 Growth media

LB medium with ampicillin, IPTG and X-Gal

LB (Luria-Bertani) medium contains 10 g/l tryptone, 5 g/l yeast extract, 5 g/l sodium chloride (NaCl) and 15 g/l agar, pH 7.0 (Sambrook *et al.* 1989). The medium was autoclaved and cooled to 50°C before adding ampicillin to a final concentration of 100 µg/ml, 0.5 mM IPTG (isopropylthio-β-D-galactoside) and 80 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside).

The medium was poured into petri dishes, allowed to solidify and stored at 4°C for no longer than 1 month. Plates were allowed to reach room temperature prior to plating out of bacterial colonies (Technical manual: pGEM®-T and pGEM®-T Easy Vector Systems, Promega Corporation, Madison, WI, USA).

SOC medium

The SOC medium contained 2 g/100ml tryptone, 0.5 g/100ml yeast extract, 10 mM NaCl, 2.5 mM KCl, 1 ml filter-sterilised Mg^{2+} stock (20.33 g/100ml $MgCl_2.6H_2O$ and 24.65 g/100ml $MgSO_4.7H_2O$) and 20 mM glucose, with the final volume adjusted to 100 ml, pH 7. The medium was filtered-sterilised (Technical manual: pGEM®-T and pGEM®-T Easy Vector Systems, Promega Corporation, Madison, WI, USA).

2.3.7 Sequencing reactions

Quarter volume sequencing reactions were performed with 2 µl ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit II (Perkin-Elmer, Warrington, UK), 3.2 pmol primer T7 or SP6 (stock 10 pmol/µl), ≤ 200 ng DNA/PCR template, with the final volume adjusted to 5 µl with ddH_2O.

Cycle conditions were in accordance to those proposed by Perkin-Elmer (Warrington, UK) for GeneAmp PCR Systems 9600 and 2400. Amplification consisted of 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min.

The PCR products were precipitated using the ethanol sodium acetate precipitated protocol of Perkin-Elmer (Warrington, UK). A 20 µl reaction mixture (quarter reactions adjusted to this volume with ultra pure water) was precipitated by 0.1 volume (v/v) 3M sodium acetate, pH 4.6 and 2.5 volumes (v/v) absolute ethanol. DNA was 'pelleted' (10 000 rpm, 30 min) before the ethanol mixture was aspirated with a micropipette. Salts were removed with a 70% (v/v) ethanol (250 µl) washing step and the invisible pellet dried under vacuum after the ethanol was removed.

Sequencing were conducted on an ABI PRISM™ 377 automated DNA sequencer.

2.3.8 Cleaning of PCR products

PCR reactions that were selected for cloning or sequencing were first purified using either the QIAquick PCR Purification Kit (Qiagen, GmbH, Germany) or the ethanol sodium acetate protocol (previous section). The protocol of Qiagen (GmbH, Germany) was used for the QIAquick PCR Purification Kit.

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CHAPTER 3

TAXONOMIC NOTES ON THE GENUS *CLIVIA* LINDL.

3.1 Introduction

The taxonomic history of the genus *Clivia* is, like most other taxonomic stories, full of twists and turns. What makes this tale more interesting is that it is one of those rare cases in history where the same genus was described—on the same day—by two different authors (Hooker 1828; Lindley 1828; Obermeyer 1972; Duncan 1985).

At present the genus *Clivia* Lindl. consists of five recognised species, *C. nobilis* Lindl. (1828), *C. miniata* (Lindl.) Regel (1854), *C. gardenii* Hook. (1856), *C. caulescens* R.A.Dyer (1943) and *C. mirabilis* Rourke (2002). The genus is endemic to southern Africa, i.e. South Africa and Swaziland (Vorster 1994; Meerow & Snijman 1998; Duncan 1999; Snijman 2000; Koopowitz 2002; Rourke 2002a).

The aim of this chapter is to supply background information on the historical and current classification of the genus *Clivia* Lindl. Diagnostic features and habitat information required to identify the various species are also noted and used in a key. Current evidence on the molecular phylogeny and interspecific hybridisation of the genus will also be reviewed.

3.2 Taxonomic notes

3.2.1 Suprageneric classification

A comparison between different suprageneric classifications of *Clivia* is supplied in Table 3.1. The suprageneric classification of Kubitzki (1998b) was adopted in this thesis and is as follows:

Class	'Monocotyledons'
Superorder	Lilianae Takhatajan (1967)
Order	Asparagales Huber (1969)
Informal group	'Higher' Asparagales Kubitzki (1998a)
Family	Amaryllidaceae J. St-Hil. (1805)
Tribe	Haemantheae (Pax) Hutchinson (1934)
Genus	*Clivia* Lindl. (1828)

Table 3.1 A comparison of different suprageneric classifications for the genus *Clivia* Lindl.

Rank	Snijman (2000)	Meerow *et al.* (1999)	Kubitzki (1998b)[1]	Takhtajan (1997)	Dahlgren *et al.* (1985)	Cronquist (1981)
Division	-	-	-	-	-	Magnoliophyta
Class	'Monocotyledons'	-	'Monocotyledons'	-	'Monocotyledons'	Liliopsida
Subclass	-	-	-	Liliidae	-	Liliidae
Superorder	Lilianae	-	Lilianae	Lilianae	Liliiflorae	-
Order	Asparagales	-	Asparagales	Amaryllidales	Asparagales	Liliales
Group (informal)	-	-	'Higher' Asparagales	-	-	-
Family	Amaryllidaceae	Amaryllidaceae	Amaryllidaceae	Amaryllidaceae	Amaryllidaceae	Liliaceae[2]
Tribe/Tribus	-	Haemantheae	Haemantheae	Haemantheae	Haemantheae	-
Genus	*Clivia*	*Clivia*	*Clivia*	*Clivia*	*Clivia*	*Clivia*

[1] Chapters by Kubitzki 1998a; Kubitzki *et al.* 1998; Meerow & Snijman 1998 were used to compile this classification.

[2] Cronquist (1981) lumps into Liliaceae amongst others the classical families Liliaceae and Amaryllidaceae.

The superorder Lilianae comprises four orders, of which the order Asparagales is the largest containing 31 families (Kubitzki *et al.* 1998; Meerow *et al.* 1999). Kubitzki (1998a) proposed the informal group 'Higher Asparagales' for those families in which microsporogenesis is successive and steroidal saponins are common (except in Amaryllidaceae).

The family Amaryllidaceae occurs worldwide, mainly throughout warm temperate and tropical regions. It encompasses approximately 60 genera, representing an estimated total of 850 species. About 18 of the genera are represented in southern Africa—one of the three centres of diversity—with about 280 species. The other two centres of diversity are the Andean region in South America (28 genera) and the Mediterranean (8 genera) (Meerow & Snijman 1998).

Amaryllidaceae inflorescences are pseudoumbels. In the case of the flowers, the perigone consists in all cases of two whorls, each with three petaloid tepals. The tepals are usually connected basally to form a tube, with those of the outer whorl usually slightly longer than those of the inner whorl. Stamens are opposite the tepals and occur usually in two whorls (3 + 3). The gynoecium is syncarpous and tricarpellate. All genera have septal nectaries in their ovaries. Seedlings are distinguished by a bifacial cotyledon (Meerow & Snijman 1998, and references therein).

Haemantheae is an African baccate-fruited tribe and the only tribe of the Amaryllidaceae that contains rhizomatous genera, namely *Clivia*, *Scadoxus* and *Cryptostephanus*. Members of this tribe have fleshy, recalcitrant seeds that lack phytomelan (Meerow & Snijman 1998; Meerow *et al.* 1999). Meerow *et al.* (1999) showed that the tribe is strongly supported by both molecular and morphological evidence, thus rendering the subtribe Cliviinae (for *Clivia* and *Cryptostephanus*) of Müller-Doblies, paraphyletic.

The genus *Clivia* is characterized by evergreen, rhizomatous plants with distichous, firm, strap-shaped leaves. Inflorescences are pseudo-umbels borne on solid, compressed scapes that usually produce red (yellow for some forms of *C. miniata* and *C. caulescens*)

subglobose berries with fleshy, ivory-white seeds embedded in a soft yellow pulp (Meerow & Snijman 1998; Snijman 2000; Rourke 2002a).

3.2.2 Infrageneric classification

The taxonomic history of the different *Clivia* species is briefly described in this section.

Clivia nobilis Lindl. (1828)

The first scientific collection of a member of the genus *Clivia* dates back to September 1815. The collection was made near the mouth of the Great Fish River, Eastern Cape, South Africa by William J. Burchell (Vorster 1994; Duncan 1999).

It was James Bowie, however, who brought the new species to the attention of botanists in England. He collected specimens in the same area as Burchell in the early 1820s and brought them to England in 1823 (probably to Kew Gardens and Syon House) (Hooker 1828; Lindley 1828; Obermeyer 1972; Duncan 1992; Vorster 1994; Duncan 1999). According to the review by Smith & Van Wyk (1989) of the collecting journeys of Bowie, he most probably collected *C. nobilis* during the course of his fourth journey (May 1821–December 1822). Bowie, being notorious for insufficient and misleading details, gave the Orange River as the locality of *C. nobilis*. Commercial interests—with Cape plants being highly fashionable in Europe at that time—could have been the reason for his misleading notes (Smith & Van Wyk 1989). In correspondence to Hooker, Mr. Aiton (patron to Bowie) gave the Great Fish River as the locality (Hooker 1828).

Bowie wrote to W.J. Hooker (before returning to the Cape) informing him of a 'Cyrtanthus-like plant' that he had collected previously in the Cape. He included specimens of flowers and a leaf, both collected in the wild. In his letter he requested Hooker that if the plant flowered, it should bear as specific name, that of his patron, Mr. Aiton (Hooker 1828).

A specimen indeed flowered that year (October 1827?). Mr. Forrest, who was in charge of the plant collection at Syon House, requested that a drawing of the flowering plant be

made. The Duke of Northumberland granted permission for the plant to be sketched. At the same time, Mr. Aiton, sent a drawing, some fruits, and some extracts of Bowie's notes on the habitat of the new genus, to Hooker. The sketch ordered by the Duke was copied and together with the other information supplied by Mr. Aiton, was used in the article by W.J. Hooker (October 1828) in which he described the new genus. He named it *Imatophyllum aitoni* as requested by Bowie (Hooker 1828). *Imatophyllum* was later miss-spelled by Hooker, when he described *Imantophyllum ? miniatum* (Koopowitz 2002)

Mr. Forrest meanwhile, had discussed the interesting plant that was in his care with the botanist John Lindley. The plant flowered for the second time (July 1827?) at Syon House and Mr. Forrest informed Lindley (Lindley 1828) of this. A drawing of the plant was made (probably the same one as used by Hooker) and in October of 1828, John Lindley described the species as *Clivia nobilis* (Lindley 1828; Duncan 1999). In Lindley's article (1828) it is clear that he was uncertain about the collector and original locality. He nevertheless credited Bowie as the collector but the exact locality remained speculative. He named the genus *Clivia* after the Duchess of Northumberland, Lady Charlotte Florentine Clive. In his article he thanked her for the opportunity to publish the new species (Lindley 1828; Duncan 1999). Lindley commented that, at first, the new species appeared to be a member of *Cyrtanthus*, but later questioned whether it should be placed in the family Amaryllidaceae [=Amaryllideae]. He classified it in this family because it resembles *Haemanthus*, some members of which have imperfect bulbs (Lindley 1828).

Lindley and Hooker's publications did not only appear in the same year, but also on the same day in October 1828 (Obermeyer 1972; Duncan 1992). In 1830, Roemer and Schultes, according to literature, chose *Clivia nobilis* as the type species, thus reducing Hooker's *Imatophyllum aitoni* to a synonym (Obermeyer 1972; Duncan 1992). *Clivia nobilis* is illustrated in Figure 3.1.

Obermeyer (1972) stated that rumours had it that Lindley obtained the plant he used to describe the new genus, 'surreptitiously' from Kew. In Lindley's article describing the new genus *Clivia*, he does not mention once the word Kew (Lindley 1828). He refers to

Mr. Forrest and Syon House as sources of information, but doesn't state clearly from where he obtained the material he used to describe the new species. He thanked the Duchess for allowing him to name the species after the family Clive (Lindley 1828). I suspect that Lindley's request regarding the naming of the genus was probably either directed at the Duchess (Lady Clive) or was intercepted by her. Mr. Forrest could have acted on Lindley's behalf, asking the Duchess rather than the Duke, for permission. Mr. Forrest knew that Hooker was busy naming the new species. At the same time Aiton,

Figure 3.1 *Clivia nobilis* in cultivation.

Bowie and probably the Duke gave permission to Hooker to describe his new species.

***Clivia miniata* (Lindl.) Regel (1854)**

Clivia miniata* var. *miniata

This species was discovered in the early 1850s and due to its beautiful flowers, it was on public display even before it was named (Duncan 1985, 1992; Koopowitz 2002).

Initially, flower shape led different taxonomists to place it in various genera. According to Koopowitz (2002), Lindley described it in 1854 as a possible species of *Vallota*, namely *Vallota ? miniata*, the question mark indicating his uncertainty (Koopowitz 2002). Hooker

described the same species—again in the same year as Lindley—but placed it in his genus *Imantophyllum*. He described it as *Imantophyllum* ? *miniatum* in Curtis's Botanical Magazine, 1854 (Duncan 1985, 1992; Pole Evans 1921; Koopowitz 2002). Again, he was unsure about the generic identity of the taxon. Koopowitz (2002) refers to another name change made by Koch, during which the species were placed in yet another genus, namely *Himantophyllum miniatum*.

Regel discarded Hooker's name *Imantophyllum miniatum* ten years later on the basis of the recognition by Roemer & Schultes (1830) of Lindley's *Clivia nobilis* as type species. According to Duncan (1985, 1992) Pole Evans (1921) and Koopowitz (2002), Regel changed the name of Hooker's species to *Clivia miniata* (Lindl.) Regel in *Gartenflora*, 1864, p. 131, t. 434. He argued that the different flower form (mainly perianth shape) was insufficient to place the species in its own genus and that Lindley's earlier description had priority, therefore justifying the reduction of both Koch's *Himantophyllum miniatum* and Hooker's *Imantophyllum* ? *miniatum*, to synonyms under *Clivia miniata* (according to Koopowitz 2002). *Clivia miniata* cultivar is depicted in Figure 3.2.

Figure 3.2 *Clivia miniata* cultivar.

***Clivia miniata* var. *citrina* Watson (1899)**

The earliest date for the discovery of a yellow form of *C. miniata* is given to be around 1888 in Zululand, KwaZulu-Natal, by Phillips (1931) when he described the variety *C. miniata* Regel var. *flava* E.Phillips. Subsequently, one or two yellow-flowered specimens of *C. miniata* were collected in the Eshowe Forest, Zululand, by Mr. C.R. Saunders of Melmoth, Zululand. A specimen was send by Mr. B. Nicholson to the National Herbarium, Pretoria, from which Phillips described the new variety (Phillips 1931).

Gooding (1964), however, mentions a yellow/cream form of *C. miniata* discovered in Eshowe, Zululand in 1892. Sir Melmoth Osborne's (the Commissioner at the time) Zulu cook discovered a pale yellow-coloured *Clivia* while searching for firewood. According to Gooding (1964), Sir Osborne shared offsets of the plant with his friends, giving one to his assistant, Sir Charles Saunders. Sir Charles sent some of the 'bulbs' (rhizomes), including a flower, to his mother. Mrs. J.H. Saunders. She made a drawing of the flower and sent it with a 'bulb' (rhizome) to the Royal Botanical Gardens at Kew where notes of the drawing were made before it was returned to her. A first collection date of around 1888 claimed by Duncan (1985, 1999) and Phillips (1931), therefore may well be nearer 1892 and it is likely the collector accredited should either be the Zulu cook or Sir Melmoth Osborne.

According to a communication between Wessel Marais (Herbarium, Royal Botanical Gardens, Kew) and Mrs. Mauve (Botanical Research Institute, Pretoria) in 1964, an earlier note reporting a yellow form of *C. miniata* exists, namely in *Wien. Illustr. Gartenzeit*, 1888: 275. The yellow form was referred to as *Clivia sulphurea*, a novelty displayed at some show. According to the communication, this name is a *nomen nudum* and therefore has no nomenclatural standing. This, however, places the date of discovery definitely before 1892 and almost certainly before 1888.

Subsequently, various other yellow-flowered specimens of *C. miniata* (Figure 3.3) were discovered in both the Eastern Cape and KwaZulu-Natal, but in most cases precise

localities are not known. The picture is further clouded by spontaneous mutations and hybrids that were produced in cultivation over the years (Duncan 1985, 1992, 1999).

Formal description of a yellow form of *C. miniata* followed some years after its first collection. W. Watson published an article containing reference to the yellow variety of *Clivia miniata* in volume 25 of *The Gardener's Chronicle* in 1899. He formally described the variety as *C. miniata* Regel var. *citrina* W.Watson in *The Garden* 1899:56 (Duncan 1985, 1999).

Varietal names like *C. miniata* Regel var. *flava* E.Phillips (Phillips 1931) and *C. miniata* var. *aurea* Hort. (described in *The Gardener's Chronicle* 35: 301 and *The Garden* 65: 330, both dated 7th May 1904—according to the communication between Mauve and Marias) are later homonyms and should be discarded. As already mentioned, *C. sulphurea* is a *nomen nudum* and should also be discarded.

Figure 3.3 *Clivia miniata* yellow cultivar.

***Clivia gardenii* Hook. (1856)**

Clivia gardenii (Figure 3.4) was discovered and collected by Major Robert J. Garden of the 45th Regiment. He collected specimens while stationed in Natal and sent them to the Royal Botanical Gardens at Kew. It was here that Sir W. Hooker, being third time lucky, named the new species *Clivia gardenii* when it flowered in 1856, in honour of the collector Major Garden (Hooker 1856; Obermeyer 1972; Duncan 1985, 1992, 1999; Koopowitz 2002).

Figure 3.4 *Clivia gardenii* var. *gardenii*.

Clivia gardenii* var. *citrina* Swanevelder et al. *ined.

In this dissertation a yellow form of *Clivia gardenii* Hook. is described as a variety (Figure 3.5).

Clivia gardenii var. **citrina** *Swanevelder, A.E.van Wyk & Truter*, var. nov. floribus pallide luteis vel citrinis, non aurantiacus vel rubris ut in varietate typico distinguitur. TYPE.—KwaZulu-Natal, 2731 (Louwsburg): Ngome Forest (–CD), *Swanevelder & Truter* ZH10 (PRU, holo.).

The holotype was collected in Ngome Forest (Ngotshe District, KwaZulu-Natal) by Z.H. Swanevelder and J.T. Truter on 22 June 2002 (permit no. 30443/2002, collection no. ZH10) The visit confirmed previous reports of a population of yellow-flowered *C. gardenii*. This stands in contrast to the single specimen of *Clivia miniata* var. *citrina* Watson that was available when the variety *citrina* was described. We therefore consider the establishment of a new yellow-flowered variety *Clivia gardenii* var. *citrina* as fully justified.

Figure 3.5 *Clivia gardenii* var. *citrina* Swanevelder, A.E.van Wyk & Truter, *ined.*

The first published reference to the yellow-flowered form of *Clivia gardenii* is by Obermeyer (1972). In that publication a specimen of a yellow-flowered plant, collected by Dr. L.E.W. Codd in April 1956, was used to illustrate *C. gardenii*. The specimen was also from Ngome Forest and was planted in the Pretoria Botanical Gardens where it flowered in September 1965. A plate was prepared by the artist C. Letty in that same year. It was used by Obermeyer (1972) in her publication '*Clivia gardenii*' in *The Flowering Plants of Africa* 42: t.1641. A second publication—a photograph by G. Duncan—was published on page 37 of the *Clivia Yearbook 2* (*Clivia* Club, Kirstenbosch Botanical Garden, 2000).

***Clivia caulescens* R.A.Dyer (1943)**

The first *Clivia* species to be scientifically described and named in its country of origin was *Clivia caulescens* (Figure 3.6). This pendulous-flowering species, similar to *C. gardenii* and *C. nobilis*, was under observation for several years before it was formally described as a new species, by Dr. R.A. Dyer in 1943 (Dyer 1943; Duncan 1999).

Figure 3.6 *Clivia caulescens.*

In his publication Dr. Dyer acknowledged two collectors of the new species, namely Dr. F.Z. van der Merwe and E.E. Galpin. The specimen collected by Dr. Van der Merwe was used to prepare the plate in Dyer's publication. Galpin was credited with collecting a specimen as early as October 1890 (Dyer 1943).

***Clivia mirabilis* Rourke (2002)**

Clivia mirabilis is the most recent species described in the genus and the second to be described in South Africa. Mr. J. Afrika, a game guard at the Oorlogskloof Nature Reserve near Nieuwoudtville, Northern Cape Province, drew the attention of Mr. Wessel Pretorius, the Reserve Manager, to this species. Mr. Pretorius collected and sent a

specimen to the Compton Herbarium, Cape Town, as part of a batch of material that needed identification. In February 2001, Dr. J.P. Rourke identified the genus and determined that it was an undescribed species after visiting a flowering population of the plants in the Oorlogskloof Nature Reserve. The new species was subsequently described as *Clivia mirabilis* Rourke in May 2002 (Rourke 2002a, b).

Clivia 'Robust' *gardenii*

The so-called 'Robust' form of *C. gardenii* (Figure 3.7) was first proposed as a new species after genetic data analyses of several collections of *Clivia* by Ran *et al.* (2001a, 2001b). According to Ran *et al.* (2001b), the RAPD results showed enough statistical significant differences between *C.* 'Robust' *gardenii* and the other species, to merit a new taxon. This taxon only occurs in a specific phytogeographical region, the Pondoland Centre of Endemism (Chapter 4, Van Wyk 1994; Van Wyk & Smith 2001).

Figure 3.7 *Clivia* 'Robust' *gardenii* in habitat.

3.3 Notes on the identification of *Clivia* species

Though the genus *Clivia* comprises of five species, *C. miniata* is the only one that is quite readily distinguishable with its large trumpet-shaped flowers, arranged unmistakably in an upright umbel. The four pendulous-flowered species all appear very similar at a glance and a combination of features must therefore be used to make a positive identification. Natural variation further complicates identification because the diagnostic features are not constant and can vary greatly within a species (Duncan 1999). The inadequacy of existing distribution maps for the various species also renders the identification difficult (Chapter 4, Winter 2000).

These factors have led to much confusion and wrong identifications abound in herbaria. Publications based on such wrong identifications have further led to confusion among both the scientific community and the public. This snowball effect is clearly evident in a publication of Vorster (1994) in which he attributed the so-called *C.* 'Robust' *gardenii* to *C. nobilis*. Though the *C.* 'Robust' *gardenii* was not yet recognised as a potentially new taxon at the time, his specimen should at least have been called *C. gardenii* and not *C. nobilis*. Diagnostic characters and habitat information required for positive identification of the different species of *Clivia* are summarised in Table 3.2 and are also supplied in the form of a key. With this information it should be possible to identify at least most specimens of *Clivia* from the wild to species or infraspecific taxon.

Table 3.2 Key diagnostic characters for the identification of *Clivia* species.[1*]

Character	*Clivia caulescens*	*Clivia gardenii*	*Clivia miniata*	*Clivia mirabilis*	*Clivia nobilis*	*Clivia* 'Robust' *gardenii*
Flowering time	September–November (Spring)[2*]	May–July (late Autumn–mid Winter)	August–November (Spring–early Summer)[2*]	October–mid-November (late Spring)	August–January (Spring–Summer)[2*]	Late March–early August (Autumn–Winter)
Flower: number	14–50	10–20	10–40	20–48	20–50	15–40
Umbel: form	Usually tight & flattened on one side	Usually loose, flattened to one side, slightly rounded on other side	Forming big round umbels, almost globose	Forming a tight umbel, judged from published pictures	Dense, compact, round umbel	Variable, usually loose, slightly globose
Distance stigma protrudes from tip of perianth tube	< 7 mm	Prominent, > 7 mm	Variable	Slight, judged from published pictures	< 6 mm	Variable, stigma pushed out beyond anthers
Degree anthers protrudes from tip	Slight	Always prominent	Variable	Slight	Variable	Slight–prominent
Flower length (perianth and ovary length)	30–35 mm	40–52 mm	Variable, depending on flower shape	35–50 mm	24–40 mm	30–55 mm
Pedicels: orientation	Stiff, erect, drooping near flower	Stiff, erect/sub-erect	Stiff and erect	Drooping	Slightly curved along length/drooping	Stiff, erect/sub-erect
Pedicels: colour	Usually green	Usually tinged red or orange	Green	red/orange during flowering, green when fruiting	Usually green	Variable

Character	*Clivia caulescens*	*Clivia gardenii*	*Clivia miniata*	*Clivia mirabilis*	*Clivia nobilis*	*Clivia* 'Robust' *gardenii*
Pedicels: length	15–35 mm	20–40 mm	± 30–70 mm	25–40 mm	20–40 mm	15–60 mm
Flowers: Orientation	Drooping	Drooping on stiff pedicels	Erect	Drooping	Drooping	Drooping on stiff pedicels
Flowers: Perianth shape	Tubular and curved; inner petals re-curved	Tubular and curved (falcate) downwards; inner petals re-curved	Open, funnel-shaped with spreading flower segments	Tubular, linear to curved, tubular with increasingly flaring at the apex	Tubular and linear with straight inner petals	Tubular, somewhat falcate with an increasingly flaring apex
Leaf sheath: colour	Green–light red	Green–light red	Green–light red	Prominent, flushed deep carmine maroon	Purplish	Green–light red
Leaves: Orientation	Arching	Recurved	Arching	Stiff, erect	Stiff, sub-erect	Arching–erect
Leaves: length × width (mm)	300–400(–900) × 35–50(–70)	350–450(–900) × 25–50(–60)	400–500(–900) × (25–)50–65(–70)	600–1200 × 30–50	300–700(–1000) × 25–45	300–800(–1200) × 30–70(–90)
Leaves: margin	Rarely serrated	Cartilaginous, minutely toothed	Usually entire	Entire, cartilaginous, usually smooth	Serrated	Cartilaginous and dentate
Leaves: apex	Obtuse–acute	Obtuse–acute	Acute	Obtuse-acute	Retuse and oblique	Abruptly rounded/ retuse
Leaves: special characteristics	-	-	-	Prominent white stripe in centre of leaf	White stripe absent or present	White stripe absent or present
Aerial stem	Usually present when mature; up to 3 m long	Rarely present; very old specimens	Rarely present; very old specimens	Not yet reported	Absent	Usually present for swamp forms

Character	*Clivia caulescens*	*Clivia gardenii*	*Clivia miniata*	*Clivia mirabilis*	*Clivia nobilis*	*Clivia* 'Robust' *gardenii*
Seed: number	1–4	Usually 1 or 2	1–4(–25)	(1)2–4(–7)	1 or 2(–6)	1 or 2(–4)
Seed: maturation time (months)	± 9	± 9–12	± 9–12	± 4–6	± 9–12	± 9–12
Seed: size (diameter)	Medium, ± 12 mm	Large, ± 18 mm	Medium, ± 12 mm, Transkei and Eastern Cape forms larger	Small, ± 10 mm	Small, ± 9 mm	Large, 10–18 mm
Endocarp: colour	Colourless	Colourless	Colourless	Colourless	Red-pigmented	Colourless
Distribution	Limpopo Province (Soutpansberg) Mpumalanga Province and Swaziland	KwaZulu-Natal Province	Eastern Cape Province (Transkei), KwaZulu-Natal Province, Swaziland, Mpumalanga Province	Northern Cape Province	Eastern Cape Province	Southern KwaZulu-Natal Province, Eastern Cape Province (Pondoland Centre of Endemism)

[1*] Information included in this table based on: Mr. J. Truter pers. comm.[1]; Mr. A. Hardinge pers. comm.[2]; Mr. R. Dixon pers. comm.[3] Hooker 1828, 1856; Pole Evans 1921; Dyer 1943; Obermeyer 1972; Duncan 1999; Malan 2000; Koopowitz 2002; Rourke 2002a, b; personal observations by author.

[2*] May flower sporadically throughout the year.

[1] J.T. Truter, PO Box 5085, Benoni South 1502, South Africa
[2] A. Hardinge, PO Box 14964, Margate 4275, South Africa
[3] R. Dixon, PO Box 1041, Pyramid 0120, South Africa

3.4 Key to the identification of *Clivia* species

1a Perianth lobes open to form a trumpet-/funnel-shaped flower; flowers erect (Eastern Cape Province, KwaZulu-Natal Province, Mpumalanga Province and Swaziland).... **2**

2a Flowers yellow ..... ***C. miniata* var. *citrina***

2b Flowers orange, red or pastel shades of these colours..... ***C. miniata* var. *miniata***

1b Perianth lobes parallel to form tubular flowers; flowers pendulous **3**

3a Leaves stiffly erect or sub-erect; leaf sheath prominently coloured (carmine-maroon or purplish); seed diameter small (< 10 mm); umbel dense and compact with high flower numbers (usually 30 or more)..... **4**

4a Endocarp pigmented (reddish); leaf apex retuse and oblique; leaf sheath purplish; white stripe in centre of leaf present or absent; pedicels green at anthesis; summer rainfall area (Eastern Cape Province) ..... ***C. nobilis***

4b Endocarp unpigmented; leaf apex obtuse-acute; leaf sheath prominent, flushed deep carmine; white stripe in centre of leaf present or absent; pedicels orange-red at anthesis; winter rainfall area (Northern Cape Province)

..... ***C. mirabilis***

3b Leaves flexible, arching, recurved or arching-erect; leaf sheath not prominently coloured; seed diameter medium to large (> 10 mm); umbel densities vary, variable flower numbers..... **5**

5a Flowering in spring or early-summer; older specimens with prominent stems; seeds usually with a diameter of ± 12 mm; perianth tube rarely curved (falcate); stigma and anthers may protrude slightly from perianth (Limpopo Province (Soutpansberg), Mpumalanga Province and Swaziland)

..... ***C. caulescens***

5b Flowering in autumn or winter; prominent stems present or absent; seed usually large with a diameter of ± 18 mm; perianth tube usually curved (falcate); stigma and anthers usually protrude from perianth..... **6**

6a Flowering in autumn and winter; sometimes in swampy habitat—with a prominent stem; high flower numbers (15–40); leaf apices becomes abruptly rounded/retuse; plants robust (southern Kwazulu-Natal Province, Eastern Cape Province: Pondoland Centre of endemism)

..... ***C.* 'Robust' *gardenii***

6b Flowering in late autumn and mid winter; usually in a well-drained habitat, rarely with a prominent stem; flower numbers low (10–20); leaf apices obtuse-acute; plants less robust (KwaZulu-Natal Province) 7

7a Flowers yellow ..... ***C. gardenii* var. *citrina***

7b Flowers shades of orange and red ..... ***C. gardenii* var. *gardenii***

3.5 Interspecific hybrids

The genus *Clivia*, notably *C. nobilis* and *C. miniata*, has received much horticultural attention since being introduced to Britain during the early and mid 1800s. With only the two species known at the time, horticulturists were quick to attempt interspecific hybrids. According to Vorster (1994) the first hybrid between these two species was published in 1856 in *Revue Horticole* 8: 258–260. The author(s) of this interspecific hybrid, *C. miniata* × *C. nobilis*, named it *Himantophyllum cyrthanthiflorum* (Vorster 1994). *Himantophyllum miniatum*, a synonym for *C. miniata*, was used as source for genus name during the naming of this hybrid (Koopowitz 2002). Another well known interspecific hybrid, possibly between *C. miniata* and *C. nobilis*, was produced by Charles Raes in the late 1850s and published by Van Houtte as *Clivia* Cyrtanthiflora in 1869 (Duncan 1999).

Koopowitz (2000) refers to *C.* Cyrtanthiflora—published as *Imantophyllum* Cyrtanthiflorum (1877)—as the only valid 'grex name' published at the time. According to Article 4.6 (including Note 4, Art. 4) of the *International Code of Nomenclature for Cultivated Plants* 1995 (ICNCP), the designation 'grex' is reserved for the Orchidaceae (Trehane *et al.* 1995). Article 4 (ICNCP) stipulates that the designation 'cultivar-group' should be used for assemblages of similar cultivars—either within a genus, hybrid genus (nothogenus), species, hybrid species (nothospecies) or other denomination class (Trehane *et al.* 1995). It is here recommended that the cultivar-group name, *Clivia* Cyrtanthiflora Group, be used for all hybrids between these two species.

It is also recommended here that the series of 'grex names', erroneously designated as such by Koopowitz (2000), be replaced by the designation cultivar-group. The cultivar-group names for the existing hybrids, including the names of the parent taxa, are therefore as follows:

Parentage	Cultivar-group name
C. gardenii × *C. caulescens*	*Clivia* Caulgard Group
C. miniata × *C. nobilis*	*Clivia* Cyrtanthiflora Group
C. Cyrtanthiflora × *C. miniata*	*Clivia* Minicyrt Group
C. gardenii × *C. miniata*	*Clivia* Minigard Group
C. miniata × *C. caulescens*	*Clivia* Minilescent Group
C. nobilis × *C. caulescens*	*Clivia* Nobilescent Group
C. gardenii × *C. nobilis*	*Clivia* Noble Guard Group

Though a particular cultivar-group name is used for any cross between two particular taxa (Trehane *et al.* 1995; Koopowitz 2000), there are uncertainties as to which parents have been used as berry- or pollen parent to date. Literature research has yet to produce proof of the following hybrids (berry parent listed first): *C. caulescens* × *C. gardenii*, *C. caulescens* × *C.* 'Robust' *gardenii*, *C.* 'Robust' *gardenii* × *C. nobilis* and *C.* 'Robust' *gardenii* × *C. gardenii* (Table 3.3 and references therein). Conflicting statements between different sources, poor record keeping and misidentification of the pendulous species, prompts one to question the reversal of some of the yet unsuccessful hybrids, listed as successful (e.g. *C. gardenii* × *C. caulescens*, Table 3.3). The extent to which interspecific hybridisations have been attempted on numerous individuals from different localities, are not normally indicated. Hybrids produced between very fertile individuals of different species, which might be separated hundreds of kilometres from each other, may produce offspring. Such hybrids would probably not occur in nature.

Clivia miniata appears to be very compatible with the other species and is the only one that has been successfully hybridised with all the known species—the one exception being *C. mirabilis*, which was only described in 2002 (Rourke 2002a). Attempts to use *C. mirabilis* in hybridisation will undoubtedly be attempted soon. Natural hybrids between *C. miniata* and other *Clivia* species are also known for some populations where two *Clivia* species occurs together. These include *C. miniata* × *C. nobilis* (*C.* × *cyrtanthiflora*),

C. miniata × *C. gardenii* and *C. miniata* × *C. caulescens* (J.T. Truter pers. comm.[1]; Winter 2000).

Table 3.3 Known interspecific hybrids in the genus *Clivia*.

♂ Species ► / ▼ ♀ Species	*C. caulescens*	*C. gardenii*	*C. miniata*	*C. mirabilis*	*C. nobilis*	*C.* 'Robust' *gardenii*
C. caulescens	-	NO[1]	YES[1]	?	YES[1, 4]	NO[1]
C. gardenii	YES[1, 2, 4]	-	YES[1, 2]	?	YES[1, 2]	YES[1]
C. miniata	YES[1, 2, 4]	YES[1, 4, 3]	-	?	YES[1, 2, 4]	YES[1]
C. mirabilis	?	?	?	-	?	?
C. nobilis	YES[1, 2]	YES[1]	YES[1]	?	-	YES[1]
C.* 'Robust' *gardenii	NO/YES[1]	NO/YES[1]	YES[1]	?	YES[1]	-

[1]J.T. Truter pers. comm.; [2]Koopowitz (2000); [3]Ran (2001c) & [4]Anderson (2001).

3.6 Systematics of the genus *Clivia* Lindl.

In recent years, molecular evidence became increasingly popular as the method of choice for reconstructing phylogenetic relationships amongst taxa. This can clearly be seen when one reviews the number of publications published yearly in the field of plant systematics. At least three such studies on *Clivia* have already been published (Ran *et al.* 2001a; Ran *et al.* 2001b; Conrad & Reeves 2002).

Meerow *et al.* (1999) placed the genus *Clivia* into its family context by using sequence data, generated from the rbcL and trnL-F regions, of the chloroplast genome. The results suggested that *Cryptostephanus* is the genus nearest to *Clivia* and further supports the tribe Haemantheae.

The work by Ran *et al.* (2001a; 2001b) was the first to look at the infrageneric relationships of *Clivia*. In a phylogenetic analysis of the genus (Ran *et al.* 2001a), they utilized the ITS and the 5S non-transcribed spacer regions of the genome. The trees generated by the two data sets were identical. This corresponds with another publication

[1] J.T. Truter, PO Box 5085, Benoni South 1502, South Africa

by this research group (Ran *et al.* 2001b), in which RAPDs (randomly amplified polymorphic DNAs) were used to infer relationships within the genus. Ran *et al.* (2001a; 2001b) showed with their molecular work that the so-called *C.* 'Robust' *gardenii*, is sufficiently different from the other species to justify naming it as a new taxon. The general relationships between the different species as indicated by the work of Ran *et al.* (2001a; 2001b), are shown in Figure 3.8.

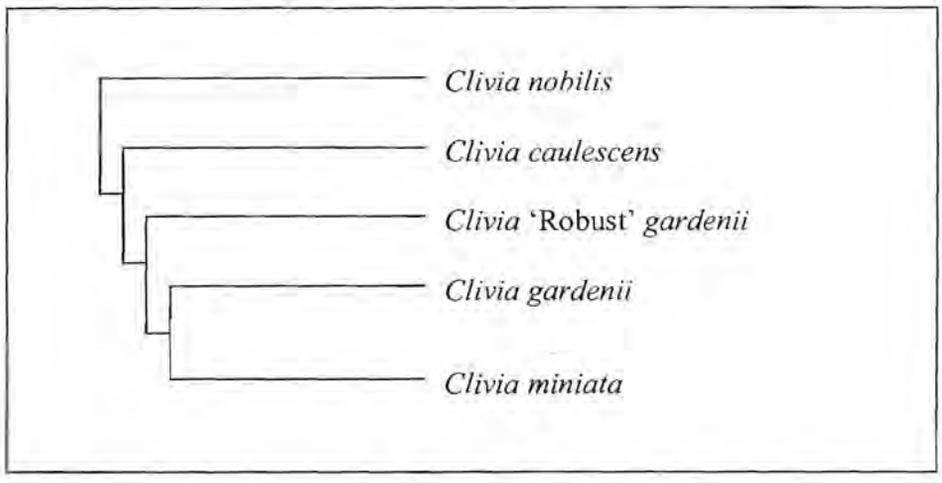

Figure 3.8 A tree proposed by Ran *et al.* (2001a; 2001b).

Conrad & Reeves (2002), like Meerow *et al.* (1999), targeted the plastid genome. They used sequence data generated by two intergenic spacers (rpoB-trnC and trnL-F) and two introns (rps16 and trnL) in their analysis. They showed that *C. mirabilis* was sister to all the other taxa (Figure 3.9) followed by *C. nobilis* and then *C. gardenii*. Unfortunately they did not include *C.* 'Robust' *gardenii* into their analysis. *C. caulescens* and *C. miniata*, according to the resultant tree, are more closely related, with *C. gardenii* sister to these. This pattern is different from relationships suggested by the trees of Ran *et al.* (2001a; 2001b), all of which grouped *C. miniata* and *C. gardenii* together, with *C. caulescens* being sister in three of their four trees. These differences in the trees could be due to numerous factors, including different methods of analysis, different mutation rates of the regions under investigation, intraspecific variation among localities from different areas (Chapter 6), the latter which is not accounted for by the small sample sizes usually used in molecular studies.

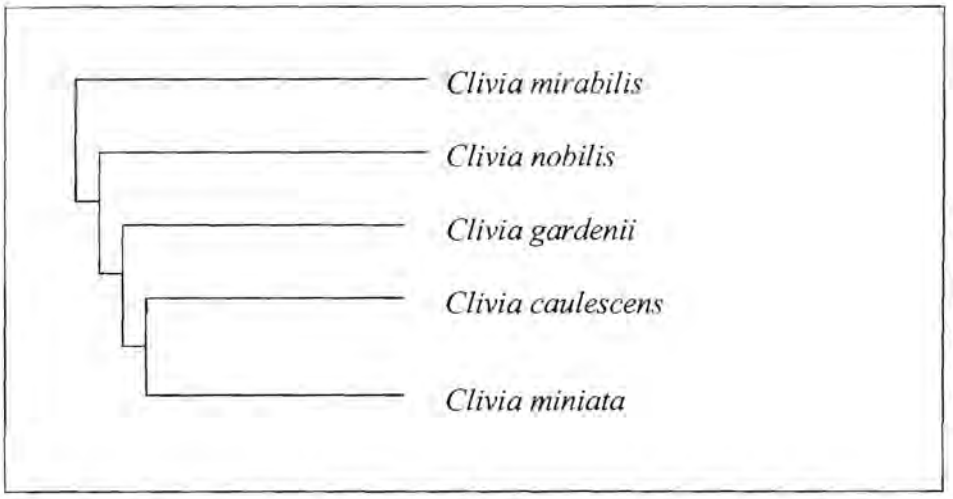

Figure 3.9 Tree proposed by Conrad & Reeves (2002) using plastid regions in their analysis.

A hypothetical tree (Figure 3.10), based on the distribution records (Table 3.2 and Chapter 4), proposes the possible relationships between the different species within the genus *Clivia*.

Figure 3.10 Hypothetical tree based on phylogenetic and distribution records of the different species within the genus *Clivia*.

Clivia mirabilis is placed basal, followed by *C. nobilis*, *C.* 'Robust' *gardenii* and *C. gardenii*, with *C. miniata* and *C. caulescens* filling the terminal clade. This tree corresponds best with the tree proposed by Conrad & Reeves (2002). This could be

attributed to the maternally inherited chloroplast regions they used (Chapter 6). Maternally inherited regions will only be distributed though seed. With speciation occurring through isolation, one expects higher nuclear variation (2001a; Ran *et al.* 2001b), but lower variation in more highly conserved regions like the chloroplast genome.

3.7 Acknowledgements

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CHAPTER 4

CLIVIA POPULATIONS: DISTRIBUTION, ECOLOGY & CONSERVATION STATUS

4.1 Introduction

The recent horticultural attention received by *Clivia miniata* (Chapter 5) has emphasised our lack of knowledge regarding the natural ecology of the genus. In one of the few reports on the distribution and ecology of *Clivia*, Winter (2000) emphasized the lack of herbarium records of this group in the herbaria of the National Botanical Institute of South Africa. He believes that due to the inaccessibility of most *Clivia* populations, the discovery of additional localities for the various species is highly likely.

This chapter aims to contribute towards our understanding of *Clivia* ecology and its distribution range. A brief historical overview of early Africa and its climate precedes an hypothesis, aimed at explaining the current distribution pattern of *Clivia* species. The conservation status of the genus is also reviewed using existing/future threats and present distribution patterns. The distribution of infraspecific taxa (e.g. yellow-flowered varieties) will not be considered in this chapter.

4.2 Materials and methods

Maps presented in this chapter are compiled from more than 250 herbarium records, confirmed visual reports and records in living collections (Appendix I). Herbarium records in the C.E. Moss Herbarium (University of the Witwatersrand), Compton Herbarium (National Botanical Institute, Cape Town), Lowveld National Botanical Gardens Herbarium (National Botanical Institute, Nelspruit), H.G.W.J. Schweickerdt Herbarium (University of Pretoria), Natal Herbarium (National Botanical Institute, Durban), National Herbarium, including PRECIS (National Botanical Institute, Pretoria) and Selmar Schonland Herbarium (Rhodes University, Grahamstown) were used.

All records were sorted according to the different species before analysis. Grid and GPS references available were plotted using the program *Arc View GIS*. Records lacking this information were located using the locality descriptions, maps and the search function of *Arc View GIS*.

The difficulty experienced by most people in identifying the pendulous-flowered species prompted the questioning of some locality records. Record identity was deemed unreliable when no duplicate record existed for that locality (either visual, herbaria or living collection) and the record fell outside a grid already containing a known record or it was outside the previously accepted distribution range/habitat of that species. Collections made before species description, were deemed incorrectly identified (species unknown at time of collection) when they did not fulfil the above criteria. Sightings were only included as records if they were confirmed by at least two individuals or by a herbarium/living collection record. Records lacking the necessary information to establish the grid references were discarded.

4.3 Distribution and ecology of Clivia

4.3.1 Introduction

Clivia is known to grow in diverse habitats, from coastal forest and secondary coastal dunes, to swamps, riverbanks and rock screes. Specimens are even found as epiphytes in some localities (Duncan 1999; Winter 2000). *Clivia* populations normally occur as rather inaccessible, isolated colonies. According to Winter (2000), these populations are extremely old, with some populations producing very little seed. The only hope for these populations to survive is their ability to regenerate vegetatively.

Species prefer to grow in cool, shaded, well drained habitats, located in the summer rainfall area, with the exception of *Clivia mirabilis*, which has a localized distribution in a semi-arid area, with Mediterranean climate and winter rainfall (Duncan 1999; Winter 2000; Rourke 2002a, b).

Clivia is believed to be endemic to South Africa and Swaziland, with unconfirmed reports of sighting in Mozambique and as far north as Kenya and Uganda (Winter 2000; Rourke 2002a). The distribution of *C. miniata*, *C. nobilis*, *C. caulescens* and *C. gardenii* is along the coastal and inland Afromontane forests of southern Africa, with *C. mirabilis* growing among relictual evergreen Afromontane forest elements in the southwestern corner of the Northern Cape (Rourke 2002a). The genus' distribution extends in an

eastwards direction, from the coastal areas of the Eastern Cape Province in the south, through KwaZulu-Natal Province, Swaziland and Mpumalanga Province to the Soutpansberg in Limpopo (=Northern) Province (Duncan 1985, 1992; Vorster 1994; Duncan 1999; Snijman 2000; Winter 2000; Rourke 2002a). *Clivia mirabilis*, however, is only known to occur in one area near Nieuwoudtville in the Northern Cape Province (Rourke 2002a).

4.3.2 *Clivia nobilis* Lindl.

The distribution of *Clivia nobilis* has been described as within the Eastern Cape (including Transkei) and southern KwaZulu-Natal (Vorster 1994; Duncan 1999). Malan (2000) reported localities from west of Grahamstown, in the Albany and Bathurst Districts, along the Eastern Cape coastal belt to the east of Kei River Mouth. Winter (2000) described the distribution as from Alexandria Forest, northwards along the coast to Nqabara River, with occasional populations inland as far as Grahamstown.

The present study found that *Clivia nobilis* is only distributed in the Eastern Cape (Malan 2000; Winter 2000) and not in southern KwaZulu-Natal as previously reported by Vorster (1994) and Duncan (1999). Populations are concentrated towards the coast, from just north of the Sundays River Mouth, extending up along the coast to the Mbashe River area, with colonies occurring as far inland as the vicinity of Grahamstown (Figure 4.1).

The distribution range of *C. nobilis* is located in the Albany Centre (AC) and the southern part of the Maputaland-Pondoland Region (MPR) of endemism (Figure 4.1). The Albany Centre is a mosaic of different vegetation types, displaying floristic elements of the five different phytochoria converging on it. Biomes such as Savanna, Forest, Thicket, Grassland, Fynbos and Nama-Karoo are found in this centre (reviewed by Van Wyk & Smith 2001). According to Van Wyk (1994), the AC represents a southwards extension of the MPR, but with the presence of Cape floristic and Karroid elements giving the centre its own distinct character. The MPR and AC also contain enclaves of Afromontane forests (Van Wyk & Smith 2001).

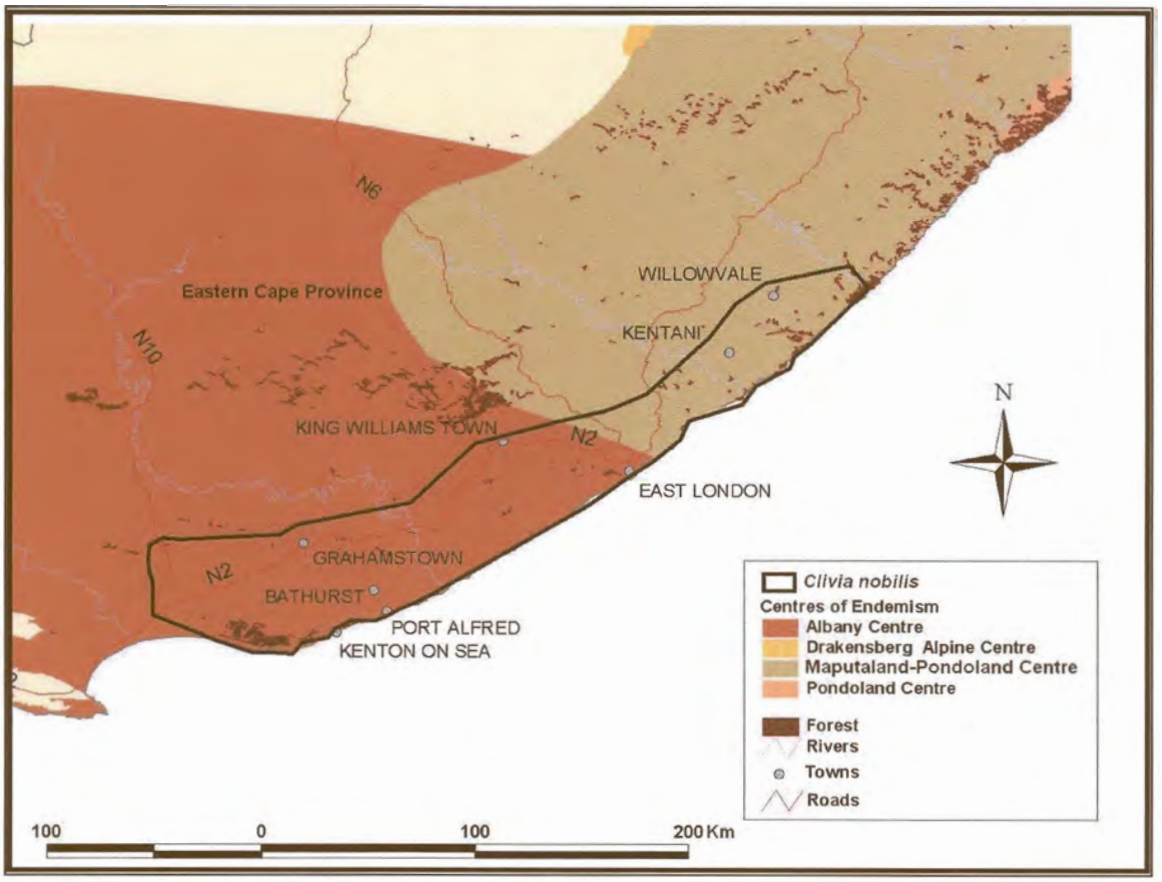

Figure 4.1 Natural distribution range of *Clivia nobilis*.

The Afromontane Archipelago-like Region of Endemism is a series of isolated floristic areas distributed from the Cape Peninsula, northwards along the southern and East African uplands, to northeastern Africa (White 1983). The region has a temperate climate and receives mostly high rainfall, anything from 700 mm to 2000 mm annually. In southern Africa, this region is centred in the Lesotho and KwaZulu-Natal Drakensberg and Midlands, extending north- and southwards along the Great Escarpment. A second centre, containing the largest contiguous block of forest in the subcontinent, is located on the coastal platform of the southern Cape (Lubke & Mckenzie 1996; Cowling & Hilton-Taylor 1997). Dominant canopy species in Afromontane forest are 30–40 m high, with those in the Albany Centre 2–10 m in height (Lubke & Mckenzie 1996; Midgley *et al.* 1997).

The MPR encompasses, like the AC, a diverse array of vegetation types, including grassland, forest (tropical/sub-tropical and Afromontane), savanna, thicket and aquatic elements (Goldblatt 1978; Cowling & Hilton-Taylor 1997; Van Wyk & Smith 2001). Van Wyk (1994) mentions that the southern part of this region contains mainly grasslands, with indigenous forest covering less than 1% of the entire MPR. The coastal forests are floristically related to the coastal forests of East Africa and secondarily to the tropical rainforests of Guineo-Congolian Region (Goldblatt 1978; White 1983; Cowling & Hilton-Taylor 1997). Midgley *et al.* (1997) mapped Subtropical Thicket as a vegetation type mainly confined to the Eastern Cape. Climate in this region ranges from subtropical/tropical in the low-lying areas, to more temperate on the higher ground (with frost in winter). Annual precipitation varies from 400–1 200 mm or more and occurs predominantly during summer (Goldblatt 1978; Van Wyk 1994; Cowling & Hilton-Taylor 1997; Van Wyk & Smith 2001).

The coastal areas of the Albany Centre have a mild climate (9–25°C) and receive 600–900 mm rainfall annually. Inland areas have frost/snow in the winter to 45°C maximum summer temperatures and ± 250 mm mean annual precipitation (Midgley *et al.* 1997; Van Wyk & Smith 2001). *Clivia*, being a shade-loving genus, will grow in the higher rainfall areas where there is appropriate canopy cover—clearly evident in the distribution pattern and habitat of the species (Figure 4.1).

Clivia nobilis is found under evergreen forest, low bush (thicket) and amongst dune vegetation (Duncan 1999; Malan 2000; Winter 2000). Inland populations are found in wooded kloofs where they grow on riverbanks, rocky outcrops and along forest margins (Malan 2000; Winter 2000). Indications are that *C. nobilis* is not competing very successfully with the current climax forest communities. Populations are usually more exposed on primary coastal dunes with their low canopy cover (2–5 m) (Duncan 1999; Malan 2000).

A visit to the Kei River area confirmed the habitat of a *C. nobilis* coastal population. This locality appears to contain two ecotypes. At the base of the dune away from the sea, long-leaved, large *C. nobilis* plants with long extended root systems, growing under a high, closed canopy of 5–10 m, are found. Mid-way up the dune, short leaved plants with smaller rooting systems are found under a low 2–3 m canopy. Plants were in flower or in bud. The colony grows in sea sand with lots of humus/decomposing leaves originating from the canopy overhead. Some plants on the top of dunes grow in full sunlight with no apparent damage.

Many seedlings, growing either together in groups or scattered, gives a seedling to mature plant ratio of 3:1. Some plants even grow epiphytically. Seedlings of every size were seen in the colony. Single, 2, 3 and multi-stemmed mature plants were recorded. There were a number of scapes and leaves damaged by presumably insects (caterpillars). During the time of the visit (October 2001), at least 40% of the adult plants were in flower, with more still in bud. Individuals that flowered earlier (out of season) produced fruit with seed on almost every pedicel; fertilization and seed-set are high. This population appears to be actively growing and reproducing, sexually and especially asexually.

Leaves of the plants were fairly narrow (25–45 mm), with plants not markedly stunted. There was some flower colour variation, generally light orange with some green at the tips to darker red with/without green tips, pastel orange and pink-red. From 10 to 40 flowers per umbel were noted. No significant signs of large herbivory were detected, but some plants had been removed from the colony. The plants were well spaced and there appeared to be little competition with undergrowth. The above observations are similar to those of Malan (2000).

The accuracy of some herbarium specimens in NBI herbaria was questioned by Winter (2000). *Clivia gardenii* (*C.* 'Robust' *gardenii*) and *C. caulescens* specimens were sometimes misidentified as *C. nobilis*.

4.3.3 *Clivia gardenii* Hook. & *Clivia* 'Robust' *gardenii*

The Eastern Cape Province and KwaZulu-Natal Province are currently regarded as the distribution area of *Clivia gardenii*. Scattered populations occur from Port St Johns (Eastern Cape) in the south, to Ngome Forest (KwaZulu-Natal) in the north (Hooker 1856; Obermeyer 1972; Vorster 1994; Duncan 1999; Winter 2000).

The distribution pattern presented in this study coincides more or less with the currently accepted distribution for *Clivia gardenii* (Figure 4.2). However, known records clearly divide the distribution into three separate areas. The southernmost of these, extends from Port St. Johns in the south to the Mzimkulu River in the north. The second area begins around Durban, progressing northwards to Empangeni and inland as far as the Howick area. The most northern area is confined to Ngome Forest. No linking records were found to connect the different areas.

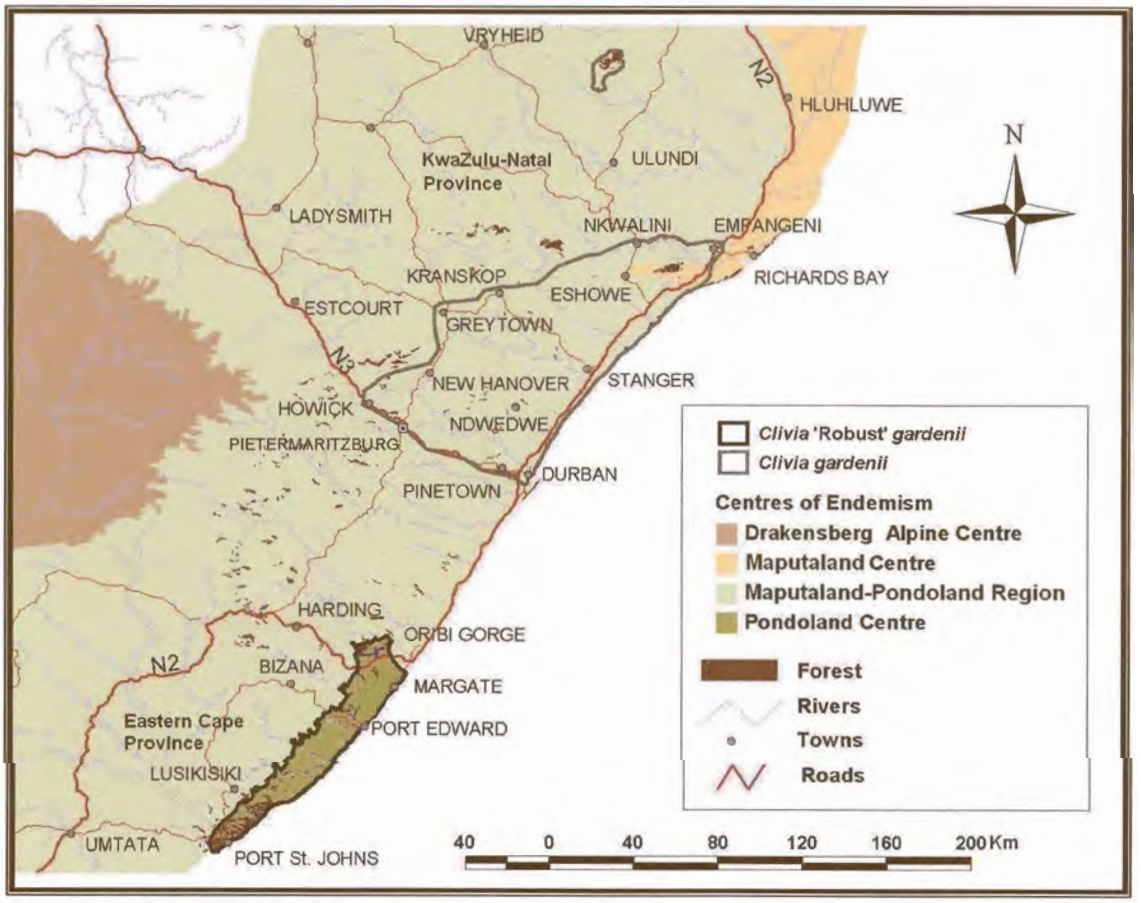

Figure 4.2 Natural distribution range of *Clivia gardenii* and *Clivia* 'Robust' *gardenii*.

Clivia ' Robust' *gardenii* has not yet been formally described as a separate species, therefore specimens of this taxon are filed together with *C. gardenii* in herbaria. In this section, both *Clivia gardenii* and *Clivia* ' Robust' *gardenii* records were used to produce a distribution map for *C. gardenii* in a broad sense. Sightings of *Clivia* 'Robust' *gardenii* were compared to the habitat descriptions on herbarium specimens and localities of *C. gardenii*. In doing this, it became obvious that the distribution area for *Clivia* 'Robust' *gardenii* is limited to the southernmost area on the *C. gardenii* distribution map (Figure 4.2). Furthermore, all sightings of *Clivia* 'Robust' *gardenii* are restricted to this southernmost distribution area. If this southern area is accepted as the distribution range for *Clivia* 'Robust' *gardenii*, then the distribution of *C. gardenii* in a strict sense, appears to extend only from the Durban area northwards.

Molecular evidence produced by Ran *et al.* (2001b) (Chapter 3) has already suggested that the *C.* 'Robust' *gardenii* is a new taxon. This is supported by the proposed distribution pattern, with *C.* 'Robust' *gardenii* geographically isolated (allopatric) from *C. gardenii*. Flowering times coincide during late autumn and early winter (Chapter 3), but genetic isolation is further enhanced when one takes into account that the pendulous species are essentially selfers (Rourke 2002a). Artificial attempts to hybridise these two taxa, also produce conflicting results (Chapter 3, Table 3.3). These, including some morphological differences (Chapter 3, Table 3.2), concur with the requirements of the biological species concept and supports the recognition of *C.* 'Robust' *gardenii* as a new species (Mayr 1992).

The distribution range of *C.* 'Robust' *gardenii* corresponds to that of the Pondoland Centre of endemism. The Pondoland Centre (PC) forms part of the larger Maputaland-Pondoland Region (Tongaland-Pondoland Region of Goldblatt (1978);(Van Wyk 1990, 1994; Van Wyk & Smith 2001). This centre encompasses a 1 880 km^2 large outcrop of Msikaba Formation sandstone, covering the area between the Mzimkulu River (southern KwaZulu-Natal) and the Ntsubane region (Egossa Fault) in the Eastern Cape, including smaller sandstone outcrops such as those at Port St Johns and Uvongo (Van Wyk 1994; Van Wyk & Smith 2001). Msikaba Formation sandstone was previously grouped together

with the Natal Group sandstone, but are now recognised to be unrelated in origin (Van Wyk & Smith 2001, and references therein).

Topographically the PC is characterised by rugged plateaus (100–500 m above sea level) that are deeply dissected by narrow river gorges. Isolated forest patches, containing mixed tropical and Afromontane elements, are confined to the protection of these deep gorges. Mean annual rainfall varies from 1 000–1 200 mm and occurs mainly in the summer months with the southern part receiving an expected 50 mm monthly. The mean annual temperature along the coast is around 20°C. Soils in this centre are usually sandy, acidic, highly leached and often shallow (Van Wyk 1994; Van Wyk & Smith 2001).

The distribution range of *Clivia gardenii* lies within the Maputaland-Pondoland Region of endemism, between the Pondoland Centre and the Maputaland Centre. The most northern locality at Ngome Forest is situated in the KwaZulu-Natal Midlands and is part of the mistbelt Afromontane vegetation. This forest annually receives approximately 1 530 mm of rain and is found on sandstone, dolerite and shale. Afromontane trees are responsible for a 20 m high canopy (Midgley *et al.* 1997). More detailed notes on this forest follow later in this section. Information on the MPR has already been supplied in the previous section (4.3.2, *Clivia nobilis* Lindl.).

The grouping of *Clivia gardenii* and *C.* 'Robust' *gardenii* in the literature makes it difficult to establish the exact habitat for these two taxa. It would, however, appear as if *C. gardenii* prefers in general a well drained habitat, though populations have been recorded in marshy environments. *C.* 'Robust' *gardenii* occurs commonly in a marshy environment, with some populations growing in seepage areas below/on cliffs (pers. comm. J.T. Truter[1] and A. Hardinge[2]). Various diagnostic characters support a distinction between these two taxa (Chapter 3). It is here proposed that *C.* 'Robust' *gardenii* is an endemic to the Pondoland Centre and its outliers, whereas the typical *C. gardenii* occurs

[1] J.T. Truter, PO Box 5085, Benoni South 1502, South Africa
[2] A. Hardinge, PO Box 14964, Margate 4275, South Africa

in the Maputaland-Pondoland Region from Durban northwards, with an outlier distributed in the Ngome Forest.

In communications between Mr. A. Hardinge[2] and the author, various environmental features of *C.* 'Robust' *gardenii* were mentioned. Mr. Hardinge, familiar with many populations of this taxon, reported that though *C.* 'Robust' *gardenii* occurred quite often in natural wetlands/swamps, there are also examples of communities growing in humus-rich soils, sides of cliffs or on rocks. A personal communication with Mr. J.T. Truter[1], suggests that plants most likely occurred in the wetter drainage portions at the base of cliffs. Mr. Hardinge reported that plants occurred under natural forest cover, in or near water. Population densities vary from 5 or 6 plants per 10 m^2 up to 20 plants per square metre, with higher densities observed in wetter areas. In swampy places, plants occurred in big clumps with individuals as high as 1.8 m with buttress roots, but those in dryer, rocky habitat are noticeably 'stockier'. Seed production appears to vary between communities. Removal of plant material from natural habitat, probably for 'muti' purposes, has been reported (Mr. A. Hardinge[2]).

In the course of this study, Ngome State Forest was visited and the environment of a population of *C. gardenii* in this forest was investigated.

Ngome Forest, situated between Vryheid and Nongoma, is part of the Ntendeka Wilderness Area. A population of *C. gardenii* was located under a tall, closed canopy of evergreen trees in the forest, occurring in small colonies with a patchy distribution, and preferring eastern or western aspects, with southern aspects apparently too cool and wet and northern aspects too hot and sunny.

Most *C. gardenii* plants were recorded on steeper slopes, usually 45° and even in cracks of the cliff face/river embankment. Here they form colonies all along the steep crest and ledges of the cliff/embankment. Some of these colonies are so inaccessible that only experienced rock climbers with the necessary equipment could possibly reach them. In this population, some colonies tend to flower more profusely than others. The induction

of flowering was shown to be temperature related in *Clivia miniata* (De Smedt *et al.* 1996; Honiball 2000). No similar studies on any of the other species are currently known. Could flowering be triggered by light and/or day length in *C. gardenii*? This hypothesis stems from the observation that colonies that are exposed to light, flower more profusely in the Ngome location.

Different colour forms, ranging from pastel to darker orange, were seen. Average flower number per umbel varies from 20 to 25. The population is estimated to contain 1 000–2 000 or more mature individuals, of which an estimated 10–15% were in flower. Seed production was high (50–60% of population) with all the colonies that were in flower having fruit of the previous year. Usually one or two large seeds are found in each berry. With the slope being very steep and with some colonies seemingly without seedlings, it is proposed that colonization takes place lower down the embankment—if no other dispersal vector is present. Due to the inaccessibility of the terrain, this hypothesis could not be verified. An abundance of different size plants, ranging from seedlings through to mature flowering plants, were seen in some of the colonies.

Clivia gardenii has an extended flowering season, with some plants already setting seed while others are still in bud. The plants prefer well-drained loamy soil. Signs of leaf-miner activity were present in many plants, with white 'tunnels' visible on the leaf surfaces. Apical damage was also observed. Lichens were mainly found on plants growing in lighter, wetter parts of the forest. No fungal or viral diseased were noted.

The population in Ngome is healthy. Seed are produced in abundance and a number of seedlings were seen. A high percentage of the mature plants produce flowers. The inaccessibility of some colonies within the population ensures the survival of the population as it provides some protection against plant collectors. The size and extent of the forest ensure that there are many similar microhabitats within the forest system.

Herbarium records indicating *Clivia gardenii* localities in both Swaziland and in the Barberton District are wrong and were rejected in accordance to the criteria set out in the beginning of this chapter. These specimens belong to *C. caulescens*.

4.3.4 *Clivia caulescens* R.A.Dyer

Clivia caulescens's distribution is here accepted to be within the eastern part of the Mpumalanga and the Limpopo Provinces (formally the Northern Province)(Vorster 1994; Duncan 1999; Winter 2000). Winter (2000) recorded the most northern localities in the Soutpansberg, Limpopo Province and the most southern in the Sodenza Range on the border of Swaziland and Mpumalanga (Winter 2000).

Records employed in this study place the most southern community of *C. caulescens* in the Mbabane District, Swaziland, and confirmed the most northern locality in the Louis Trichardt (Makhado) area, Soutpansberg (Figure 4.3). This distribution range covers three Afromontane centres of endemism, namely the Barberton Centre (BC), Wolkberg Centre (WC) and the Soutpansberg Centre (SC). These three centres combine at higher hierarchical levels of phytochorology.

The Barberton Centre comprises the crescent-shaped mountain ranges northeast, east, southwest and south of Barberton. This rugged, mountainous region receives 800–1400 mm of precipitation during the summer months. A temperate climate, with mild to cool winters, is experienced with fog common at higher altitudes. Rock of the Barberton Supergroup forms the substrate for this centre (Van Wyk & Smith 2001).

Vegetation of the BC is broadly classified as North-Eastern Mountain Grassland, with scattered Afromontane Forest and Sour Lowveld Bushveld (Van Wyk & Smith 2001). Forests, occurring as pockets, are confined to sheltered ravines, moist valleys and incised valley heads. The Barberton Centre forms part of the Afromontane Region and has close floristic links to the Wolkberg Centre. It is proposed that the Barberton Mountain Land acted as a refuge for Afromontane flora, a hypothesis supported by the high levels of local endemism (Van Wyk & Smith 2001).

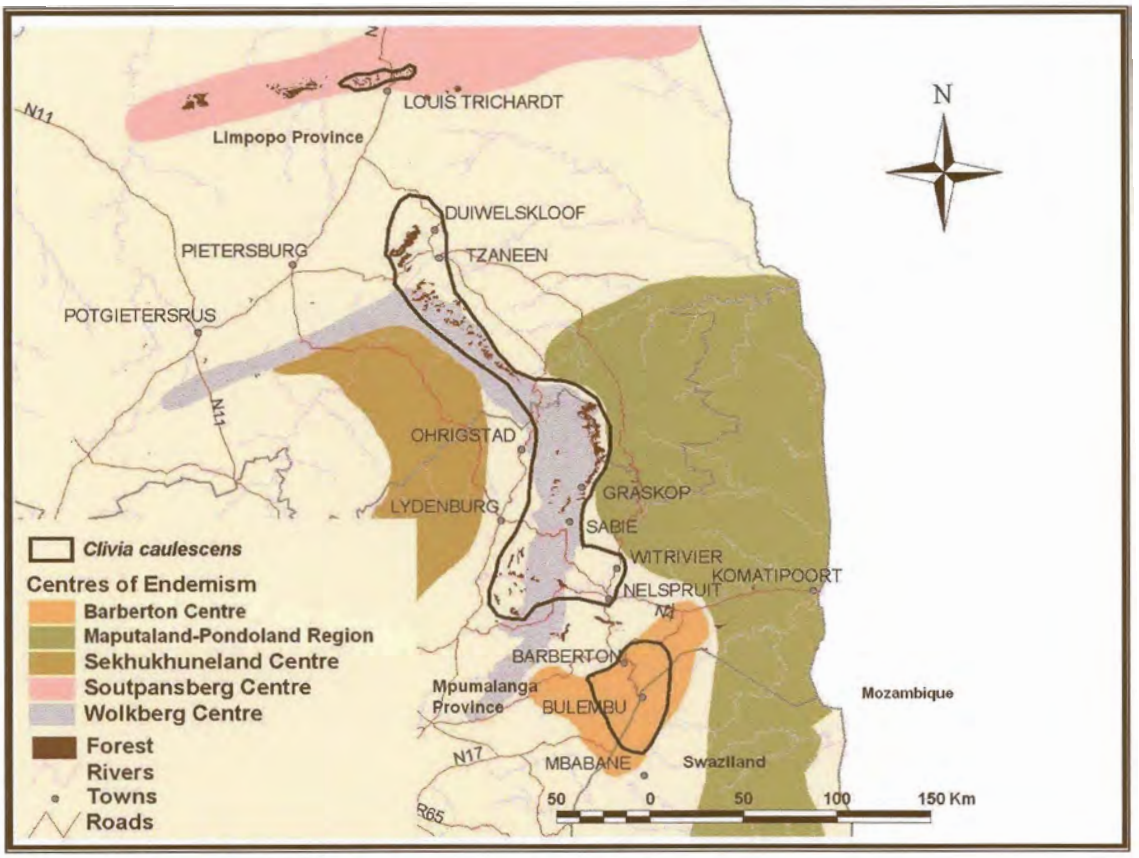

Figure 4.3 Natural distribution range of *Clivia caulescens*.

Situated in Limpopo and Mpumalanga Provinces, the Wolkberg Centre is defined by rocky outcrops of the Transvaal Supergroup. These outcrops extend from Kaapsehoop in the south to near Tzaneen in the north and westwards to the Strydpoort Mountains ending near Potgietersrus. Two subcentres have been proposed for the WC, the Blyde Subcentre (south of the Olifants River) and the Serala Subcentre (north of the Blyde subcentre) (Van Wyk & Smith 2001). Records indicate that *C. caulescens* grows in both these subcentres.

The WC is characterised by hot, wet summers (drier to the west) that is more temperate at higher altitudes and dry, cool winters with frost (higher altitudes). Mean annual precipitation is in excess of 1 000 mm and may reach 2 000 mm in some places. Various

soil types occur in this centre, for example sandy soils derived from quartzite (Van Wyk & Smith 2001).

Vegetation is predominantly montane grassland with Afromontane Forest confined to pockets along the slopes of the main escarpment, sheltered ravines and incised valley heads. These Afromontane Forests form part of the Afromontane Region and has floristic links with BC, SC and Chimanimani-Nyanga Centre. Links to the mountains of the Albany Centre and Cape floristic elements are conspicuous at higher altitudes (Van Wyk & Smith 2001).

The SC encompasses the Soutpansberg and the adjacent Blouberg Massif with rocks of the Soutpansberg Group as main substrate. Southern slopes of the mountains are wetter (up to 2 000 mm) than the northern ones (370 mm). A temperate climate is experienced at higher altitudes with a more subtropical climate in the lowlands. Fog is common at high altitudes and frost is usually absent. Sandstone and quartzite are the two main rock types (Van Wyk & Smith 2001 and references therein).

The vegetation of the SC consists largely out of bushveld and thicket, with well-developed pockets of Afromontane Forests on the wetter slopes and grassland at higher altitudes. Wetter parts form part of the Afromontane Region and link this centre to the WC. Weak floristic links also exist with the Eastern and Western Cape (Van Wyk & Smith 2001 and references therein)

Clivia caulescens grows like the other *Clivia* species in association with evergreen forest, where it occupies the forest floor, either on moss and lichen covered rocks or tree trunks. According to the literature, populations have been found in association with sandstone (Duncan 1999; Winter 2000). Herbarium records and literature references suggest that this species occurs at high altitudes, with some even above the snowline (Winter 2000). Specimens have been seen growing in full sun to light shade and on slopes of 5–45°. *Clivia caulescens* has been noted growing in sandy loam derived from Wolkberg quartzites (*F. Venter*, PRU 81136, 1986/4/8).

The sketchy available descriptions in the literature of *C. caulescens* ecology prompted various visits to different localities in an attempt to fill these gaps. Five different localities were visited and observations are summarised below.

Bearded Man Mountain is on the Swaziland border with Mpumalanga Province and 1 337 m above sea level. Plants grow on a WSW aspect, on a slope of 45–55°, under a close canopy in what can be described as a 'dry forest' (based on the scarcity of epiphytes). Many saplings were observed in this forest. At this locality, *C. caulescens* grows in close association with *C. miniata* (also see section 4.3.5 for more detailed description of habitat). Identification was hampered with only a single *C. caulescens* in flower. No competition was observed where both species are growing, with an increase in undergrowth resulting in fewer plants. The *C. caulescens* plants have distinct stems and no obvious hybrids were identified. Only a few plants were seen, therefore limited assumptions can be made about this *C. caulescens* population.

The grass-covered mountainous terrain of the Sodenza Mountains, Songimvelo Game Reserve, has many natural valleys and seepage areas. In these natural drainage areas woodland patches occur. Two *C. caulescens* communities associated with the elaborate drainage system of the mountain, were investigated, namely a forest patch around Malandweni Cottage and a forest-covered kloof, Lobodtlyana.

Lobodtlyana Kloof, a forest patch, consists of sheer cliff faces connected by moderate steep slopes, ensuring excellent drainage. The forest canopy is high and open, but the undergrowth increases as one moves towards the exposed edges of the forest. Again *Clivia miniata* and *C. caulescens* were found growing together.

Clivia caulescens plants are mainly found on rocky outcrops that form part of the higher, steeper parts of the slopes. These well-drained areas contain humus rich soil in which the plants grow and are also exposed to more light than surrounding areas. Inaccessibility confined the exploration to the valley, with the steeper slopes and lower areas not fully

investigated. This would clearly influence estimates, probably to a minimum value rather than an average. The *C. caulescens* population size is estimated to be around 100 plants of which 50% are seedlings. The number of positively identified individuals, based on the presence of inflorescence and leaf morphology, also influenced this estimate. Plants grow as small colonies or as individuals dispersed throughout the *C. miniata* community and had a dark red flower colour.

Lichens grow on both *C. caulescens* and *C. miniata*, but seem to prefer the *C. miniata* that grows in area with more light. No fruits were seen in both these species. Although human activities were noted, this site is well protected by its seclusion. This community is categorized as healthy and actively growing. Although no seed-set was observed, large numbers of seedlings were present.

Malandweni Cottage was built in one of the woodland patches of the natural drainage system and is surrounded on three sides by a closed-canopy forest that grows along a V-shaped drainage area. *Clivia caulescens*, in the forest patch that surrounds the cottage, has deep red flowers. Many of the *C. caulescens* plants formed multi-stems at their bases. This is attributed to abscission rot—older stems tend to break-up due to localized rot.

The valley floor was severely trampled by animals visiting the stream. *C. caulescens* plants were some of the casualties and many of the remaining plants are located on rocks within the stream (majority) and in other areas where the animals cannot disturb them (growing next to trees on the denser outskirts of the forest). This *C. caulescens* population contains to approximately 50 to 60 individuals. Seedlings account for around 10% of the population. Very few plants were in flower (5–10% of the population), but it was still very early in the flowering season.

Although only a limited number of individuals were found, we believe that *C. caulescens* is more abundant and would be found in similar interconnected forest patches that occur within the reserve. The Malandweni Cottage's valley is indeed connected to other similar

seepage areas and forms part of a larger "interconnecting seepage forest system" occurring along the mountain. Similar *C. caulescens* habitats are therefore common.

The last two communities are in the vicinity of God's Window and Kowyns Pass near Graskop. The God's Window population is on a hiking trail, with plants growing from open sunny spots at the top of the valley, to cool forest shade at the bottom. At the top, this area is located 1 730 m above sea level, with most plants growing on level ground.

The majority of the plants (almost 100%) were single-stemmed, very old plants, with no obvious suckers on them. Plants with aerial stems up to 3 m long (i.e. stems reclining on rocks/forest floor and against trees) were seen. Flower colour was mostly orange to deep red-orange, with up to 50 flowers per umbel. At least 40% of the plants were in flower/in bud/pushing inflorescences. Very few seedlings, in comparison to the mature plants, were seen. Seeds were present together with clear indications of seed-set in old inflorescences. Some of the plants in open settings show yellow, scorched leaves. Plants grow in the humus layer as well as epiphytically on rocks (lithophytes). This is an extremely large population (at least 2 000 mature plants) with very inaccessible communities. In general, the population consists of big, very old, mature plants. Indications of plant removal by humans were obvious, an action clearly facilitated by the presence of a hiking trail traversing the population.

In the Kowyns Pass area, plants grow on the top, sides and floor of the valley. Seedlings and younger plants were abundant, with older plants largely limited to the higher portions of the valley/boulders. Streaks of white/yellow on the foliage gave the plants a 'variegated' appearance, but it is possibly a viral infection. Approximately 30% of the adult plants were in flower with 20–35 flowers per umbel.

4.3.5 *Clivia miniata* (Lindl.) Regel

Currently *Clivia miniata* is accepted to occur from the Kei River in the south, through the Eastern Cape and KwaZulu-Natal Provinces, with the most northern localities in the Sondeza Mountains, between Swaziland and Mpumalanga Province (Vorster 1994; Duncan 1999; Winter 2000).

The present study also places the most northern limits of *C. miniata* in the Barberton area (Figure 4.4). Communities were also recorded in Swaziland, near the border with Moçambique and in the Lebombo Mountains, northern KwaZulu-Natal Province (Figure 4.4). The most extensive distribution area begins in the Hluhluwe District, KwaZulu-Natal Province, from where it extends southwards along the coast to the most southern locality at the Koek-Koek River, Eastern Cape Province. The far northern localities show a disjunct pattern (Figure 4.4).

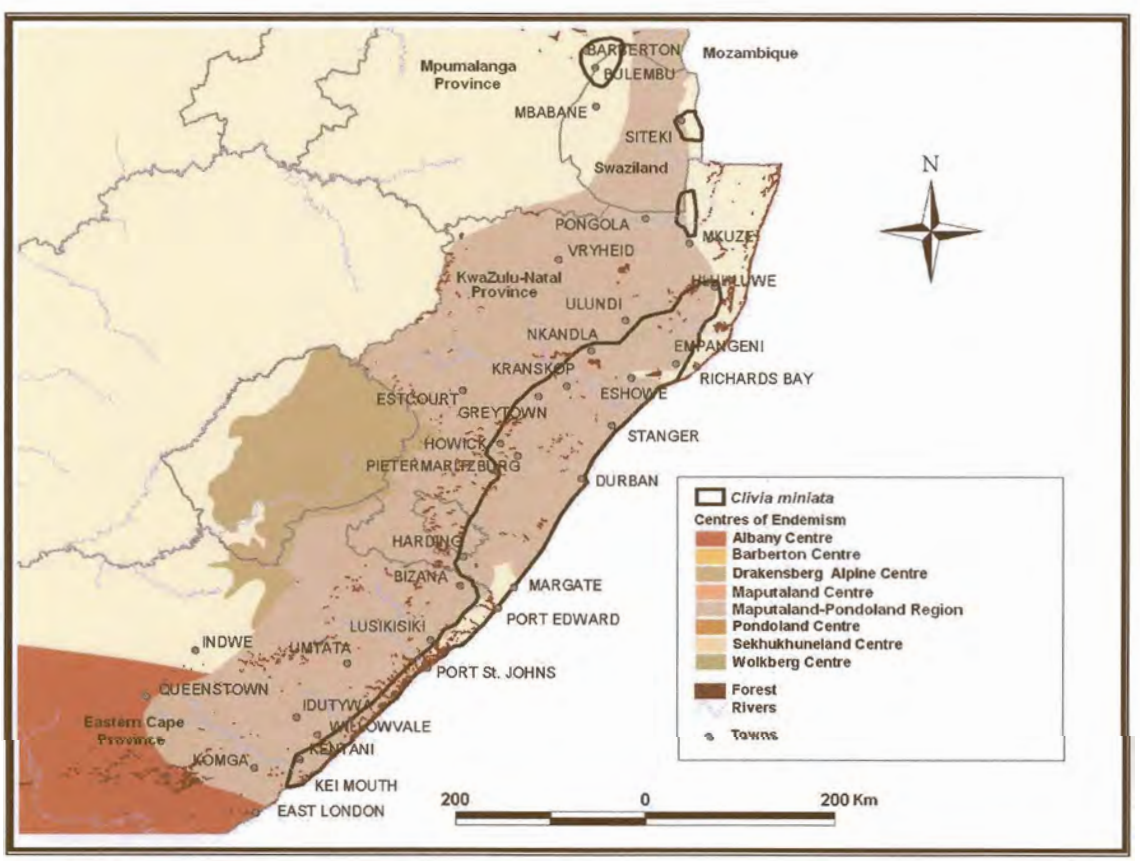

Figure 4.4 Natural distribution range of *Clivia miniata*.

This disjunct pattern is probably not real if one considers that the mountainous terrain limits botanical exploration and that it is very likely that favourable habitats exist between these localities.

The distribution range of *C. miniata* falls within four regions/centres of endemism, namely the Barberton Centre (see section 4.3.4), Maputaland-Pondoland Region (see section 4.3.2), Maputaland Centre and Pondoland Centre (see section 4.3.3). Natural hybrids have been reported in cases were *Clivia miniata* grows in close proximity to other members of the genus (Chapter 3).

Clivia miniata communities have been reported on sandstone and dolerite (Winter 2000). It usually occurs in large colonies in evergreen forest. Populations have been noted along shaded watercourses, ravines and ledges. *Clivia miniata* occasionally grows epiphytically on rocks and in tree forks (Duncan 1999; Winter 2000).

In the present study three different populations of this species were investigated, namely in the vicinity of Port St Johns, Bearded Man Mountain and Lobodtlyana Kloof, Songimvelo Game Reserve.

The Port St Johns locality, part of the Pondoland Centre, is midway up a mountainous slope. At this locality *C. miniata* plants form a green 'sea', with small boulders or trees appearing as enclosed islands therein. Closer observations revealed the plants were growing in the debris (humus) accumulated between the boulders of a rock scree. Plants grow on the south-facing aspect of the mountain at a slope of 30–40°. A high canopy, comprised out of larger than 20 m tall trees, allowed for a high light intensity environment. Holes in the canopy further increased the light intensity. *Clivia miniata* plants grow without any competition in this habitat, with the population covering a strip estimated to be at least 30 m in width and 50–80 m or more in length. The boundaries of the colony are formed by shadier undergrowth.

Plants formed both multi-stems, with on average 2 or 3 stems per plant, or occurred as single-stemmed individuals. Caterpillar damage was visible on some plants and other insect activity was also noted (beetles). Lichens, which covered almost the entire leaf surface exposed to the sun, were frequently observed. Plants in more shady areas had few or no lichens.

The plants grow shallowly/loosely on top/between rocks, with some roots running into cracks. Growth substrates are mainly rotting bark/detritus/leaves and other humus. Boulders were estimated to be ± 500 mm to 2 000 mm in diameter.

Population size is well over 200 mature plants (plant clusters were counted). Semi-broad to narrow-leaved plants (35–50 mm) made-up the colony, some leaves as wide as 70 mm. Many plants were markedly stemmed (old stems). The stems were sometimes rotten away, but with new suckers being produced. One plant grew epiphytically in the hollow trunk of a *Ficus* spp. This community seems to reproduce mainly asexually via suckers or 'rotting stems'. Some sexual reproduction was observed but this clearly is of minor significance.

Flowers were narrow-petalled, pastel pink to orange, with very faint colouring in the centre (which could be absent). Average number of flowers per umbel was estimated to be between 10 and 15. Little variation between flowers was noted. There is, however, some variation in general plant morphology and leaf width.

Humidity of the understorey was high (it had rained earlier) with damp undergrowth. However, water seeped away from the plants due to the high number of boulders—thus a well drained, though damp environment.

Very few seedlings (less than 3% of total individuals) were located. This is to be expected if the tendency is for only 15–20% of the mature plants to flower per season. Poor seed-set was noted.

The population seemed limited to the old disturbed area (rock fall). Though genetic recombination had occurred (very low), it is not clear if the population is healthy with only vegetative reproduction.

The second population investigated was at Bearded Man Mountain, on the border between Swaziland and Mpumalanga (see also section 4.3.4). Plants occurred along a strip of approximately 200 m in length, up the mountain on a slope of 30–45°. *Clivia caulescens* were growing alongside *C. miniata*.

Most of the *C. miniata* plants were in flower. Few flowers (4–8) per umbel were produced, but the flowers were usually large and hibiscus-shaped. Flower centres were either white or yellow with flower colour ranging from light orange and pink pastel to darker orange. Approximately 600 to a 1 000 individuals formed this community. Seedlings account for approximately 20% of the population. Most of the population looked like old, mature plants.

At this locality *C. miniata* grows in the humus layer, with shallow, surface roots. There were a few boulders/rocks with an average width of 0.5 m. Lichens covered some leaves and 'leaf-miners' were also present. Plants grow as individuals, with clumps seldom seen. Some individuals produced marked/aerial stems, as in *C. caulescens*, but not to the same extent.

The Lubodtlyana Kloof population (also see section 4.3.4) contains both *C. miniata* and *C. caulescens* communities, with *C. miniata* clearly dominant. The *C. miniata* community consisted of approximately 300 individuals, with an estimated 20% seedlings. *Clivia miniata* plants are found on both the steeper slopes and the valley floor, where they grow in well-drained humus-rich soil.

Plants grow as individuals or small colonies throughout the kloof. Individuals in flower were largely limited to the higher, lighter parts of the valley. An estimated 10% of the population flowered. Seed-set was noted, though no seeds were found. Flower colour and

shape varied from a closed reddish flower to an open pastel-pink flower. Flower number ranged from 4–15. Leaf shape also varied.

4.3.6 *Clivia mirabilis* Rourke

Clivia mirabilis is only known from the eastern part of Oorlogskloof Nature Reserve, Northern Cape Province (Rourke 2002a). There are no other records or confirmed reports of other populations outside the reserve (Figure 4.5).

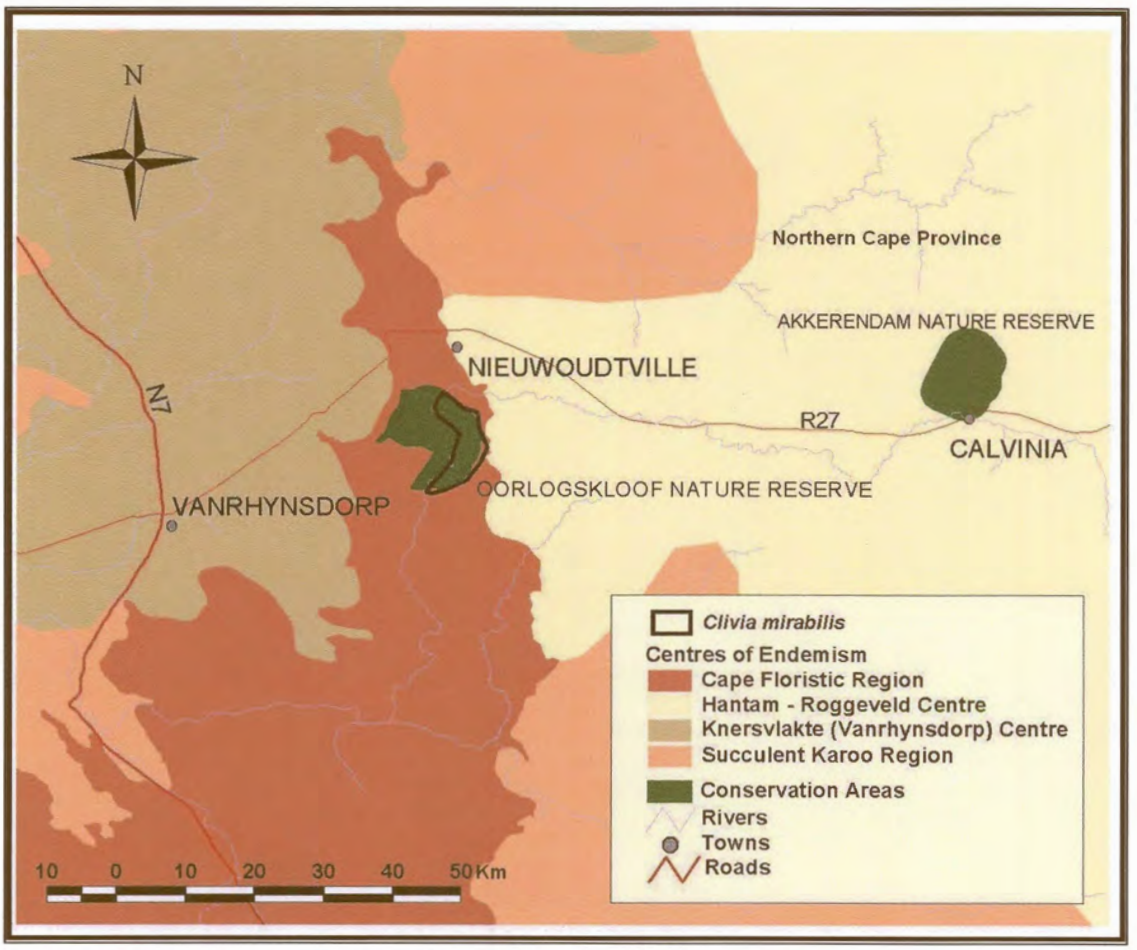

Figure 4.5 Natural distribution range of *Clivia mirabilis*.

The Oorlogskloof Nature Reserve falls within the Cape Floristic Region (CFR). Recognised by some as one of the six Floristic Kingdoms, this region covers approximately 90 000 km^2. In this relatively small area, the highest extratropical concentration of higher plant taxa in the world are contained, confirming its status as one

of the earth's 25 species diversity hotspots (Cowling & Hilton-Taylor 1997; Van Wyk & Smith 2001).

Average annual precipitation for the CFR is between (250–)300–2 000(–5 000) mm, with mean annual temperatures of 15–16°C (coast) to 17–18°C (inland) (Cowling & Hilton-Taylor 1997; Van Wyk & Smith 2001). The main geological formation the Cape Supergroup, with the quartzites of this group giving rise to acidic, low-nutrient, coase-grained soils (Van Wyk & Smith 2001). The Cape Floristic Region contains five broad vegetation types, namely Afromontane Forests, Subtropical Thicket, Fynbos, Renosterveld and Succulent Karoo (Van Wyk & Smith 2001, and references therein).

The Oorlogskloof Nature Reserve receives an average precipitation of 414 mm annually, strictly during the winter months. Characteristic of this area is its semi-arid Mediterranean climate. Light frost is probably experienced briefly during these months—a consequence of being 100 km inland and at an elevation of 850–900 m (Rourke 2002a, b). A more detailed ecological description of the habitat of *C. mirabilis* is given by Rourke (2002a).

In this harsh environment, *C. mirabilis* plants are found growing in humus, caught between cracks produced by the erosion of Nardouw Formation Sandstone. Coarse sandstone talus screes, the result of erosion, contain relictual Afromontane elements. *Clivia mirabilis* occurs as solitary or grouped/clumped specimens on these rock screes, usually under the relictual Afromontane woodlands, though occasional clumps have been noted to grow in full sun. The only known population is estimated to be well over 1 000 individuals, distributed over several hectares.

4.4 Biogeography of *Clivia*

4.4.1 Introduction

One can only speculate about the past distribution and migration of *Clivia* and its predecessors. This section focuses on the establishment of the current distribution patterns for the various *Clivia* species—taking into account environmental alterations brought about by both past climatic and topographic changes.

4.4.2 Development of Africa and its flora

The Cretaceous period featured the onset of dramatic floristic change, eventually resulting in the predominance of the angiosperms. During the early Cretaceous, angiosperms achieved importance in low palaeo-latitudes, mainly in northern Gondwana. At the time the climate was warm and moist (tropical). Separation of Africa and India from the super-continent, Gondwana, occurred through continental drift by mid-Cretaceous times. Angiosperms developed tolerance to cold and spread into middle and high palaeo-latitudes, resulting in an explosion of diversity towards the end of the Cretaceous period. Australia, New Zealand, Antarctica and South America separated during this latter part of the Cretaceous period. Worldwide, angiosperms were well established by the mid-Cretaceous (Bredenkamp *et al.* 2002, and references therein).

The late Cretaceous and Paleocene had Africa located approximately 15° south of its current position, placing northern Africa over the equator. Westerlies probably resulted in a cool, wet climate in the southern third of the continent, with warm, humid climate and low relief in the rest of the continent. This resulted in tropical forest extending from coast to coast over northern and eastern Africa (which was not elevated then) (Goldblatt 1978; Partridge 1997; Bredenkamp *et al.* 2002, and references therein). Bredenkamp *et al.* (2002) refer to pollen records suggesting extensive forest cover even as far south as southern Africa. The temperate forests located in the Cape today are possible relics of the forest flora that covered the southern third of the continent during this time (Goldblatt 1978).

The Cenozoic period is regarded as the time during which the current biomes developed. The Neogene coincided with considerable climatic changes within southern Africa. Tectonism elevated the southern African central Highveld and parts of the eastern Great Escarpment. These geographic changes brought about cooler and drier conditions. Older ecosystems became restricted, eventually being replaced by newer ones. These vegetation types progressively expanded to adapt to the drier climate (Bredenkamp *et al.* 2002).

The Eocene-Oligocene was marked by the separation of other continents, resulting in the development of a circum-Antartic current that led to a drop in temperature in the high southern latitudes. Towards the end of the Oligocene, Antarctica was torn from the remaining landmass and drifted southwards. The cold Benguela current, flowing along the west coast of Africa, originated in ice covered Antarctica. Factors such as the destruction of the Tethys sea (altering the major latitudinal circulation), the closure of the Panamanian portal, formation of the Mexican plateaux, tectonic uplifts producing the Alps and Andes, resulted in an increase in aridity to the former moist west coast of Africa. This seasonal dry climate promoted selection for drought resistance. Forests were pushed into sheltered ravines that contained sufficient moisture, forming discontinuous forest patches (Goldblatt 1978; Bredenkamp *et al.* 2002, and references therein).

Quaternary times encompassed major climate changes in response to glacial-interglacial periods. Pleistocene interglacial periods stimulated forest expansions and major plant migrations, with interglacial periods reducing these communities (Goldblatt 1978; Bredenkamp *et al.* 2002, and references therein).

4.4.3 Proposed migration of *Clivia* species

Biogeographically, *Clivia* evolved and moved as part of the family Amaryllidaceae. Meerow *et al.* (1999) support previous hypotheses that the Amaryllidaceae originated in western Gondwana, using plastid DNA phylogeny. They showed the deepest topological branches originated in Africa, some with considerable innovations as typified by the Afrocentric tribes (Amaryllideae, Haemantheceae and Cyrtantheae). They concluded that the family was African in origin. Today Amaryllidaceae has three centres of diversity, in

the Andean region (28 genera), Mediterranean (8 genera) and southern Africa (18 genera) (Meerow & Snijman 1998).

The greatest diversity of the African Amaryllidaceae is concentrated in South Africa (Meerow *et al.* 1999, and references therein). *Clivia*, as part of the sub-Saharan tribe Haemantheae, corroborates this pattern with all species situated in South Africa (though two occur also in Swaziland). The origin of an ancestral *Clivia* species is therefore likely to be south-southwest Africa.

Increasing aridity, the uplift of the continental mass and quaternary fluctuations in Africa's climate, pressurised the Amaryllidaceae to diversify and adapt to increasing drought. Meerow *et al.* (1999, and references therein) stated that the adaptations in the Afrocentric tribes could be the result of an increase in radiation that occurred in Africa within recent paleoclimatic and geological history. *Clivia* adapted by developing thick, fleshy, perennial roots (Duncan 1999; Winter 2000; Rourke 2002a). Instead of growing in soil, it circumvented competition with tree roots by adapting to grow in the top humus layer of the forest floor (personal observations). *Cryptostephanus*, *Scadoxus* and *Clivia*, all genera of the baccate-fruited Haemantheae, did not form bulbs like other Amaryllidaceae, but remained forest understorey taxa and closely connected to this forest element (Meerow *et al.* 1999).

Midgley *et al.* (1997) suggest that Afromontane forests had a southern, temperate origin. One can argue that *Clivia* either developed with these forests and therefore had a more southern origin or found refuge in them while migrating to escape the increasing arid western side of Africa. Midgley and co-workers further concluded that distribution patterns of Afromontane Forests were the result of biotic interactions, rather than the influence of the historical centre of origin. Though this might be true for Afromontane forests, *Clivia*'s centre of origin may have played a major part in the current geographic distribution of the genus. This statement is substantiated by the current phylogenetic analysis of the genus (Ran *et al.* 2001a; Ran *et al.* 2001b; Conrad & Reeves 2002). *Clivia mirabilis* was shown to be sister to the other species of *Clivia*. It does not imply that

C. mirabilis occupied the 'centre of origin' of the genus. Other taxa reflecting a different 'centre of origin' may have gone extinct.

The discovery of *C. mirabilis* in Oorlogskloof Nature Reserve in the Northern Cape, supports the hypothesis that the Cape region acted as a refuge for previous tropical vegetation types in this part of Africa (Meerow *et al.* 1999; Rourke 2002a). Increasing temperatures and lower precipitation experienced in western Africa following the break-up of Gondwana and the formation of the Great Escarpment, probably forced *Clivia* (in association with the Afromonatane element) towards the wetter southern and eastern coastal areas of South Africa.

Clivia mirabilis (or rather its predecessor) found refuge in the Oorlogskloof canyon during this migration. It adapted to the increasing aridness of the surrounding landscape, as the forest environment gradually deteriorated. This isolation and adaptation eventually give rise to *C. mirabilis*. *Clivia* migrated eastwards where it reached the east coast of South Africa. Either a sub-tropical or tropical environment existed along the coast or glacial periods provided connections between the Afromontane communities. In both cases, *Clivia* moved northwards along the coast, bordered by the sea and the Great Escarpment. Fragmentation, probably as a result of inter-glacial times, isolated communities at the southern part of the distribution first. These isolated communities of the Eastern Cape Province experienced alterations in the climate that favoured the adaptation to the region of *C. nobilis*.

Van Wyk (1990) suggested that the Pondoland Centre, with its sandstone substrate, acted as a barrier to plant migrations from the east coast (mainly during the Quaternary period). This would have 'trapped' species to the south of this region during the contraction or advancement of vegetation. He proposed a closer connection between the various sandstone formations and vegetation. The Msikaba Formation Sandstone (Pondoland) together with the Natal Group sandstone, have been shown to be related to the Table Mountain Group, with the latter closely related to rocks of the Cape Supergroup that is closely associated with the Cape Floristic Region. Various endemics connect these

regions and support this hypothesis (Van Wyk 1990; Van Wyk & Smith 2001, and references therein).

Van Wyk (1990) proposed that the sandstone outcrops of Pondoland and KwaZulu-Natal acted as edaphic islands to migratory vegetation (Van Wyk 1990). *Clivia* probably dispersed further northwards via the Natal Sandstone Group outcrops, to the Afromontane areas of Mpumalanga and the Limpopo Provinces. Van Wyk (1990) mentions various taxa in a similar pattern of distribution, probably during Plio-Pleistocene epoch. Subsequent fragmentation and isolation of the different communities, would explain how the current *Clivia* species originated, but this doesn't always correspond to hitherto proposed phylogenies (Ran *et al.* 2001a; Ran *et al.* 2001b; Conrad & Reeves 2002).

The proposed origin of the different species as explained in the previous paragraphs, correlates to the phylogeny of Conrad & Reeves (2002) but not to those proposed by Ran an co-workers (Ran *et al.* 2001a; Ran *et al.* 2001b) (Chapter 3). Conrad & Reeves (2002) utilised the plastid genome for their analysis, while Ran and co-workers used a random technique on total genomic DNA (RAPDs) as well as sequence information from the ITS region (Ran *et al.* 2001a; Ran *et al.* 2001b). According to both Ran *et al.* 2001a and 2001b, *C. caulescens* is basal to *C.* 'Robust' *gardenii*, the sister to both *C. miniata* and *C. gardenii*. These phylogenetic trees suggest that *C. nobilis* evolved first, followed by *C. caulescens*, *C.* 'Robust' *gardenii*, *C. gardenii* and *C. miniata*. If vicariance is assumed then this biogeographic pattern is possible. The ancestral species may have been more widespread in the past prior to the fragmentation of its range within the summer-rainfall region. The fragmentation may not have proceeded in a northerly direction but could have been at peripheral sites in the north and south.

The phylogenetic tree proposed by Conrad & Reeves (2002) suggests that the species started to differentiate as one moves up along the coast, with first *C. nobilis*, then *C. gardenii* and finally *C. caulescens* and *C. miniata*. Unfortunately they did not distinguish between *C. gardenii* and *C.* 'Robust' *gardenii* in their study.

Chloroplast DNA diversity in *C. miniata* (Chapter 6), suggests an origin for the species in the northern part of Pondoland or in southern KwaZulu-Natal. From this location, *C. miniata* migrated along the coast in both a northerly and southerly direction. This would fit in with the assumed phylogeny, with *C. miniata* developing from a *C.* 'Robust' *gardenii* or *C. gardenii* like ancestor.

4.5 Conservation status

The previous sections highlighted the limited geographic range of the various *Clivia* species, all more or less confined to certain regions/centres of endemism. This limited distribution, further restricted by microhabitats associated with patchy Afromontane forests, raises the question of the conservation status of the various infrageneric taxa.

At present, some *Clivia* species are mainly placed under Lower Risk-Least Concerned, Lower Risk-Near Threatened and Vulnerable headings (Table 4.1) (Golding 2002; Lötter & Krynauw 2002). This seems insufficient when one considers the data presented in the previous sections.

Both environmental and human-induced factors are threatening the survival of *Clivia*. The main environmental factor is the microhabitat to which *Clivia* is evolutionary connected, namely the Afromontane Forest of southern Africa.

Afromontane Forest occupies approximately 6 000 km^2 in South Africa and Swaziland, of which only 17.64% is conserved (Lubke & Mckenzie 1996). Geographical distribution of these forests are determined by a single key-limiting factor, namely water, restricting them to wetter kloofs and gullies. Fire is also important in maintaining the forest boundaries especially within the grassland and Fynbos vegetation—with forest mainly confined to fire safe refuges (Lubke & Mckenzie 1996).

Table 4.1 Current published conservation status of the genus *Clivia*.

Clivia taxon	Specific distribution area used for risk assessment	2001 IUCN Red List Category	Reference
C. nobilis	KwaZulu-Natal, Eastern Cape	Lower risk-least concerned	Golding 2002
C. miniata	KwaZulu-Natal, Eastern Cape	Lower risk-least concerned	Golding 2002
C. caulescens	Eastern Cape[1]	Lower risk-least concerned	Golding 2002
C. gardenii	KwaZulu-Natal	Lower risk-least concerned	Golding 2002
C. caulescens	Devils Bridge	Data deficient	Golding 2002
C. miniata var. *citrina*	?	Data deficient	Golding 2002
C. nobilis	Swaziland[1]	Data deficient	Golding 2002
C. miniata	Swaziland (Lebombo, Piggs Peak area)	Lower risk-near threatened	Golding 2002
C. miniata	Mpumalanga	Vulnerable (VU B2abii, iii, v)	Lötter & Krynauw 2002

[1] Distribution record incorrect

Clivia species, in conjunction with Afromontane Forest, show a patchy (disjunct) distribution pattern (Lubke & Mckenzie 1996; Cowling & Hilton-Taylor 1997). A restricted ecological niche within Afromontane Forest—usually a well drained area—limits the available microhabitat that *Clivia* species can successfully colonise. Microhabitats, though restricted in number, are not always exploited by *Clivia* species. This might indicate inadequate seed dispersal. In certain microhabitats, usually without competition from other taxa, *Clivia* communities are sometimes termed 'locally abundant' due to their high numbers. Unfortunately, the use of this term may lead to the conclusion that once a *Clivia* species has established itself in an area, it becomes the climax taxon. This might be true in some cases, but with low seed production and recalcitrant seed, such climax communities might take several generations (years) to establish. Any changes in environmental conditions, with an effect on microhabitat, will have a serious impact on the survival of this hardy genus.

Human factors threatening the survival of this genus are mainly habitat destruction and illegal removal of specimens from nature. Habitat destruction is the result of woody material being removed for fuel or agricultural purposes and urbanisation (Chubb 1996;

Duncan 1999). Plants collected for horticultural or medicinal properties are probably the most serious threat to natural *Clivia* populations (Chubb 1996; Duncan 1999; Lötter & Krynauw 2002). The high demand by traditional healers for *Clivia* plants was clearly evident when Mander (1998) identified *C. miniata* as the tenth most sought after medicinal plant to be traded in Durban, KwaZulu-Natal. Williams *et al.* (2001) found *Clivia* species in 70% of the Witwatersrand '*muti*' shops they surveyed.

Clivia populations are currently located in several nature/game reserves across the country. This can, in part, be attributed to the geographic range of the various species, most of which have communities in centres/regions of endemism that currently boast some areas that receive special protection. Will this be sufficient to save *Clivia* species, or will the inaccessibility of *Clivia* communities and their disjunct distribution pattern eventually prove to be the key to their survival?

Current conservation status classifications for *Clivia* seem insufficient when one considers the data presented, therefore we propose a new conservation classification for the different members of the genus (Table 4.2).

Clivia mirabilis is placed as Critically endangered (CR) on the basis of a single location with its extent of occurrence being less than 100 km^2 and area of occupancy is estimated to be less than 10 km^2. This species is currently receiving maximum protection, thanks to its confinement to a rather inaccessible part of the Oorlogskloof Nature Reserve.

Clivia 'Robust' *gardenii* is categorised Endangered (EN) on the grounds of the populations being highly fragmented within its limited extent of occurrence (estimated to be less than 5 000 km^2) and area of occupancy (less than 500 km^2).

Table 4.2 Proposed conservation status of *Clivia* species.

Clivia species	Distribution	Proposed Category[1]
C. miniata	Eastern Cape, KwaZulu-Natal, Mpumalanga Provinces, Swaziland	Vulnerable (VU), A3d
C. caulescens	Mpumalanga, Limpopo Provinces, Swaziland	Vulnerable (VU), A3d
C. gardenii	KwaZulu-Natal Province	Endangered (EN) B2a
C. nobilis	Eastern Cape Province	Endangered (EN) B2a
C.* 'Robust' *gardenii	Eastern Cape, southern KwaZulu-Natal (Pondoland) Provinces	Endangered (EN) B1a+2a
C. mirabilis	Northern Cape Province	Critically Endangered (CR) B1a+2a

[1] According to 2001 IUCN Red List Categories, Version 3.1, as in Golding 2002.

Clivia gardenii and *Clivia nobilis* are categorised as EN based on an area of occupancy that is estimated to be severely fragmented and less than 500 km^2. *Clivia miniata* and *C. caulescens* are labelled vulnerable (VU) with an estimated population size reduction of 30% or more, projected/suspected to be met in the following three generations as a result of actual/potential levels of exploitation. While the remaining *C. miniata* populations are usually rather inaccessible, some *C. caulescens* populations are more readily accessible, placing more pressure onto this taxon.

4.6 Conclusions

Distributions maps for the different *Clivia* species showed the geographical ranges of all to fall within various Centres/Regions of floristic endemism (Table 4.3 and Figure 4.6). The geographic isolation of *Clivia* 'Robust' *gardenii* supports molecular evidence produced by Ran *et al.* (2001b), in establishing this form as a new species. The biogeographical patterns displayed by the species, environmental information from the present and the past, as well as available molecular phylogenies, were used in the formulation of a hypothesis to explain the current distribution of the genus.

Table 4.3 *Clivia* species and the regions/centres of endemism to which they are mainly confined.

***Clivia* taxon**	**Centres of plant endemism**
Clivia caulescens	Barberton Centre Soutpansberg Centre Wolkberg Centre*
Clivia gardenii	Maputaland-Pondoland Region
Clivia miniata	Albany Centre* Barberton Centre Pondoland Centre* Maputaland-Pondoland Region
Clivia mirabilis	Cape Floristic Region*
Clivia nobilis	Albany Centre* Maputaland-Pondoland Region
Clivia 'Robust' *gardenii*	Pondoland Centre*

*Regarded global 'hot-spots' of biodiversity (Cowling & Hilton-Taylor 1997)

Forests are reported to constitute less than 1% of the total land cover of southern Africa (Cowling & Hilton-Taylor 1997). The relevant environmental factors, together with the biogeographical distribution, were used to propose IUCN conservation statuses for the different *Clivia* taxa.

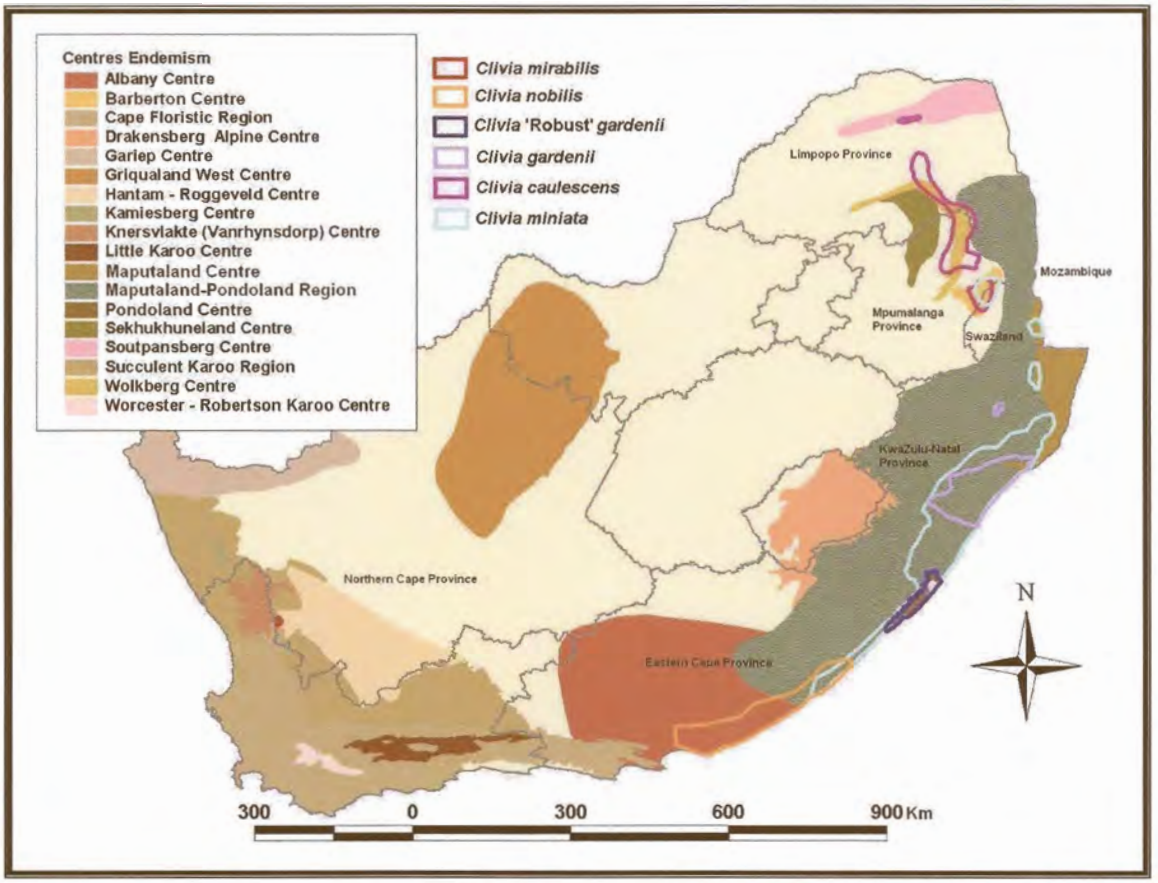

Figure 4.6 The distribution range of *Clivia* taxa and the regions/centres of endemism to which they are mainly confined.

4.7 Acknowledgements

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CHAPTER 5

HORTICULTURAL SIGNIFICANCE OF *CLIVIA MINIATA*

5.1 *Clivia miniata*: an international industry

The horticultural history of *Clivia* started when the first specimen of the genus, namely *Clivia nobilis* Lindl., was taken to Britain for identification during the early 19th century (Chapter 3.2.2). However, it was the introduction of *Clivia miniata* (Lindl.) Regel that captured the imagination of the gardeners in Europe (Chapter 3.2.2). Today, *Clivia* is horticulturally truly cosmopolitan—even though it is native to South Africa and Swaziland only.

Clivia miniata is the main driving force behind the horticultural success of the genus. *Clivia miniata* cultivars and hybrids come in an assortment of colours, ranging from lemon–yellow, peach, pink, apricot, salmon, orange to brick–red. Unusual cultivars like *C. miniata* 'Striata'—with cream stripes on orange flowers—the yellow flowering *Clivia miniata* 'Sir John Thouron' or specimens with variegated foliage, are highly sought after. The variety found in flower form and colour is one of the reasons for the horticultural success of the species. The ease with which *Clivia* is grown and hybridised, together with the big showy flowers and a high tolerance for abuse, makes it an ideal plant for amateur botanists, landscape designers and gardening hobbyists (Martin 1994; Bryan 1995). The long 'seedling stage' and lengthy waiting period for sucker production, in conjunction with human impatience, facilitates the trade of this species.

The *Clivia* industry is dominated by *Clivia miniata*. The genus name, *Clivia*, is sometimes incorrectly used for referring to *C. miniata*—with the species name reserved to clarify multi-species situations. With no international monitoring institutions known, there are no official estimates available to determine the extent of the industry. No comprehensive worldwide studies on the *Clivia miniata* industry, covering aspects such as annual production, monetary value and breeding aims, are known. This type of study is complicated by the fact that a large part of the industry is owned by the private sector. The present study has shown that private growers are responsible for a significant share of the total income generated by the industry.

This chapter focuses on the horticultural significance and trade in *Clivia miniata* worldwide. Furthermore, the breeding aims and horticultural history of *C. miniata* in the different regions are discussed. The principal aim is to highlight the horticultural significance of *C. miniata* as a desirable entity for international plant collectors.

Information presented in this chapter was gathered through an extensive survey of the literature, the World Wide Web—including a related news group (http://groups.yahoo.com/group/clivia-enthusiast/) and websites of mail ordering nurseries—and personal communications with various *Clivia* growers. Where possible, estimates are supplied to highlight the horticultural significance of *C. miniata* internationally. This is the first known attempt to try and quantify the horticultural significance of *Clivia* worldwide.

5.2 South Africa

5.2.1 Introduction

South Africa is the chief producer of commercial *Clivia* hybrids in Africa. This is to be expected, since the genus is endemic to the country (Rourke 2002). Surprisingly, South Africa only became a role player in *Clivia* cultivation in about 1998. The growth of the *Clivia* industry in the region is largely due to the formation of the *Clivia* Club in 1992 (Duncan 1999). Rare clones that were in the hands of a select few (Holmes 2001), were now being actively promoted and marketed to a wider audience. The increase in public awareness and knowledge, promoted largely by the *Clivia* Clubs and their shows, led to a boom in the local *Clivia* industry, with plants being actively propagated to satisfy the increasing demand.

In 2002 the *Clivia* Club was replaced by the *Clivia* Society, with numerous Clubs and interest groups under its umbrella. These include the Cape *Clivia* Club, Northern *Clivia* Club, KwaZulu *Clivia* Club, Eastern Province *Clivia* Club as well as interest groups such as Border, Southern Cape, Northern KwaZulu-Natal, Metro and the Waterberg Bosliekklub (from the inside cover of *Clivia* Four, published by the *Clivia* Society). Shows, newsletters and discussion sessions organized by these Clubs and Groups, as well

as the Society's annual publication (*Clivia* Yearbook), are contributing to the success of the industry in South Africa.

5.2.2 Market trends

The South African market focuses on a broad range of plant characters, which include flower form, flower colour, leaf width, leaf variegation and interspecific hybrids. With material becoming readily available and a large natural genetic pool at the disposal of *Clivia* growers in South Africa, new hybrids are actively being produced. Weather conditions that prevail virtually throughout the country are ideal for the promotion of *Clivia miniata* as a garden plant. The other species of *Clivia*, however, enjoy limited interest in South Africa.

In recent years, the local demand for rare hybrid lines produced in other countries like Japan and China, have increased considerably. This creates new possibilities for breeders to hybridise different cultivars in an industry where anything new is highly valued.

5.2.3 Value of the South African *Clivia* industry

Private growers dominate the South African market. This makes it difficult to obtain reliable estimates of the actual number of plants being produced annually. Income generated by seed and plant sales is virtually impossible to calculate without production figures. Annual production is estimated to be anything from one to five million plants. Supposed plant production figures are around one million, with plants sold for an average of R30 per plant; the revenue generated would be in the vicinity of R30 million. Although comprehensive production statistics are unavailable—some known figures, obtained from different sources, are given here to highlight the market size.

The income statement of the Northern *Clivia* Club of the *Clivia* Society of South Africa was used to calculate sales at the Club's 2001 annual show. This revealed that over R187 000 worth of plants and seeds were sold over the two-day period of the show (Northern *Clivia* Club: Financial Report at 30 September 2001). The Club's policy of charging a percentage (15%) from the income generated by sales during the show, was

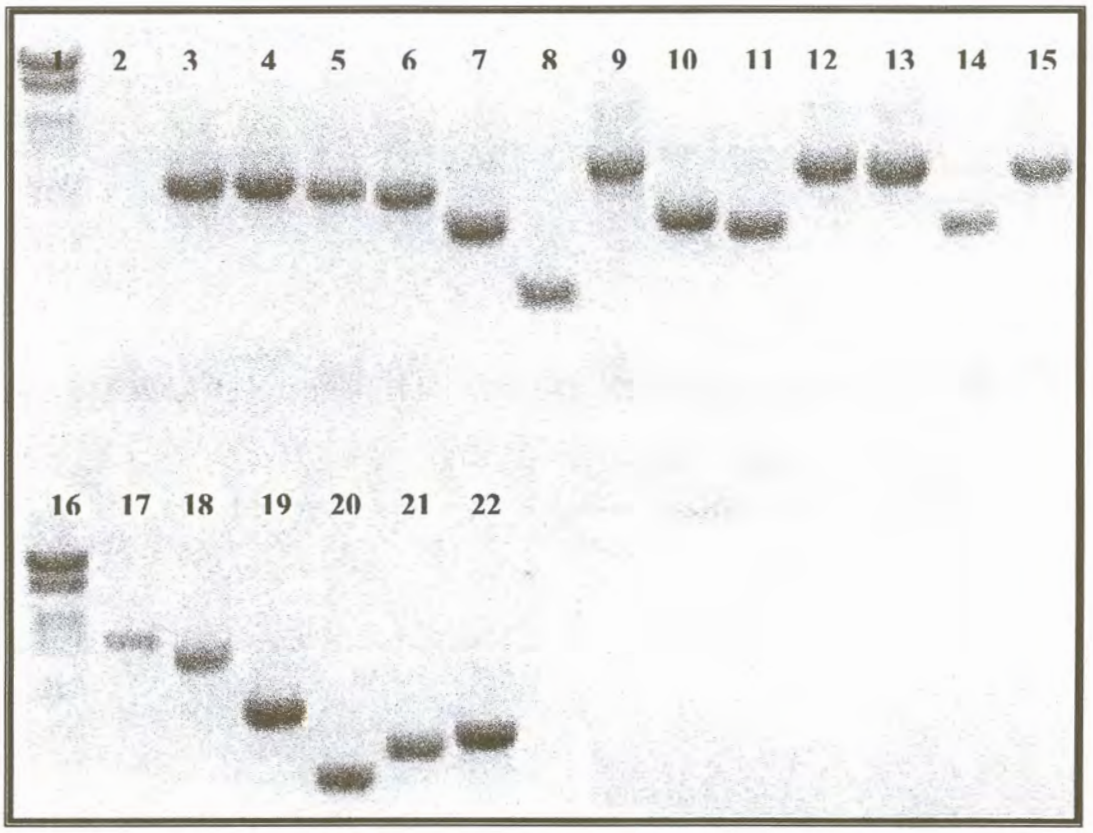

Figure 7.1 A colony PCR of cloned fragments originating from the primers HVH$(GTG)_5$ (lanes 3–7), DHB$(CGA)_5$ (lanes 8–14), DBD$(CAC)_5$ (lanes 15, 17 & 18), DBV$(CAT)_5$ (lanes 19–22). Lanes 1 and 16 are lambda III molecular weight markers. Lane 2 is the negative control of the PCR.

Three of the microsatellites were pure, with one a mononucleotide repeat (A_{11}), one dinucleotide repeat ($(GT)_9$) and one trinucleotide repeat $(TCG)_6$. Most of the remaining microsatellites were interrupted mononucleotide repeats (T_5GT_5, T_4AT_6, A_6CA_4 and $T_5GCT_4CT_3$) and one interrupted trinucleotide repeat, $(CCT)_2TCT(CCT)_2CGT$. Due to insufficient flanking sequences, only four microsatellite primer sets could be developed (Table 7.2).

used to re-calculate the total sales. At the 2002 show a fixed amount was charged per stall, making it impossible to determine sales for this particular event (J.W. de Kock pers. comm.[1]).

Information received from Mr. K. Fargher, the manager of the mail-order World Wide Web based nursery 'The *Clivia* Store' (www.clivias.com), gives an indication of the size of the export market of the South Africa *Clivia* industry. From January 2001 till middle December 2002, sales of approximately US$52 000 was generated. Seeds and plants were exported to, amongst others, the USA, Australia, Canada, UK, Sweden, Netherlands, Japan, France, New Zealand, Denmark, Malta, Peoples Republic of China, Finland, Spain, Belgium, Portugal, Israel, Germany, Puerto Rico (USA), South Korea, Costa Rica, Brazil and Mexico (K. Fargher pers. comm.[2]).

The market still appears to be unsaturated—even though prices have decreased over the last couple of years for certain varieties. High prices are still being paid for top quality and rare specimens—clearly evident from the recently held auction by the KwaZulu-Natal *Clivia* Club. The auction sold 81 plants for a staggering R91 695. A *C. miniata* plant was sold for R13 600 and a yellow *Clivia caulescens* for R8 600 (Vermaak & Nuss 2002).

5.3 Europe

5.3.1 Historical overview

The début of *Clivia miniata* into Europe's horticultural markets during the 1850s launched the genus into instant stardom. This species was so spectacular that specimens were publicly displayed, even though still undescribed. Increasing popularity made *C. miniata* a very successful indoor plant during the Victorian era. This species is regarded by some as one of the most highly priced plant varieties during the 19th century (Pole Evans 1921; Duncan 1985, 1992; De Coster 1998a; Van Huylenbroeck 1998; Koopowitz 2002).

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[2] K. Fargher, 21 Murray Street, Waverley, Johannesburg 2090, South Africa

5.3.2 The European market: trends and value

The popularity of *Clivia miniata* has maintained its reign in the European market ever since its introduction, largely due to numerous new cultivars being produced. The most popular being broad-leaved, compact types—though types with high flower counts were also popular. The market briefly lost interest in this pot plant after the World War II (1960s), mainly because of its long production period (less profitable in a market looking for inexpensive plants) and an old image sentiment. Commercial interests have been renewed in recent years (De Coster 1998a, b; Van Huylenbroeck 1998; Duncan 1999).

Clivia miniata is currently being grown throughout Europe, especially in Belgium, Denmark, Finland, France, Germany, Italy, Netherlands, Portugal, Spain, Sweden and the United Kingdom (K. Fargher pers. comm.[1]; B.M.J. Zonneveld pers. comm.[2]; De Coster 1998a, b). Compact plants flowering within three years, with umbels above the foliage, have been the latest market trend in the northern European countries. Southern European countries appear to prefer the larger varieties (De Coster 1998a, b; Van Huylenbroeck 1998; Duncan 1999).

Belgium's annual production for 1997 is estimated at 700 000 flowering plants, making it the largest producer in Europe at that time. A large portion of Belgian plants (278 000 plants) was sold during 1997 at the Dutch auctions for an average price of US$3.9 per plant. The Netherlands produced around 200 000 plants for the corresponding period. Production figures for Italy, Germany and France were substantial (De Coster 1998b; Van Huylenbroeck 1998). Europe therefore produced more than 1 million plants in the 1997 season. In 2003 at Dutch auctions, orange-flowered cultivars were sold for €4 a plant and the yellow-flowered cultivars for €6. Retail prices are usually two to three times the auction price for the same cultivar (B.J.M. Zonneveld pers. comm.[2]).

No current estimates of the size of the industry could be obtained, but if the 1997 production figures are used, the wholesale income is estimated to be 4–6 million Euro for

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[2] B.J.M. Zonneveld, Institute of Biology, Leiden University, 64 Wassenaarse weg, AL Leiden 2333, The Netherlands

the 2002 season. The retail income is therefore in the vicinity of 4–12 million Euro per annum, depending on the number of yellow-flowered cultivars sold. With these estimates, the gross revenue generated by the industry is calculated at between 8–18 million Euro for 2002.

5.4 Asia

5.4.1 Introduction

The main focal point of the *Clivia* industry in Asia is the Far East, notably China and Japan. *Clivia* is also grown in other Asian countries, e.g. Israel, North Korea and South Korea, but details regarding the size of these markets are unknown (K. Fargher pers. comm.[1]; Duncan 1999).

Outlined in this section is a detailed discussion of two of the world's most important *Clivia* markets, namely China and Japan. Due to the unfamiliarity with these markets in Western countries, this discussion is more elaborate.

5.4.2 *Clivia* in Japan: Daruma

Introduction

Clivia was introduced into Japan during 1854 (Xue *et al.* 2000). This was probably *C. nobilis* as *C. miniata* was only discovered in the early 1850s (Chapter 3). *Clivia miniata* was most probably introduced to Japan from Europe in the 1870s (Men & Poa 1997).

The general trend in Japanese horticulture to produce dwarf hybrids, with broad leaves or variegated foliage, stems from the Edo Period in Japanese history (1603–1867). During this period various wild mutations of highly priced plants were collected. The Japanese model for *Clivia* is based on two plants, the Nippon lily or omoto, *Rohdea japonica* (Thunb.) Roth (Convallariaceae or Liliaceae *s.l.*) and Japanese wind orchid, *Neofinetia falcata* (Thunb.) Hu (Orchidaceae). The Daruma cultivar-group corresponds to these

[1] K. Fargher, 21 Murray Street, Waverley, Johannesburg 2090, South Africa

species in leaf shape and variegation form—commercial Daruma names even correspond to these species' cultivar names (Sasaki 2001).

The Japanese *Clivia* industry

Clivia miniata is highly commercialised in Japan. The other species is not well known. In Japan, the emphasis is placed on the plant rather than its flowers. Selection of plants is based solely upon foliage features; beautiful flowers are a bonus. *Clivia miniata* plants with symmetric, fanlike appearance, half round leaf apexes, visible contrasts between leaf veins and blades as well as the overall shape of the leaves, are all highly desirable features in both Japan and China (Nakamura 1998; Duncan 1999; Rourke 2000).

Japanese horticulturists focus their breeding programs on the production of short and broad-leaved plants. Selections with the correct leaf width to leaf length ratio have the cultivar-group name Daruma. Subtle differences in leaf shape and variegation are used to distinguish between the various commercial cultivars. The Daruma cultivar-group name is generally accepted to have its origin in Buddhism—either from a small doll named *Daruma* that is being sold at temples (as a symbol of happy fortunes), or from a monk named Daruma (Sasaki 2001).

Clivia prices in Japan ranges from US$10–30 for orange-flowered plants, to US$80 for a yellow or selected hybrid (Toshiyuki Hosoya pers. comm.[1]). Precise statistics regarding the Japanese *Clivia* industry are unavailable, but production and sales are estimated to be considerable.

5.4.3 *Clivia* in China: Junzi-Lan

Clivia: a part of the Chinese history

The main introduction of *Clivia* into China only occurred in the early 20th century when Japan invaded China. Legends in the southern region of Liaoning Province claim that a German brought some plants from the Drakensberg to Qing Dao after the Opium War (1840) and a Danish missionary brought plants to Liaonan around the same time (Mr. Wu

[1] Toshiyuki Hosoya, 507-1 Aterazawa, Ooemachi Yamagata 990-1101, Japan

Jin, pers. comm.[1]; Men & Poa 1997; Xue *et al.* 2000). These plants were called '*German-lan*' and '*Danish-lan*', respectively. Denmark is pronounced in this region as '*Darma*', therefore, the Chinese term '*Darma-lan*' is sometimes used for *Clivia* (Men & Poa 1997).

The Japanese policy of 'opening the country and learning from books' was largely responsible for the introduction of *Clivia* into China during the Japanese occupation. A Japanese, named Tamura, brought plants to the Manzhou Royal Palace in Changchun, the capital of China at the time. The Japanese emperor also gave rare *Clivias* to the last Chinese emperor of the Qing Dynasty, Aixin Jue Luo Pu-yi. At this time, *Clivia* was regarded as a noble plant and was only publicly displayed at state functions. Just a small number of Japanese, royals, courtiers and high-ranking government officials could enjoy them, with the ordinary people not even knowing of their existence (Wu Jin pers. comm.[1]; Men & Poa 1997; Xue *et al.* 2000; Van der Linde 2001).

In 1942, Pu-yi, while mourning the death of his wife, the Empress Tan Yu Ling, ordered a pot of *Clivia* to be displayed 'before her spirit' at the temple Hu Quo Bo Ye Ji (*Guardian Wisdom Temple*). The *Clivia* was not returned to the palace after the 49-day funeral. P'u Ming, a monk at the temple, kept the plant and started to cultivate it. This plant eventually gave rise to the cultivar Monk ('He Shong') when it reappeared in 1963 (Men & Poa 1997; Xue *et al.* 2000). It was the end of World War II in 1945 that was responsible for the release of *Clivia* to the general populace. T'ao Men and Wang Yung Pao (1997) reported that historical records indicated that there were only 'two *Clivia* pots' preserved after the collapse of the government. An old florist (gardener) of the court, Chang Yu T'i, preserved a specimen that was subsequently presented to Changchun Park (Zhang Chun Sheng Li Park). In celebration of the victory of '*Peoples' Liberation*' against Japan; this specimen was named 'Sheng Li', i.e. '*Great Victory*'. The other specimen was taken by the royal chef and presented to the manager of the Changchun Tung Hsing Dyeing Factory (Dong Xing Dyeing Factory), Ch'en Kuo Hsing—a cultivar today named '*Dyer*' or '*Dyeing Factory*' (Men & Poa 1997).

[1] Wu Jin, Xuzhou Medical College, 84 West Huaihai Road, Xuzhou 221002, Jiangsu Province, P.R. China

In the 1960s it was realised that *Clivia* could be produced from seed. This led to intensive breeding programs and the development of various new, highly desirable cultivars, i.e. '*Engineer Huang*' and '*Painter*'. Then the Cultural Revolution came about (1966 to 1976). With *Clivia* regarded as a mark of distinction, i.e. elitist, many prominent growers faced prosecution and even death in Changchun. During this time many rare *Clivias* were destroyed (Men & Poa 1997; Xue *et al.* 2000; Van der Linde 2001).

The current Chinese name used for *Clivia*, 'Junzi-lan' (noble orchid), originates from a Japanese publication in 'Horticulture Illustrated 1931'(according to Men & Poa 1997). In this publication, *Clivia* was referred to as '*Kunshi-ron* Orchid'. '*Kunshi-ron* Orchid' refers to the original Latin meaning of *Clivia*, namely noble, good and strong, but was later modified to '*Kunshi-ran*'. The Chinese later adopted these ideographs, but pronounced them as '*Jun-zi-lan*'. In Chinese, '*Jun-zi*' means noble or 'those with ability & morality' and '*lan*' means orchid. '*Kun*' in Japanese, similarly as '*Jun*' in Chinese, would be added to the end of a persons name to express respect (Wu Jin pers. comm.[1]; Men & Poa 1997; Xue *et al.* 2000). T'ao Men and Wang Yung Pao (1997) illustrated the Chinese view of *Clivia* with the statement; "The naming of *Junzi-lan* added the graceful and dignified *Clivia* to rare lines in the flower kingdom".

Flower cultivation became a fashionable trend after the Cultural Revolution—breathing new life into *Clivia* cultivation. This eventually caused an outbreak of '*Clivia*-fever' all over the country in the mid 1980s. In October 1984 *Clivia* was officially named the official flower of the city of Changchun by the '*Changchun People's Congress*'. Large-scale cultivation projects by nationalised companies, private groups and individuals—all coming together in the search of commercialised companies and *Clivia* enhancing activities, were started. This was probably the beginning of Chinese institutions like the *Clivia* Business Association (Changchun, Anshan), the *Clivia* Union (Beijing), the *Clivia*

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Study Society (Shenyang) the *Clivia* Project Association (Harbin) and the China *Clivia* Association (Jilin, Liaoyang, Daliang) (Men & Poa 1997).

Big expositions and exhibitions further stimulated the development of new and highly desirable hybrids (Xue *et al.* 2000; Van der Linde 2001). Recently, a 5-day competition held in the city Changchun, capital of the northeast Province of Jilin, attracted 50 000 *Clivia* entries from more than 3 000 participants. Only ten plants regarded as 'superior' won the title 'the king of flowers'. Approximately 100 000 visitors attended this competition (http://www.chinatradenews.com.cn/ 20000223/07.htm, date of access 17/03/2003).

The Chinese market

The Chinese *Clivia* industry is essentially situated in the northeastern part of China, in the Jilin Province. Changchun, the capital, is regarded as the cradle of the Chinese *Clivia* industry. In Anshan, *Clivia* breeding has also been popular since the 1990's (Wu Jin pers. comm.[1]).

In the mid 1980s the *Clivia* industry reached its peak when a 5-leaved sucker from the *Monk* cultivar-group, was sold for 100 yuan—approximately 3 months wages of an ordinary worker (Wu Jin pers. comm. [1]). The price stayed around 100 yuan till the early 1980s (Van der Linde 2001). However, in the mid 1980s, top quality plants were sold for anything from 60 000 to 200 000 yuan per plant (60 000 yuan equals about US$20 000 at that time) (Wu Jin pers. comm.[1]; Men & Poa 1997; Van der Linde 2001). The prices dropped dramatically during the latter part of 1985, with some as much as 99%. Today ordinary mature *Clivia* plants can be bought for as little as 30–100 yuan, but rare cultivars still demand high prices, i.e. a top quality plant can reach anything from 10 000–200 000 yuan (US$2500–5000) (Wu Jin pers. comm.[1]; Men & Poa 1997; Van der Linde 2001). Top quality one-leaf seedlings can even reach US$250 each (Wu Jin pers. comm.[1]).

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Annual production of mature Chinese *C. miniata* cultivars is estimated to exceed a million, excluding one to two year old seedlings. This assessment, though not official, is regarded as a rough figure of the total volume traded. Exact volumes would be hard to obtain due to the size of China. The estimated income generated by the industry, is believed to be well over US$100 million annually (Wu Jin pers. comm.[1]).

5.4.4 Conclusions

Clivia is clearly a part of Japanese and Chinese culture. Huge interest in the cultivation and horticultural development of *Clivia*, evident from the numerous institutions and websites devoted to the group, make these countries world leaders in *Clivia* production. They are responsible for various production techniques, including tissue culture protocols (e.g. Miyoshi & Co., Tokyo, Japan) and other vegetative multiplication techniques; research on all aspects of plant growth, development and chemical constituents, are conducted in both countries (Min & Jinsheng 1984; Men & Poa 1997; Chapman 1999; Xue *et al.* 2000).

The Chinese market is believed to be one of the largest, if not the largest, market for *Clivia* cultivars. The true value of this market is probably in the vicinity of US$200 million annually.

5.5 Australia and New Zealand

5.5.1 Introduction and historical overview of the Australian *Clivia* market

The first introduction of *Clivia* into Australia occurred in 1844 when J.C. Bidwill, an early director of the Sydney Botanical Gardens, brought some *C. nobilis* on board the *Arachne* to Sydney (correspondence by Mr. G. Callcott to the *Clivia*-enthusiast group, dated July 26, 2001). More introductions followed, with imports of new hybrids and *C. miniata* occurring regularly (Smith & Henry 1998).

[1] Wu Jin, Xuzhou Medical College, 84 West Huaihai Road, Xuzhou 221002, Jiangsu Province, P.R. China

Clivia miniata is very popular in Australia. The similarity between the Australian and South African climate makes it possible for *Clivia* growers to grow plants in shade houses or in shady spots in the garden. Hence, the plants are a favourite of landscapers and gardeners alike (K. Smith pers. comm.[1]; Smith & Henry 1998). *Clivia miniata* is the most widely cultivated *Clivia* species in Australia, grown in both South Australia and the southwestern corner of Western Australia (K Smith pers. comm.[1]).

A frustration, however, is the frequent misidentification of hybrids and species. This causes great confusion in the Australian market. Features in popular garden magazines, often repeating previous mistakes (some published as recently as 1996), are contributing towards this problem (Smith & Henry 1998).

5.5.2 Australian *Clivia* industry

The *Clivia* industry in Australia focuses on the broad-leaved cultivars. In Queensland nurseries, these are produced in their thousands. Red, variegated and peach are currently the cultivars in demand. *Clivia miniata* plants are sold as pot plants during the flowering season, but unlike in Europe, these plants are planted into the garden (K. Smith pers. comm.[1]).

Market prices for *Clivia* in Australia have dropped in recent years. Mature yellow-flowered plants that were sold in the past for AUS$100–150 are now difficult to sell at retail stores, even though prices are reduced. Plants over AUS$50 are hard to sell. The price drop could be attributed to an increase in producers, e.g. during 1995 there were only 2 or 3 mail order nurseries offering *Clivia*, but in 2002 this number more than doubled to 7 or 8 (K. Smith pers. comm.[1]). Growing imports (from USA, South Africa, Europe, China, Japan and especially from New Zealand), an increase in maturing plants produced from previously imported/produced seeds and higher local production, all attribute to this drop in the market.

[1] K. Smith, 593 Hawkesbury Road, Winmalee NSW 2777, Australia

Collectors, however, are still prepared to pay high prices (>AUS$150) for guaranteed cultivars. Variegated plants are priced from AUS$150 to AUS$1500. It is therefore the known producers and collectors in selective sectors of the market that are profiting (K. Smith pers. comm.[1]; Smith & Henry 1998). The exact production figures and total income of the industry in Australia are unknown.

5.5.3 Future prospects

The Australian *Clivia* Society, with 140 members to date (June 2003) (K. Smith pers. comm.[1]), is still in its infancy. Collaboration with other similar organizations in the world could play an important role in marketing this highly desirable horticultural subject to the Australian public.

5.5.4 New Zealand: an emerging market

New Zealand is one of the emerging markets of the *Clivia* industry. Seed and plants are currently being produced for both the local and the Australian market, with focus on yellow-, cream-, red- and orange-flowered plants. Internal production of seed/plants and a growing interest among the local population, are currently driving the market. Seeds have been imported from countries such as Japan, South Africa and Europe, raised and sold to the local market, for several years already (K. Smith pers. comm.[1]; K. Hammett pers. comm.[2]).

On 15 March 2003 the New Zealand *Clivia* Club was formed with 69 founding members. A month and a half later, the club has grown to 100 members. The aim is to convert the club into a Society in years to come (K. Hammett pers. comm.[2]).

5.6 Americas

5.6.1 Introduction

Clivia has increased in importance on commercial markets during the last few years. *Clivia miniata* is grown from Canada to Brazil, including several states in the United

[1] K. Smith, 593 Hawkesbury Road, Winmalee NSW 2777, Australia
[2] K. Hammett, 488c Don Buck Road, Massey, Aukland 8, New Zealand

States of America (Indiana, Michigan, California, Kentucky, etc.), Mexico and Puerto Rico (K. Fargher pers. comm.[1]; K. Smith pers. comm.[2]; M.J. Morri pers. comm.[3]; Koopowitz 2000, 2002; Heilenman 2003).

5.6.2 Trends and prices in an emerging market

Clivia material is being imported from South Africa, Japan and China. Californian growers specialize in flower varieties that are more colourful than those in South Africa and China. According to Mr. M.J. Morri (*Clivia* Creation Nursery, Michigan), his customers seek *C. miniata* plants with both attractive foliage and flowers, with no special preference to either. Mr. Morri specializes in unique plants that are hard to find. His projected sales for 2004 are double those of 2003 (M.J. Morri pers. comm.[3]). *Clivia* prices are still relatively high due to the increase in demand. A mature orange-flowered variegated plant sells for US$100–150 and a yellow-flowered variegated plant for US$400–450 (M.J. Morri pers. comm.[3]).

Various other companies sell plants in the USA, with prices ranging from US$50 for mature orange-flowered plants to hundreds of US$ for mature yellow-flowered plants (Koopowitz 2002). In 1998, yellow *C. miniata* plants were on offer for US$950 by White Flower Farms (Pennsylvania, United States of America). Seed prices range from US$0.60–20.00 a seed, depending on the demand (Chapman 1999).

There are no figures available for any of the American markets to date. Considering initial tendencies of other countries that grow *Clivia*, prospects for the US market look very promising.

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[2] K. Smith, 593 Hawkesbury Road, Winmalee NSW 2777, Australia
[3] M.J. Morri, *Clivia* Creations Nursery, PO Box 192, Union City, Michigan 49094, USA

5.7 Concluding remarks

Clivia miniata is currently grown worldwide. The surveys used in compiling this chapter give some indication of the considerable size of the *Clivia* industry. The number of mature *C. miniata* hybrids produced annually is estimated to be around 6–10 million plants. Income generated by the trade in these plants, including seedling sales, is conservatively estimated to be US$200 million to US$400 million annually. The Chinese *Clivia* market is the largest in the world. However, figures obtained from USA growers indicate that there is a substantial market in the USA waiting to be developed. In Table 5.1, estimates are given for the different regions where *C. miniata* is grown on a substantial scale. Note that these are estimates calculated from a small percentage of the market and could therefore be somewhat distorted, most likely towards the conservative side.

Clivia economics is firmly based on supply and demand, the two variables that drive all trade. The possibility of high returns on investments stimulates an increase in demand. Naturally, an increase in the production is stimulated, including an increase in producers trying to get returns on their investments. The market becomes saturated and the prices decrease. In trying to counter this trend, producers develop new cultivars or hybrids. This forces the buyers to become knowledgeable about cultivars and their monetary value, resulting in an educated clientele with very specific needs and demands.

Another way of countering the drop in price of cultivars is through exports. The local clients' demand for new and improved cultivars is the driving-force behind imports. This puts pressure on local producers to have the latest cultivars on offer. Only those producers with good reputations and products are able to stay in business.

Better communication between growers stimulates the market. That is, with new hybrids becoming more rapidly known, the demand for them increases. The market is therefore driven by the latest cultivars. Older cultivars rapidly become outdated as new cultivars are produced. The serious collector is prepared to pay high prices, usually for the new and improved cultivars. What is important is that the market has become knowledgeable,

demanding breeding records, genetically identical material and improved cultivars of known origin.

Table 5.1 Horticultural important production regions for *Clivia miniata*: estimated production figures and revenues, including breeding trends and markets.

Production area	Breeding trends and markets	Estimated annual production & revenue
South Africa	Broad and variegated leaves, intra- & interspecific hybrids, flower forms and colours, pot- & bedding plant	1–5 million plants US$1–5 million
Europe	Compact, small, broad-leaved plants flowering fast, greenhouse & pot plants	>1 million plants US$8–15 million
The Far East (China, Japan, Korea, etc.)	Foliage: broad-leaved, variegated plants with symmetric fan-like appearance, greenhouse & pot plants	>2 million plants > US$200 million
Australia and New Zealand	Broad-leaved & variegated plants, intra- & interspecific hybrids, flower colour variations, pot- & bedding plants (Aust.)	<1 million plants US$1–5 million
USA	Broad-leaved & variegated plants, intra- & interspecific hybrids, flower form and colours, greenhouse & pot plants	1–5 million plants US$50–100 million

The development of a sustainable and stable industry depends on growers identifying their target markets, for example pot plant market, collectors, general public, landscapers, etc. Take Europe, for example, where *Clivia* is mainly grown as a pot plant. It is enjoyed for a season and a new plant is bought the next year. This market is limited in size, with too high a production forcing prices down and making it commercially non-viable. This markets' profitability is directly linked to quality and the markets' trends—both contributing to the sustainability of the market. In China, *Clivia* is part of the people's culture. Both serious collectors and the general public enjoy *Clivia* in China. Producers could therefore exploit both these sectors of the market.

The future of the *C. miniata* industry is very promising. There are still substantial new markets to develop such as the Americas. *Clivia* growers have not yet explored the cutflower markets of the world. This sector could be highly profitable for those who are first to venture into it—to date it is still more profitable to produce and sell seed than to produce cutflowers. Intraspecific and interspecific hybrids have yet to reach their full potential. Biotechnological manipulation of floral colour holds considerable potential. The industry is still growing and with new hybrids being produced worldwide, the future looks prosperous indeed.

5.8 Acknowledgements

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CHAPTER 6

INTRASPECIFIC CHLOROPLAST DNA POLYMORPHISMS WITH GEOGRAPHIC STRUCTURE DETECTED IN *CLIVIA MINIATA* (LINDL.) REGEL (AMARYLLIDACEAE) BY PCR-RFLP

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Intraspecific chloroplast DNA polymorphisms with geographic structure detected in *Clivia miniata* (Lindl.) Regel (Amaryllidaceae) by PCR-RFLP

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6.1 Introduction

Clivia, a small genus of horticulturally significant perennials in the tribe Haemantheae (Amaryllidaceae), consists of five species. The genus is largely confined to the Afromontane forests of South Africa and Swaziland, with *Clivia miniata* having the widest range. The distribution range of *Clivia miniata* overlaps with that of all the species, except *C. mirabilis* (Duncan, 1999; Rourke, 2002). Recently molecular evidence was used to study the phylogeny (Ran *et al.*, 2001a) and genetic relationships (Ran *et al.*, 2001b) among and within the various species of the genus. To date, however, there are no reports on any members of *Clivia* regarding the degree of interspecific variation at molecular level of natural populations.

The chloroplast genome has been extensively used during the last 25 years in various molecular analyses of plants, including phylogenetics, molecular systematics, gene flow studies, population studies and classification at higher taxonomic levels (Harris *et al.*, 1991; McCauley, 1995; Olmstead *et al.*, 1994; Palmer, 1987; Palmer *et al.*, 1988; Soltis *et al.*, 1992b). This utilisation was mainly driven by its usual uniparental inheritance, limited or no ability to undergo recombination and a different evolutionary mutation rate than nuclear DNA (Birky *et al.*, 1992; Harris *et al.*, 1991; McCauley, 1995; Palmer, 1987; Palmer *et al.*, 1988; Soltis *et al.*, 1992b; Wolfe *et al.*, 1987). Chloroplast DNA intraspecific variation is today known in various species (for reviews see Harris *et al.*, 1991; Soltis *et al.*, 1992b). Although intraspecific cpDNA is frequently observed, Soltis *et al.* (1992b) showed that intraspecific cpDNA variation is rarely very large. McCauley (1995) however, listed several large-scale population studies that revealed different levels of intraspecific cpDNA variation. In population studies, the chloroplast genome,

when used in conjunction with nuclear markers, can be used to evaluate the influences that seed and pollen dispersal have on the total gene flow (McCauley, 1995). To date, intraspecific cpDNA variations have been used in studies of genetic diversity within and among populations, introgression, domestication of crops and in the demonstration of multiple origins for polyploids and diploids (reviewed by Soltis *et al.*, 1992b). This re-confirmed the usefulness of the chloroplast genome in studies of closely related taxa and populations.

Chloroplast PCR-RFLP is a single-locus screening approach focusing on a haploid plastid genome. This method generates co-dominant data, making this approach more robust and useful in precise analyses. Although the marker system has a low to moderate overall variability, it can be readily transferred to other taxa with the development of various universal primer sets (Sunnucks, 2000). The data generated from PCR-RFLP studies can also be compared directly with other similar studies (Sunnucks, 2000).

In this paper we document for the first time intraspecific cpDNA variation in a member of the genus *Clivia*. We also demonstrate that cpDNA variation in *Clivia miniata* is geographically structured to a centre of diversity.

6.2 Materials and methods

Plant material

Leaf material of *Clivia miniata* was obtained from plants of known provenances in the Kirstenbosch National Botanical Gardens[1] or collected from nature. Material from nature was kindly donated for this study by Mr. M. Exelby[2], Mr. A. Hardinge [3] and Mr. J.T. Truter[4]. Voucher specimens of all collections are deposited in the H.G.W.J. Schweickerdt Herbarium, University of Pretoria, South Africa (*Table 1*). DNA was extracted from a total of 153 plants from 18 localities, covering the entire natural distribution range of the species in South Africa (*Figure 1*).

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DNA analysis

Total DNA was extracted for each plant using a modified method of Raeder & Broda (1985). The extraction buffer consisted of 200 mM Tris HCl pH 8, 150 mM NaCl, 25 mM EDTA pH 8, 0.5% SDS (v/w) and 1% 2-mercaptoethanol (v/v). Fresh and dried leaf disks were homogenized inside the extraction buffer for one minute, in a Bio 101 FastPrep, setting 2. The homogenized material was then cleaned using phenol and chloroform, before precipitation absolute ethanol.

DNA samples of the same concentrations, originating from a specific collection area, were pooled together and used in the subsequent PCRs (Soltis *et al.*, 1989a; Soltis *et al.*, 1989b). All restriction enzyme profiles that revealed detectable polymorphisms were reconfirmed using the original individual DNA from each DNA pool.

DNA was screened with nine universal chloroplast primer pairs (*Table 2*) (Demesure *et al.*, 1995; Dumolin-Lapègue *et al.*, 1997; Goulding *et al.*, 1996; McKinnon *et al.*, 2001; Taberlet *et al.*, 1991). PCR resided in a 25 µl reaction of 2 mM $MgCl_2$, 1x PCR buffer, 0.2 µM of each primer, 100 µM of each dNTP, 1U of Taq polymerase (Southern Cross Biotechnology) and 30 ng template DNA. PCRs were performed on Applied Biosystems GeneAmp 2700 and 9700. Thermocycling consisted of 30 cycles, of 30s at 94°C, 45s at the primer annealing temperature and at 72°C for the specific extension time of the primer (*Table 2*). All PCRs had an initial 5 min denaturing step at 94°C and a 7 min at 72°C final extension step. Touchdown PCRs were performed during the optimisation steps, in accordance to Don *et al.* (1991).

PCR products were restricted with 2U restriction enzymes for 3 hours (*Table 2*) (Sambrook *et al.*, 1989; Sambrook *et al.*, 2001). Restriction fragments were separated on 2% agarose gels using a 1x TAE buffer at a constant 2.5 V/cm for 120 min. Gels were stained with ethidium bromide and photographed under UV light. A 100 bp DNA ladder (Promega) was used as size marker.

Data analysis

Only unambiguous restriction enzyme digestions were used for data analysis and missing data were indicated. Restriction enzyme polymorphisms were scored as present or absent and used to compile a data matrix for the subsequent data analysis

(Appendix II). The polymorphism profiles generated by all the different restriction enzyme reactions were used to identify different haplotypes. The different haplotypes were scored for all the sampled individuals. Data analysis was done using the program POPGENE (Yeh *et al.*, 1997).

For sub-structuring analysis, only those samples originating from the same locality or that were geographically close (i.e. locality codes KEI, KOE and KEN, see *Table 1*), were grouped together. Localities with two or less samples were not included in this analysis. All polymorphisms were used to determine total genetic variation in *Clivia miniata*.

6.3 Results

PCR amplification and screening of pooled DNA samples

Nine primer pairs and 37 restriction enzyme reactions were used to screen 153 samples for cpDNA polymorphisms (*Table 2*). Three primer pairs and four restriction enzymes were identified as cpDNA PCR-RFLP markers useful for intraspecific studies of *Clivia miniata*. Four primer pairs, psaA/trnS, trnL/trnF, trnF/trnVr and trnfM/pbsAr gave ambiguous PCR products, with primer pairs trnC/trnD and rpoC1/trnCr not amplifying any fragments (*Table 2*). These were discarded.

Of the 37 restriction enzymes tested, only five digestions resulted in polymorphic banding patterns, i.e. *AluI*, *MspI* and *Sau3AI* (*Table 3*). They revealed a total of seven polymorphisms. Four of the polymorphisms, i.e. fragment psbA/trn2kr digested with *MspI* and fragment rpl2/trnK digested with *Sau3AI* and *Tru9I*, were restriction site mutations. The remaining mutations were assumed to be insertions or deletions in accordance to their profiles. Two point mutations were detected for the fragment amplified with psbA/trn2Kr when using *MspI*. The fragment produced by rpl2/trnK and digested with *Tru9I*, revealed a point mutation and an insertion/deletion. The identified polymorphic cpDNA PCR-RFLP markers were used to screen individual *C. miniata* samples from the various localities.

Screening of localities

A total of six haplotypes (haplotypes A–F) (*Table 3*) were identified among the 153 samples analysed. Haplotype A was found to be the most common with a frequency of

0.778, occurring throughout the whole distribution range. Haplotypes C (0.144) and haplotype F (0.0588) were less common. The remaining haplotypes (B, D and E) occurred only once.

Haplotypes C and F were found to be the sole residing haplotypes of the localities Mzamba River (MZA) and Broedershoek farm (BRO) respectively, with haplotype F restricted to only that one locality. Haplotype C was also present in one other locality, Umtamvuna Nature Reserve (UMT), where it was the foremost haplotype with a frequency of 0.615. Haplotype B was a single sample from Oribi Gorge (ORI). The haplotypes D and E occurred as single, unique entities in the localities Port St Johns (PSJ) and UMT, respectively.

Haplotype variation within localities was limited to two localities, i.e. PSJ and UMT. PSJ was represented by haplotypes A (30 samples) and D (1 sample). The locality UMT showed the highest degree of haplotype diversity, with three haplotypes (A, C and E) present.

The gene diversity value, as calculated by Nei (1973), is 0.26±0.071. Only 25% of the populations had two or more haplotypes.

6.4 Discussion

We report for the first time infraspecific cpDNA polymorphisms in a member of the genus *Clivia* and propose that the polymorphisms identified, are all concentrated in centres of plant endemism.

We found that the initial screening of the populations, using normalized pooled samples, were useful in identifying both frequent and rare polymorphisms (Soltis *et al.*, 1989a; Soltis *et al.*, 1989b). This makes it a powerful procedure for screening large populations more efficiently and cheaply, allowing one to identify rare alleles without labour intensive screening procedures. Our results from this screening step corresponds to that obtained by Soltis *et al.* (1992a). Though universal primers are readily available, we found that for *C. miniata*, not all primer pairs amplified ambiguously, making the screening of various primer pairs a necessity.

Accepted mutation mechanisms for the chloroplast genome are thought to be point mutations and insertions or deletions (Harris *et al.*, 1991; McCauley, 1995; Palmer *et al.*, 1988; Soltis *et al.*, 1992b). Various reports, in which PCR-RFLP methods were employed, indicate that the frequency of insertions/deletions is higher than point mutations (Demesure *et al.*, 1996; Desplanque *et al.*, 2000; Dumolin-Lapègue *et al.*, 1997). This pattern is not supported by the results of the present study (*Table 3*) and is similar to that obtained by Ran *et al.* (2001a) in a phylogenetic study of the genus *Clivia*. Results of the present study indicate that cpDNA PCR-RFLPs generate sufficient variation in *Clivia miniata* to warrant further studies at the species level.

McCauley (1995) reported that most cpDNA variation revealed considerable spatial structure among angiosperms. The majority of this variation was found among various populations, rather than within them. In the two larger sampled localities of the present study (PSJ & UMT), more than two haplotypes were identified. This supports the hypothesis of Soltis and co-workers (1992b) of higher variation in larger sampling numbers, but does not explain the dominance of haplotype A (frequency of 0.778) over the distribution range.

We found that the bulk of the *C. miniata* cpDNA diversity is concentrated in the Pondoland region of the Eastern Cape and southern KwaZulu-Natal Provinces, South Africa. The only exception, Broedershoek, is located in the Tugela River Basin. The Pondoland region is known for its high levels of plant diversity and endemism, hence its status as the so-called Pondoland Centre of plant endemism (Van Wyk & Smith, 2001). This region with an estimated surface area of 1 880 km^2, harbours around 1 800 vascular plants species, of which at least 120 are endemic/near-endemic to the region (Van Wyk, 1994; Van Wyk & Smith, 2001). Following the rifting and break-up of Gondwana, subsequent episodes of up-lifting and erosion—during the establishment of an effective drainage system for the Great Escarpment—exposed a particular sandstone formation in Pondoland which now acts in the manner of an edaphic island (Partridge *et al.*, 2000). The centre is characterised by rugged grass-covered sandstone plateaus, dissected by deep, narrow, more or less parallel-aligned river gorges. Isolated forest patches, covering an estimated 200 km^2, are chiefly contained within these gorges (Van Wyk, 1994; Van Wyk & Smith, 2001). *Clivia miniata* is confined to these isolated

forest 'islands'. With this degree of isolation from surroundings regions, high levels of infraspecific variation are to be expected within Pondoland, a pattern supported by our data. We propose that once the *Clivia miniata* populations were established, geneflow patterns between these different localities were restricted, allowing each population to develop more independently from each other and induce genetic bottlenecks. More investigations in this area, covering more isolated populations and individuals, are needed to verify this hypothesis.

Further development of cpDNA PCR-RFLPs and larger sampling numbers will aid in studies of the different evolutionary processes within this species. We hope that with an increase in our knowledge of the chloroplast profiles from different localities, we would be able to determine the origins of unknown commercial lines, e.g. some yellow-flowered forms of *Clivia miniata*. This would be possible due to the uniparental inheritance of chloroplasts in *C. miniata* (Koopowitz, 2002) through which we can trace the maternal line. Markers like these, in conjunction with the appropriate nuclear markers, will allow us to investigate the influences that seed and pollen dispersal have on the gene flow among and within *Clivia miniata* populations.

6.5 Acknowledgements

We are grateful to the Kirstenbosch National Botanical Garden (National Botanical Institute) and private collectors for supplying locality-specific material. Our thanks to the Mpumalanga Parks Board, Ezemvelo KZN Wildlife and The Eastern Cape Department of Environment and Tourism, for the necessary plant collecting permits. Mr. A. Grobler kindly compiled the distribution-map. Special thanks to Mr J.T. Truter for his pleasant company during collecting trips. This project was funded in part by the South African National Research Foundation.

Table 1 Localities, sample sizes, haplotypes and accession numbers.

Locality code	Locality	Number of samples	Haplotypes	Accession & collection numbers
BMM	Bearded Man Mountain[1, 2]	5	A	2096, ZH 5
BRO	Broedershoek Farm[3]	9	F	431/99
DON	Donkeni[3]	9	A	327/00
HOW	Howick Falls[4]	1	A	ZH 3
KAR	Karkloof[1, 2]	5	A	3014
KEI	Kei River Mouth[2]	1	A	3107
KOE	Koek-Koek River[2]	2	A	3108, 3284
KEN	Kentani Area[2]	1	A	3611
LEB	Lebombo Mountains[2]	1	A	3213
MBA	Mbashe[3]	14	A	524/98
NQO	Nqobara River[3]	10	A	520/98
NTO	Ntomeni Forest[3]	11	A	435/99
MZA	Mzamba River[5]	6	C	PRU91195
ORI	Oribi Gorge[2]	1	B	3619
PSJ	Port St Johns[1,3]	31	A, D	ZH1, PSJ729/96
QOR	Qora[3]	10	A	720/96
NGO	Ngoye Forest[3]	10	A	436/99
UMT	Umtamvuna Nature Reserve[3, 5]	26	A, C, E	515/98, PRU91194

Collectors: Z.H. Swanevelder[1], J.T. Truter[2], Kirstenbosch National Botanical Garden[3], M. Exelby[4], A. Hardinge[5]

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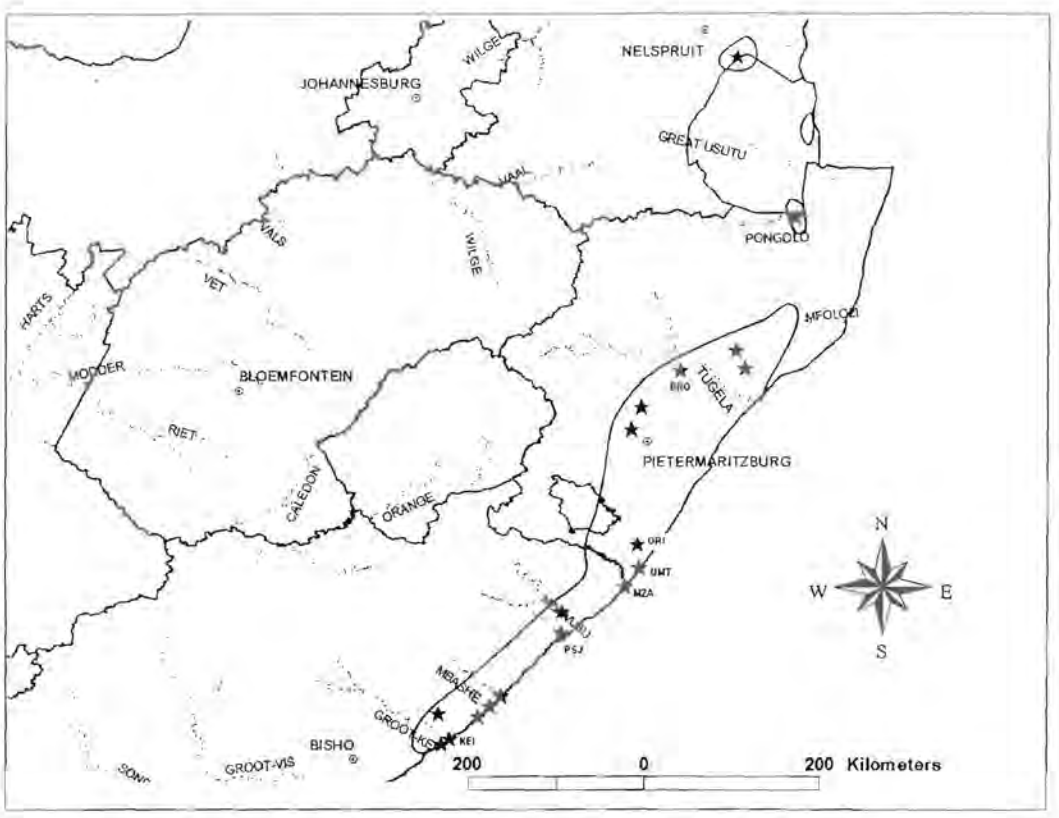

Figure 1 Map showing the distribution range of *Clivia miniata* (outline in black) and sample sites (indicated by a star). For locality abbreviations see *Table 1*.

Table 2 List of the chloroplast primers, restriction endonucleases and PCR parameters used in this study.

Primer pairs (5'→3')	T_m (°C)	Extension time (min)	Restriction endonucleases	References
psbA – agacgg tttcr gtgc trn2Kr – caacgg tagagt actcgg ctttta	57	3	AluI, AvaI, BamHI, CfoI, ClaI, EcoRI, EcoRV, MspI, PvuII, RsaI, SfuI, SmaI	1 2, 3
rpl2 – gataat ttgatt cttcgt cgcc trnK – ccgact agttcc gggttc ga	63	3	AluI, CfoI, DraI, EcoRI, EcoRV, HaeII, HindIII, MspI, PvuII, RsaI, Sau3AI, Tru9I	4 2
trnV – cgaacc gtagac cttctc gg rbcLr – gcttta gtctct gtttgt gg	62	3	AluI, AvaI, ClaI, CfoI, DraI, HaeIII, MspI, NocI, PstI, RsaI, Sau3AI, SmaI, Tru9I.	5
psaA – acttct ggttcc ggcgaa cgaa trnS – aaccac tcggcc atctct ccta	57	4	Unambiguous amplification	2
trnL– cgaaat cggtag acgcta cg trnF– atttga actggt gacacg ag	50	1	Unambiguous amplification	6
trnF– ctcgtg tcacca gttcaa at trnVr– ccgaga aggtct acggtt cg	58	4	Unambiguous amplification	5
trnfM– gaaccc gtgacc tcaaggttatg pbsAr– attcgttcgccggaaccagaagt	53-48	4	Unambiguous amplification	5
trnC– ccagtt caaatc tgggtg tc trnD– gggatt gtagtt caattg gt	59-56	4	No amplification	2
rpoC1– gcacaa attccr cttttt atrgg trnCr – cgacac ccrgat ttgaac tgg	52-46	4	No amplification	5

References: 1, McKinnon *et al.* (2001); 2, Demesure *et al.* (1995); 3, Fineschi *et al.* (2000) ; 4, Goulding *et al.* (1996); 5, Dumolin-Lapègue *et al.* (1997); 6, Taberlet *et al.* (1991)

Table 3 Description of the six haplotypes identified in *Clivia miniata*. For the haplotypes all the polymorphic fragments, including the type of mutation and size of mutated fragment, are given. Restriction enzymes producing the polymorphisms are also supplied.

Haplotype Fingerprint	PsbA/trn2Kr			rpl2/trnK		TrnV/rbcLr
	AluI 310bp insertion/ deletion	MspI Point mutation 400bp	MspI Point mutation 210bp	Sau3AI Point mutation 510bp+300bp	Tru9I Point mutation & insertion/deletion 200bp(1) / 150bp(2)	AluI 950bp insertion/ deletion
A	1	1	1	1	0	0
B	0	0	0	1	1	0
C	0	0	0	1	1	1
D	1	0	1	1	0	0
E	1	1	1	1	1	0
F	1	1	1	0	2	0
Gene Frequency (mutation 1)	0.8497	0.8431	0.8497	0.9412	0.1569	0.1447
Gene Frequency (mutation 2)					0.0588	

(1) The presence of a mutation and used to determine the gene frequencies.

(2) The shorter insertion/deletion for *Tru9I*

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CHAPTER 7

ISOLATION OF SINGLE LOCUS MICROSATELLITE-MARKERS FROM *CLIVIA MINIATA* USING A 5' ANCHORED ENRICHMENT PCR METHOD

7.1 Introduction

In the early 1980s, microsatellites were detected in eukaryotic genomes (Hamada *et al.* 1982; Tautz & Renz 1984). Initially they received little interest, but with the development of PCR, the potential of microsatellites as molecular markers became obvious in the late 1980s (Jarne & Lagoda 1996). Litt & Luty (1989) proposed that, due to the widespread distribution of these regions throughout the human genome (± 50 000 copies of the $(TG)_{10-60}$ repeat), polymorphisms associated with microsatellites could be more abundant and widespread than those of minisatellites—possibly making them very useful in linkage studies of humans. The realisation that microsatellites could be one of the most influential Mendelian markers ever discovered, soon followed (Jarne & Lagoda 1996).

7.2 Microsatellites

7.2.1 Characteristics of repetitive DNA sequences: microsatellites

Microsatellites are tandemly repeated DNA sequences, with the unit of repetition between one and eight base pairs (bp) in length (Hamada *et al.* 1982; Tautz & Renz 1984; Shriver *et al.* 1993; Valdes *et al.* 1993; Arens *et al.* 1995; Goldstein *et al.* 1995; Jarne & Lagoda 1996). They are generally extremely abundant and can occur in arrays up to 100 base pairs, but are usually 6–24 repeats in length (Weber 1990; Charlesworth *et al.* 1994; Armour *et al.* 1999). Microsatellites have also been designated simple sequences (Schlötterer & Tautz 1992), simple sequence repeats (SSRs) (Depeiges *et al.* 1995; White & Powell 1997; Botha & Venter 2000), simple repetitive DNA sequences (Strand *et al.* 1993; Jurka & Pethiyagoda 1995), sequence-tagged microsatellites sites (STMSs) (Depeiges *et al.* 1995, and references therein) and short tandem repeats (STRs) (Depeiges *et al.* 1995; Hancock 1999).

Microsatellites can be classified into three categories, namely pure/perfect, e.g. $(CA)_n$, compound e.g. $(GA)_n(CA)_n$ and interrupted e.g. $(GA)_nTTCA(GA)_n$ repeats. Any combination between these different families are possible (Weber 1990; Estoup *et al.* 1993; Jarne & Lagoda 1996; Doyle 2001, and references therein). Estoup *et al.* (1993) proposed that, due to the two repeats that can mutate, compound repeats have a higher polymorphism potential than the other repeat types. In studies comparing the

polymorphisms of the different families, interruptions were shown to stabilise the microsatellite, thereby decreasing its variability (Jarne & Lagoda 1996).

Categories are further divided into sub-families, e.g. dinucleotide-, trinucleotide- and tetranucleotide repeats (Jarne & Lagoda 1996). The densities of repeats in genomes are species-dependant, with one locus normally occurring in every 30–50 kilobases (kb). The length of these individual loci or microsatellites is normally less than 30 repetitive units. Dinucleotide repeats TA and GA, with a mean repeat of 10, are abundantly present in plants (Depeiges *et al.* 1995, and references therein; Jarne & Lagoda 1996).

Trinucleotide repeats are found in both animals and plants. They are frequently found within exons, without disrupting the reading frame, and are therefore more conserved. This lower level of polymorphism makes them less useful for population genetics (Jarne & Lagoda 1996). The trinucleotide repeats $(AAT)_n$ and $(AAG)_n$ are the most common in plants (Depeiges *et al.* 1995).

Tetranucleotide-repeats are commonly found in many higher organisms, with the repetitive units GACA and GATA the most prominent members of this family. In some genomes they are known to cluster near the centromeres. They are frequently associated with compound and/or interrupted sequences. Tetranucleotide repeats have high polymorphisms, but are currently rarely used in population studies (Jarne & Lagoda 1996). Tetranucleotides and other longer repetitive unit microsatellites appear to be superior during PCR, because they are less likely to form 'stutter bands' (additional products generated during PCR around the amplified product) (Armour *et al.* 1999).

7.2.2 Mutation process

The chance for a mutation to occur in a microsatellite, that is either the gain or loss of a repeat, is extremely high (Moxon & Wills 1999). In comparison with the mutation rate of a point mutation, that is estimated to be in the order of 10^{-9} to 10^{-10}, microsatellite mutation rates are estimated to occur every 10^{-2} to 10^{-5} events per loci per generation (Hancock 1999, and references therein). This high mutation rate also allows

microsatellites to return back to their original state—quite often within a few generations (Moxon & Wills 1999).

This length-mutation rate is high due to the mutation processes of microsatellites. It is generally believed that the main contributor to length polymorphisms in microsatellites is polymerase slippage during DNA replication (Schlötterer & Tautz 1992; Jarne & Lagoda 1996; Hancock 1999). A second model suggests that during recombination of DNA molecules, unequal crossing-over can induce length mutations (Jarne & Lagoda 1996; Eisen 1999; Hancock 1999, and references therein).

The slippage model (also known as slip-strand mispairing (SSM)) proposes that during replication DNA polymerase 'slips', causing temporary misalignment between the template and replicated strand. To continue replication, these strands need to realign. Imperfect alignment of the DNA strands (i.e. binding out-of-phase) produces unpaired ends that are filled by DNA polymerase. The SSM model proposes that after DNA polymerase 'slippage', the likelihood of misalignment increases in microsatellite regions because of the presence of repeats. For example, misalignment can form looped-out structures in the template DNA, causing a fragment that will be shorter than the template (Schlötterer & Tautz 1992; Eisen 1999; Hancock 1999).

When two homologous chromosomes are misaligned during recombination, unequal cross-over (UCO) is the result. The UCO model suggests that this process occurs at elevated rates in microsatellites, where the likelihood of misalignment increases due to the presence of the repetitive units (Eisen 1999).

Eisen (1999) supports the SSM model because of the following: unaffected microsatellite stability found in mutant organisms with defective genes that usually play important roles during recombination; the copy-number changes in microsatellites without changes in the flanking regions and thus no recombination occurs; equal stability of microsatellites in both mitotic and meiotic cells, where the UCO model proposes higher instability during meiosis; a reduction in microsatellite stability in mutants with defective genes in the

DNA replication error correction pathway; and lastly, microsatellite orientation, in correlation with the leading and lagging strands of replication, influences its stability.

Accumulation of numerous point mutations in the microsatellite region will eventually cause the degradation of the repetitive region, forming a non-repetitive sequence. An intermediate state, where the sequence is made up of repetitive regions intermixed with various levels of other motifs, is also a possibility. Patterns responsible for these degraded microsatellites have been termed cryptic simplicity, commonly found in many genomes, especially in large eukaryotic genomes (Hancock 1999).

7.2.3 Microsatellites: an integrated part of evolutionary adaptation

Evidence suggest that microsatellite sequences serve a functional role as coding or regulatory elements in eukaryotic genomes (Kashi & Soller 1999, and references therein). As regulatory sequences, they are universally found upstream of promoter regions, sometimes even conserved in relation to coding sequences. In conjunction with promoter regions, they serve as enhancers in expression constructs, with deletions in microsatellite regions having a reducing effect on the enhancing activity of the expression constructs. Typically for upstream activating sequences, microsatellites have been shown to bind to proteins, with the enhancing effect a function of the number of repetitive sequences in the specific microsatellite (Kashi & Soller 1999; Moxon & Wills 1999).

As part of coding sequences, microsatellites are also found in various proteins. Studies have also indicated that length variations in microsatellites could have a phenotypic effect on the physiology and development of an organism (Kashi & Soller 1999). These functional characteristics, together with the high degree of polymorphism normally associated with microsatellite regions, prompted the proposal that these regions could be a source of evolutionary adaptation and of quantitative genetic variation. Both are requirements for populations to replenish any genetic variation lost through either selection or genetic drift, enabling specific genes to rapidly react to evolutionary demands (Charlesworth *et al.* 1994; Kashi & Soller 1999, and references therein).

In bacteria it has been show that microsatellites are responsible for the flexibility needed by these organisms to adapt to an ever-changing environment. Part of contingency-genes, they readily switch genes on or off, thus enabling at least some bacteria in a population to survive environmental change (Moxon & Wills 1999).

The function that microsatellites appear to play in organisms can have serious repercussions for models used in population genetics, some of which generally assume that tandem arrays are non-functional and therefore neutral or slightly deleterious. This means that they consider base substitutions in sequences to be selectively neutral. However, natural selections may be against increasing array size, making the neutral assumption invalid (Charlesworth *et al.* 1994).

7.2.4 Advantages of microsatellites as molecular markers

Microsatellite markers can detect high levels of genetic variation, are co-dominant and highly informative. Allelic distributions have indicated that microsatellites are neutral markers and that they can be detected at any time during the development of an organism. They require small amounts of template DNA—an advantage of PCR amplification. Visualising differences are easy using gel electrophoresis (Jarne & Lagoda 1996) (Litt & Luty 1989; Botha & Venter 2000).

Various studies have shown that microsatellites developed for a specific species can also be applied across a wide range of taxa (Moore *et al.* 1991; Schlötterer *et al.* 1991; Pépin *et al.* 1995; Primmer *et al.* 1996; Steinkellner *et al.* 1997; White & Powell 1997; Botha & Venter 2000).

High numbers of alleles that segregate at microsatellite loci results in faster differentiation of all individuals, allowing for a small number of loci and large number of individuals to be analysed—allowing an individual to be assigned to a given group on the grounds of its genotype. This multi-allelic characteristic allows for applications in population genetics, evolutionary studies, forensics, conservation genetics and stock

management (Balding 1999; Beaumont & Bruford 1999; Cornuet *et al.* 1999; Botha & Venter 2000).

Recently developed, highly conserved chloroplast microsatellite markers provide biologists with a new tool for ecological and evolutionary studies in plants (Vendramin *et al.* 1996; Provan *et al.* 2001). The non-recombinant and uniparental mode of organelle genome inheritance makes these markers ideal for the above-mentioned studies. Primers designed in highly conserved regions of the chloroplast genome allow for easy cross species, and even cross family, amplification (Provan *et al.* 2001).

7.2.5 Disadvantages of microsatellites as molecular markers

The development of microsatellite markers is time consuming and three to four times more expensive than for other molecular markers, e.g. RAPDs and allozymes. Primers are developed for the flanking regions of a specific microsatellite sequence, with any point mutation in these primer sites probably resulting in the materialization of a null allele. Frequencies as high as 15% null alleles have been reported. These variations in the flanking regions may be as high as 25% of the loci under investigation. Statistical testing for null alleles can, however, overcome this problem by comparing allele frequencies to those expected under the Hardy-Weinberg equilibrium, keeping in mind that the heterozygotic deficiencies have no other source (Callen *et al.* 1993; Ishibashi *et al.* 1996; Jarne & Lagoda 1996; Botha & Venter 2000; Doyle 2001).

A false microsatellite polymorphism, attributed to variations in microsatellite flanking sequences (i.e. variation in sequences between the microsatellite and primer), can be a problem. Although little such variation is reported, studies are still needed to establish the severity of these false microsatellite polymorphisms. A result of this problem is that different mutations are compared, with the introduction of noise during analysis of widely separated species/populations (Jarne & Lagoda 1996).

Amplification of microsatellites is usually very simple and reliable, but 'stutter bands' produce problems during the analysis of pooled DNA samples (Armour *et al.* 1999).

Microsatellites are expensive and time consuming to develop, though once developed, running costs are relatively low for the amount of information generated by these markers (Fisher *et al.* 1996; Botha & Venter 2000).

7.2.6 Applications

To date, microsatellites have been detected in every genome analysed (Hancock 1999), ranging from studies on yeast (Strand *et al.* 1993), insects (Estoup *et al.* 1993; Estoup *et al.* 1995; Goldstein & Clark 1995), fish (Estoup *et al.* 1998; Spencer *et al.* 2000; Feldheim *et al.* 2001), birds (Primmer *et al.* 1996; Hughes & Deloach 1997; Painter *et al.* 1997), mammals (Cornall *et al.* 1991; Stallings *et al.* 1991; Buchman *et al.* 1996; Wyttenbach *et al.* 1997), including primates and humans (Weber 1990; Deka *et al.* 1995; Jurka & Pethiyagoda 1995) to plants (Depeiges *et al.* 1995; Maguire *et al.* 2000; Collevatti *et al.* 2001; Fréville *et al.* 2001).

Microsatellite markers have been used in mapping programs (Cornall *et al.* 1991; Dib *et al.* 1996), genetic disease diagnostics (Moxon & Wills 1999; Shibata 1999), evolutionary relationships (Estoup & Cornuet 1999, and references therein; Kashi & Soller 1999), population studies investigating kinship or parentage (Queller *et al.* 1993; Gaggiotti *et al.* 1999; Ritland 2000, and references therein), bottlenecks (Spencer *et al.* 2000; Garza & Williamson 2001), gene flow (migration, dispersal) (Neigel 1997) and genetic or population structure (Estoup *et al.* 1995; Slatkin 1995).

7.3 Methods for development of microsatellite markers

There are various methods for isolating microsatellites from organisms—most of them include some sort of selection procedure, such as a microsatellite-enriched library (Edwards *et al.* 1996). Selection (enrichment) procedures are usually followed to lower costs involved in the development of microsatellite markers. Karagyozov *et al.*(1993) and Edwards *et al.* (1996) both used membrane bound oligonucleotide microsatellites for hybridisation of restricted genomic DNA fragments containing microsatellites, as an enrichment procedure. Hybridised genomic DNA regions containing microsatellites were

then used in the production of an enriched microsatellite library (Karagyozov *et al.* 1993; Edwards *et al.* 1996).

Witsenboer and co-workers (1997) developed a technique, the selectively amplified microsatellite polymorphic locus (SAMPL) technique, based on the PCR-AFLP technique. This technique amplified microsatellites using universal primers designed to amplify specific microsatellite regions. Unfortunately, a very low number of polymorphic loci were obtained of which even fewer were single copies.

Hantula *et al.* (1996) developed a multi-locus method that uses random amplified microsatellites (RAMs) to generate species-specific patterns in fungi. This method utilises the variation in both the microsatellite regions that are used for the primer annealing sites. Unfortunately, mutations can occur not only in the priming sites but also as insertion or deletion elsewhere in the amplified product or as variation within the microsatellite repeat number, resulting in polymorphisms. This makes it a dominant marker technique, in other words only the presence or absence of a mutation can be scored. Hantula and co-workers did, however, indicated that microsatellites were found between the two anchored primers. Leroy *et al.* (2000) used the same method, referred to as inter-simple sequence repeat (ISSR), to generate profiles for calli originating from cauliflower. Their results showed the genetic instability of *in vitro* grown cauliflower cultures. Van der Nest *et al.* (2000) used the ISSR method as an enrichment procedure for the isolation of microsatellite regions in *Eucalyptus*.

Fisher *et al.* (1996) used 5' anchored primers, similar to those that Hantula and co-workers (1996) and Leroy and co-workers (2000) used for their profiling techniques, as a microsatellite enrichment procedure. This technique produced at least two microsatellites, used as part of the primers and also to enrich for single locus microsatellites. The development of a single locus-specific primer, used together with one of the anchored primers, allows for the amplification of a specific locus. With the development of a single unique primer, the costs are reduced (Fisher *et al.* 1996).

The highly informative nature of microsatellite markers can greatly facilitate our understanding of *Clivia miniata* populations. In this chapter, the aim was to develop microsatellite markers for *Clivia miniata*. These would then be used in future studies of this species, to investigate natural populations.

7.4 Materials and Methods

7.4.1 DNA extraction

A modified version of the extraction method of Raeder & Broda (1985) (Chapter 2) was used. Leaf material was selected to cover the entire natural distribution range of *Clivia miniata*. Voucher specimens of material were deposited at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria, South Africa, and included material collected at Umtamvuna River (ref.# PRU91194, A. Hardinge), Bearded Man Mountain (coll.# 2096, J.T. Truter) and Kei River Mouth (coll.# 3107, J.T. Truter). DNA was purified and precipitated as described in Chapter 2.

7.4.2 Isolation PCR using the 5' anchored primers

A modified version of the ISSR method used by Van der Nest *et al.* (2000) was employed to isolate single loci microsatellites from *Clivia miniata* specimens.

Standard PCR conditions were as follow: 2.5 mM $MgCl_2$, 0.2 µM of the primer, 100 µM of each dNTP, 1 U Super-Therm DNA polymerase, 10× PCR reaction buffer (Southern Cross Biotechnology (Pty) Ltd., Cape Town, South Africa) and 30 ng template DNA. The reactions were performed in 25 µl reaction volumes and on Applied Biosystems GeneAmp 2700 or 9700. Primers containing a 5' degenerate anchor were used in the PCRs, namely $NBD(CA)_7C$; $NDV(CT)_8$; $HV(GT)_5G$; $HVH(GTG)_5$; $DHB(CGA)_5$; $DBV(CAT)_5$; $DBD(CAC)_5$; $HBD(GACA)_4$, with the degenerate code as follows: N = any base; B = C, G or T; D = A, G or T; H = A, C or T; V = A, C or G.

Thermocycling consisted of 30 cycles, of 30s at 95°C, 45s at 47°C (except for $HBD(GACA)_4$ with annealing temperature of 45°C) and at 2 min at 72°C. All PCRs had an initial 2 min denaturing step at 95°C and a 10 min at 72°C final extension step.

PCR products were separated on 2% (w/v) agarose gels (Sambrook *et al.* 1989) using a 1× Tris-acetate (TAE) buffer at a constant 4 V/cm for 20 min (Sambrook *et al.* 1989). The gels were stained with ethidium bromide and visualised under UV light (Sambrook *et al.* 1989). Lambda DNA molecular weight marker III was used as size marker (Chapter 2).

7.4.3 Cloning, colony PCR and sequencing

Selected PCR reactions were purified (QIAquick PCR Purification Kit, Qiagen, GmbH, Germany) and cloned into pGEM®-T Easy vector (Promega Corporation, Madison, WI, USA) before transforming *E. coli* cells (high efficiency competent cells JM109, Promega Corporation, Madison, WI, USA). The transformed cells were grown overnight on LB/ampicillin/IPTG/X-Gal selective medium (Chapter 2) (Sambrook *et al.* 1989).

Colony PCRs were carried out on positive (white) colonies with the following reaction conditions: 2.5 mM $MgCl_2$, 0.2 µM of primer T7 (5'-TAATACGACTCACTATAGGG-3') and primer SP6 (5'-TATTTAGGTGACACTATAG-3'), 100 µM of each dNTP, 0.5 units Super-Therm DNA polymerase and 10× PCR reaction buffer (distributed by Southern Cross Biotechnology (Pty) Ltd., Cape Town, South Africa). *E. coli* colonies were used as template DNA in the reactions which were performed in 10 µl reaction volumes. Colony PCR program consisted out of an initial denaturation step of 2 min at 94°C, followed by 25 cycles of 30s at 94°C, 30s at 50°C, 90s at 72°C and a final extension time of 7 min at 72°C.

PCR fragments selected (300–1 000 bp), were sequenced with primers SP6 and T7. Sequencing reactions were performed with ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit II (Perkin-Elmer, Warrington, UK) on a ABI PRISM™ 377 automated DNA sequencer.

7.4.4 Sequence analysis and primer design

Sequences containing pure microsatellites were identified and used in the development of primer sets. PRIMER 3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) was used to design the primer sets to flank the microsatellite regions and amplify fragments of 200–350 bp in length.

7.4.5 Evaluation of microsatellites

Microsatellite PCRs were performed under the following reaction conditions: 2.5 mM $MgCl_2$, 0.2 µM of each microsatellite primer, 100 µM of each dNTP, 1 unit Super-Therm DNA polymerase and 10 × PCR reaction buffer (distributed by Southern Cross Biotechnology (Pty) Ltd., Cape Town, South Africa). Template DNA (10–20 ng) was obtained from populations shown to be genetically different, namely Oribi Gorge, Kentani area, Mzamba River, Port St Johns, Umtamvuna River, Donkeni and Broedershoek farm (Chapter 2). For the primer sets CLV2 and CLV3, 480 mM 2-pirrolidinone was added, to prevent unspecific binding of these primer sets. Reaction volumes were 25 µl.

Touchdown PCRs were used to amplify the different microsatellites (Don *et al.* 1991). The first phase of the PCR consisted of an initial 5 min-denaturing step (94°C), followed by 6 cycles of 30s at 94°C, 30s at 55°C—decreasing with 1°C every cycle and 1 min at 72°C. This phase was followed by 25 cycles of 30s at 94°C, 30s at primer set annealing temperature, 1 min at 72°C and a final extension time of 7 min at 72°C. The annealing temperatures for primer set are as follow: CLV1, 53°C; CLV4, 55°C; CLV2 and CLV3, 51°C.

Microsatellite PCR products were separated on 3% (w/v) agrose gels using a 1× TAE buffer at a constant 2.5 V/cm for 60 min (Sambrook *et al.* 1989). The gels were stained with ethidium bromide (± 0.5µg/ml) and visualised under UV light (Sambrook *et al.* 1989). A 100 bp DNA molecular marker was used as size standard (Promega Corporation, Madison, WI) (Chapter 2).

PCR products were separated on 6% PAGE gels (2% (v/v) Long Ranger® gel solution, BioWhittaker Molecular Applications, Rockland, ME) to detect single amplified microsatellite products. A 10× Tris-borate buffer (TBE) (Sambrook *et al.* 1989) was used with 10% (w/v) ammonium persulfate (APS) and 0.004% (v/v) N,N,N',N'-tetramethylethylene-diamine (TEMED) added to initiate polymerisation. The gel solution was placed under vacuum for ± 5 min, poured and allowed to set for at least 30 min. All PAGE were run in 1% TBE buffer. A pre-run was conducted, with wells washed with TBE buffer before loading comenced. A 100 bp DNA molecular marker was used as size standard (Promega Corporation, Madison, WI).

Samples (± 10 µl) were loaded into the wells with a mixture of 2 µl SYBR Green I (diluted 1:500) (Roche Diagnostics GmbH, Mannheim, Germany) and 2 µl commercial 6× loading buffer (Promega Corporation, Madison, WI). Four-hour runs were done in darkness, with SYBR Green I being light sensitive, at a constant 170 V. Gels were visualised under UV light.

7.5 Results

A total of eight 5' anchored primers were used in an attempt to enrich for microsatellites. These primers produced a total of 34 clearly distinguishable bands (Table 7.1) that were cloned to form an enriched microsatellite library. Primer NBD(CA)$_7$C did not amplify at all, with primers NDV(CT)$_8$ and HV(GT)$_5$G producing smears, even with an increase in annealing temperatures.

Table 7.1 Fragments produced by the eight 5'-anchored primers used, including the usable products and the number of microsatellites found within them.

Primer sequence	Amplified products between 300–1 000 bp	Total number of bands amplified	Microsatellites identified
NBD(CA)$_7$C	None	No amplification	-
NDV(CT)$_8$	Smear	Unknown	-
HV(GT)$_5$G	Smear	Unknown	-
HVH(GTG)$_5$	3	5	0
DHB(CGA)$_5$	6	7	1
DBV(CAT)$_5$	3	7	2
DBD(CAC)$_5$	3	5	1
HBD(GACA)$_4$	3	10	4

Degenerate code: N = any base; B = C, G or T; D = A, G or T; H = A, C or T; V = A, C or G

Only 18 of the fragments produced by the primers were found to be within the desired 300–1000 bp region. These fragments, amplified from the microsatellite-enriched library (Figure 7.1) were sequenced, generating a total of eight microsatellites (excluding the 5'-degenerate primers). Four of these microsatellites were isolated using HBD(GACA)$_4$ primer, two with DBV(CAT)$_5$ primer, one with DHB(CGA)$_5$ primer and DBD(CAC)$_5$ primer (sequences in Appendix III).

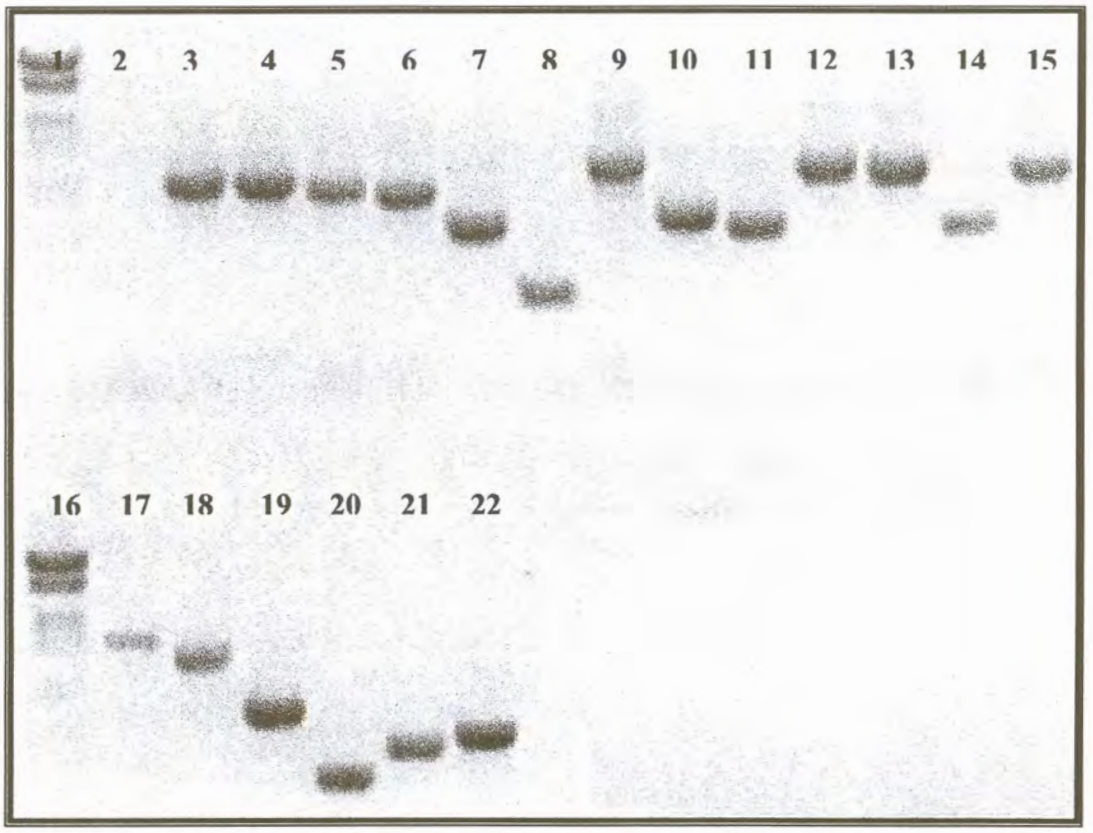

Figure 7.1 A colony PCR of cloned fragments originating from the primers HVH$(GTG)_5$ (lanes 3–7), DHB$(CGA)_5$ (lanes 8–14), DBD$(CAC)_5$ (lanes 15, 17 & 18), DBV$(CAT)_5$ (lanes 19–22). Lanes 1 and 16 are lambda III molecular weight markers. Lane 2 is the negative control of the PCR.

Three of the microsatellites were pure, with one a mononucleotide repeat (A_{11}), one dinucleotide repeat ($(GT)_9$) and one trinucleotide repeat $(TCG)_6$. Most of the remaining microsatellites were interrupted mononucleotide repeats (T_5GT_5, T_4AT_6, A_6CA_4 and $T_5GCT_4CT_3$) and one interrupted trinucleotide repeat, $(CCT)_2TCT(CCT)_2CGT$. Due to insufficient flanking sequences, only four microsatellite primer sets could be developed (Table 7.2).

Table 7.2 Primer sets designed for *Clivia miniata*, including the designed product length and primer sequences.

Primer code	Primer sequence (5'→3')	Microsatellite targeted	Designed product length
CLV1F	CAATAATGTGGCTAATGGGTTG	T_4AT_6	± 200 bp
CLV1R	CTCAAGCTATGCATCCAACG		
CLV2F	CTTGTTGTAGCTTGTAATAGC	$(GT)_9$	± 225 bp
CLV2R	CTGAACGGCAGAGGAGTTG		
CLV3F	ACAACTCCTCTGCCGTTCAG	A_{11}	± 246 bp
CLV3R	GGGTGCAGTGCACTAGTGC		
CLV4F	GCATCCCTTGCTCCTCTAC	$(CCT)_2TCT(CCT)_2CGT$	± 210 bp
CLV4R	CTCAAGCTATGCATCCAACG		

Primer sets CLV2 and CLV3 bound unspecific, resulting in numerous fragments being produced. Addition of 2-pyrrolidinone made reactions more specific, resulting in a single fragment being amplified. Unfortunately, primer set CLV3 produced a band that was approximately 800 bp longer than the designed length. Primer pair CLV4 also produced a fragment longer than the designed length. Primer pair CLV1 seems to be multi allelic, amplifying a series of fragments rather than a single one.

Evaluation of the primer sets indicated polymorphisms for the primer pairs CLV2 and CLV4 (Figure 7.2). The 6% PAGE revealed at least two length differences for primer set CLV2, a ± 230 bp fragment and a ± 250 bp fragment. Primer set CLV4 showed four fragments with length differences, two fragments larger that 1 500 bp (± 1 700 bp and ± 2 000 bp), one fragment ± 1 500 bp in length and one fragment ± 1 300 bp in length.

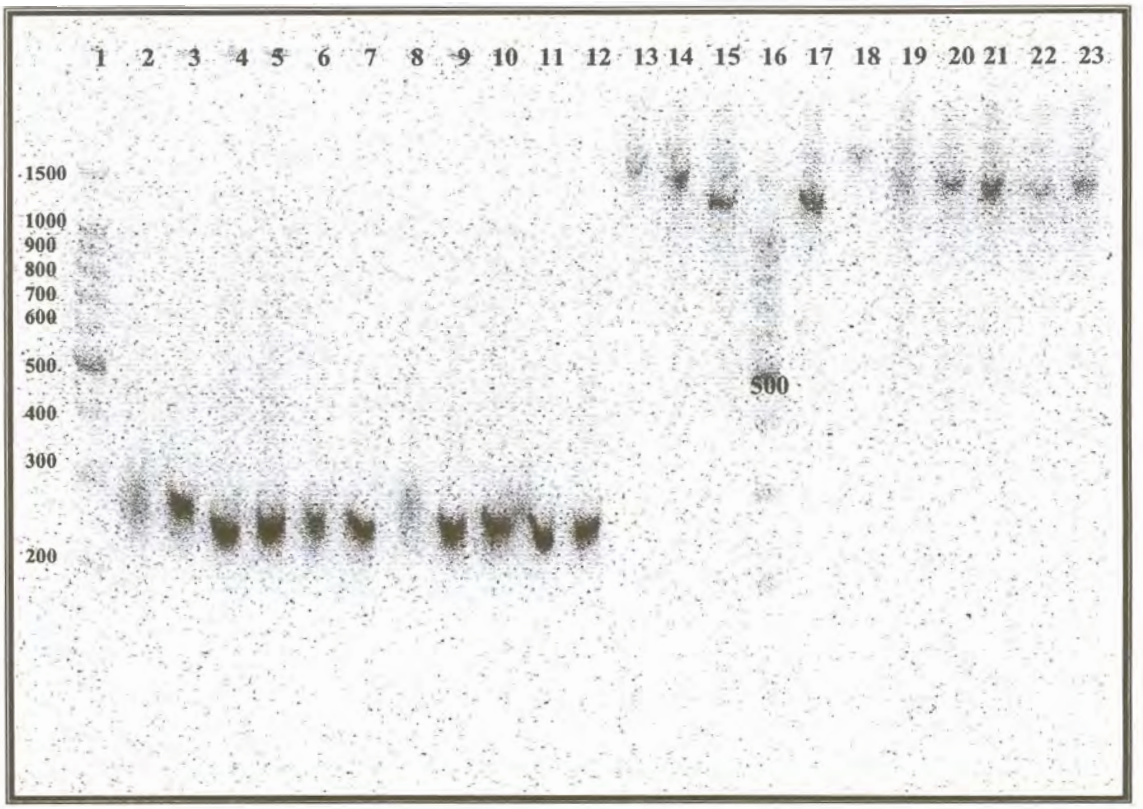

Figure 7.2 A 6% polyacrylamide gel indicating the different loci amplified using the primer sets CLV2 (lanes 2–12) and CLV4 (lanes 13–15, 17–23). Lanes 1 and 16 are lambda III molecular weight markers (Chapter 2). Lanes 2 and 13 Oribi Gorge; 3 and 14 Kentani area; 4,5, 15 and 17 Mzamba River; 6 and 18 Port St Johns; 7,8,19 and 20 Umtamvuna River area; 9,10, 21 and 22 Donkeni and 11, 12 and 23 Broedershoek (negative control not shown).

7.6 Discussion

The ISSR method for microsatellite enrichment showed that dinucleotide microsatellite regions used as primers, namely $(CA)_7$ and $(CT)_8$, appear to occur quite frequently in *Clivia miniata*, resulting in the production of smears during the enrichment PCR. An inability to produce fragments with primer NBD$(CA)_7$C during the enrichment procedure could be due to poor primer binding or the short extension period—preventing amplification of products with primer pairs that were some distance from each other. Trinucleotide primers delivered the most fragments but it was the tetranucleotide primer that produced the fragments with the most microsatellites.

The ISSR method used by Van der Nest *et al.* (2000) was successful in isolating eight microsatellites out of 18 fragments sequenced of *Clivia miniata*. This 44% microsatellite enrichment per fragment sequenced is lower than the 65% that was reported for *Eucalyptus* (Van der Nest *et al.* 2000). The number of useful flanking regions limited the design of locus specific microsatellites to only four. This lowered the total percentage microsatellites designed per number of fragments sequenced to only 22.2%, almost a third of the 65% reported by Van der Nest and co-workers.

Two primer sets, CLV 2 and CLV4, showed polymorphisms between samples from different localities. This makes them ideal for population studies of *Clivia miniata*. Though the other marker sets showed no polymorphisms between different *C. miniata* localities sampled, they might still be useful in studies of other *Clivia* species and should therefore be tested on the other members of the genus.

Though genome walking is an option to utilise the microsatellite primer sites, it would involve another cycle of PCRs, cloning, sequencing and primer design. The general belief that the mutation rate of trinucleotide microsatellites is lower than those of other families, suggests that genome walking could possibly yield fewer polymorphic microsatellites (Siebert *et al.* 1995; Jarne & Lagoda 1996). Designing a locus specific primer like Fisher *et al.* (1996), used in conjunction with the 5' anchored primer, was rejected due to this lower mutation rate of trinucleotide sequences, even though it is easier to score polymorphisms. Risks involved in attempting the isolation of these microsatellites using a genome walking technique is therefore not worth the cost.

In this study, two polymorphic microsatellite primer sets were developed using the ISSR enrichment procedure, with primer set CLV4 showing high levels of polymorphism (four different length mutations) in the 10 samples tested. This ISSR method of enrichment seemed relatively unsuccessful for *C. miniata*. It is therefore proposed that future microsatellite isolations should rather attempt another enrichment procedure, even though highly polymorphic markers have been produced with this technique.

7.7 References

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CHAPTER 8

DISCUSSION & CONCLUSIONS

8.1 Principal objectives

This study has laid the groundwork for future infraspecific and interspecific research on members of the genus *Clivia*. The main objective, to develop the necessary molecular tools for further population work within the group, was achieved by producing two different types of molecular markers, namely chloroplast PCR-RFLP markers (Chapter 6) and microsatellite markers (Chapter 7).

A second objective, to investigate the genetic diversity and structure of *Clivia miniata* populations in nature, was achieved using PCR-RFLP markers (Chapter 6). These markers were used on natural *C. miniata* populations, indicating genetic structure within and among the limited number of samples tested. This was the first infraspecific study of its kind at the population level on any member of the genus.

Other gaps in our knowledge of the genus were also addressed. A comprehensive review of the history of the different species, from the time of their discovery to the present, was given. Details regarding the taxonomy, ecology, biogeography, conservation status and horticultural significance were reviewed.

In the remainder of this chapter, the objectives of the different sections will be discussed briefly. Potential applications of the different markers will be highlighted with the focus on future population work within the group. Though the objectives of the present study were met, there are still numerous questions that need addressing in this group. These questions are briefly considered, thus highlighting the need for further work on the genus.

8.2 Chapter objectives: an overview

The different methods and materials used in this study are presented in Chapter 2. Importantly, though there are many DNA extraction protocols available, the results of Chapter 2 indicated that the altered method of Raeder & Broda (1985) was the best of all the ones tested. This method extracted large quantities of DNA, allowing small leaf samples to be used for analysis. Though fresh leaves were mainly used for extractions, this method was also successful on dried specimens, allowing DNA extractions in cases

where material is precious or limited, as in the case of herbarium specimens. The method is fast and reliable, especially when the homogenisation step is done mechanically, permitting the screening of larger sample sizes. Other methods that were optimised are given and would greatly facilitate future work on this group.

The suprageneric classifications, covered in Chapter 3, place the genus into context with other members of the Amaryllidaceae as reflected by past and currently accepted systems of hierarchical classification. This was followed by the most comprehensive overview of the infrageneric classification of the group yet, including a summary of the history and taxonomy of each member of the genus. A new taxon, namely *Clivia gardenii* var. *citrina* Swanevelder, A.E.van Wyk & Truter *ined.*, is formally proposed and a new species, *C.* 'Robust' *gardenii*, first proposed by Ran *et al.* (2001a and 2001b), is supported and discussed. An identification key and comprehensive diagnostic table (Table 3.2) addressed the practical difficulties experienced when attempting to identify the various species. The key is the first that allows identification down to the variety level.

A review of the currently known interspecific hybrids within the group, both artificial and natural, indicated possible breeding barriers among the species (Chapter 3). The designation cultivar-group, in accordance with the rules of the International Code of Nomenclature for Cultivated Plants (ICNCP), is proposed for the different inter- and intraspecific hybrids known in this genus. Current molecular evidence suggests different infrageneric phylogenies; these were used in conjunction with distribution and hybridisation information, to propose a hypothetical tree of infrageneric relationships (Figure 3.10) for the group.

In future research endeavours, the current taxonomy, including the new as yet undescribed species, *C.* 'Robust' *gardenii*, should be confirmed and used to test the phylogenetic tree proposed in the present study. With more work on the genus, the diagnostic table and identification key can be refined. Further hybridisation work is needed to confirm breeding barriers, especially between adjacent natural populations of the different species. Natural hybrids, reported by various individuals, still need

confirmation before being officially designated as such. The commercial naming of intra- and interspecific hybrids, used by the horticultural industry, seriously needs revision to ensure compliance with the stipulations of the ICNCP.

The geographical range of the different species, illustrated in Chapter 4, is the most comprehensive and accurate published to date for any member of this group. Surprisingly, all the species were shown to occur in various centres/regions of floristic endemism, supporting both newly proposed and currently accepted species on geographic isolation criteria alone. *Clivia* species that occur in more than one centre/region of endemism need further taxonomic and molecular attention, to establish whether such entities from different regions are not perhaps worthy of recognition at infraspecific level. Investigations into the possible sub-division of species are supported not only by small morphological differences between plants from different localities, but also by the highly isolated nature of *Clivia* communities—a possible diving forces for speciation in the group.

Field observations on the ecology of species made during the course of this study (Chapter 4), including descriptions of habitat, population size and current reproductive tendencies, supplement the few existing literature sources with information on these aspects. With only a few populations of *Clivia* visited and reported on in the present study, there is still considerable scope for more work on the ecology of this group. Herbarium records, most of which were collected a long time ago, need to be supplemented with new collections, reviewed and verified.

By considering the distribution ranges of the different species, in conjunction with regional climatic history, current phylogeny and literature, a hypothesis was put forward aiming to explain the current distribution pattern of members of *Clivia* (Chapter 4). This hypothesis can be refined/verified with further research into the phylogeny of the group, its distribution and relationships with taxa displaying similar disjunct patterns of distribution.

IUCN conservation statuses were compiled, taking into consideration existing and possible future threats, as well as the present distribution patterns (Chapter 4), thus highlighting the growing need for stricter conservation of the group, and the need for more research on the genus before crucial information, such as unique genetic forms within the various species, is lost forever, either as a result of human action or natural pressure.

Chapter 5 highlighted the horticultural significance of *Clivia* worldwide, especially that of *C. miniata*. In this, the first study of its kind for the genus, the history of the industry, its current breeding aims, volumes traded and estimated monetary value, are briefly reviewed to underline the considerable importance of this group of plants in international horticultural markets. *Clivia miniata* was shown to be the largest global market player of the group, with an estimated US$400 million worth of plants traded annually. The private sector produces a large portion of the value traded worldwide, with the Chinese *Clivia* market shown to be the largest. With new cultivars actively being produced, new emerging markets being explored and the high monetary value of unusual specimens, the *Clivia* industry is predicted to have a promising future. Future research into aspects covered in this chapter will identify changes in breeding tendencies and growth of the international markets—important factors for sustainable development of this horticultural industry.

In Chapter 6 the first molecular markers developed for any member in the group are reported, namely chloroplast PCR-RFLP markers. The development of these markers achieved the first objective of this study and allowed the realisation of the second objective, namely the investigation of genetic diversity of naturally occurring *C. miniata* populations. Though a limited sample size was available, biogeographic structure within and among *C. miniata* populations could still be determined. The cpDNA polymorphisms, indicated by cpDNA PCR-RFLPs within *C. miniata*, were shown to be concentrated in two centres of plant endemism, namely the Pondoland centre and the Tugela River Basin. With *C. miniata* mainly confined to 'forest-islands' within its distribution range (Chapter 4), it was not surprising to find higher levels of interspecific

variation among these often highly isolated regions. In the present study, it is proposed that once a *C. miniata* population is established, geneflow patterns between different localities become increasingly restricted, which drives local speciation within these communities. These disjunct '*Clivia*-islands' become increasingly genetically isolated from each other over time, with possible genetic bottlenecks, potentially ending in the development of new infrageneric taxa. More investigations using the developed molecular markers and a larger number of individuals from various isolated communities are needed to verify this hypothesis.

The developed cpDNA PCR-RFLP markers allow for future research into the genetics of *Clivia* populations. These markers have previously been used in genetic studies (Desplanque *et al.* 2000) to highlight the geographic structure (Soltis *et al.* 1991; Fineschi *et al.* 2000) or population structure of a species (Soltis & Soltis 1987), to estimate gene flow between and within populations (Soltis & Soltis 1987), to view evolution—using both inter- and intraspecific variation (Palmer *et al.* 1988; Soltis *et al.* 1989; Taberlet *et al.* 1991; Parducci & Szmidt 1999), to determine previous migratory routes (Soltis *et al.* 1991) and in phylogenetic studies (Palmer *et al.* 1988; Soltis *et al.* 1989), to name but a few. These markers, in conjunction with the appropriate nuclear markers (for example microsatellite markers), will allow investigations into the influences that seed and pollen dispersal have on the total gene flow among and within *Clivia miniata* populations. This chapter not only provides the necessary molecular tools for further studies, but also lays the groundwork for future research addressing the diversity of natural *Clivia* populations.

Chapter 7 describes the development of the first microsatellite markers, using the ISSR method, for a member of the genus *Clivia*. With microsatellite markers commonly being used in studies of evolutionary relationships (Estoup & Cornuet 1999; Kashi & Soller 1999), populations, with special attention to kinship/parentage (Queller *et al.* 1993; Gaggiotti *et al.* 1999; Ritland 2000), genetic bottlenecks (Spencer *et al.* 2000; Garza & Williamson 2001), gene flow due to migration/dispersal (Neigel 1997) and genetic/population structure (Estoup *et al.* 1995; Slatkin 1995), these highly informative

molecular markers can potentially greatly expand our understanding of *Clivia* populations.

As with most other scientific endeavours, this study provides more questions than answers. It nevertheless provides a basis for further research into this horticulturally important genus. With numerous still unanswered questions (such as: Which organisms serve as pollinators? How is the seed dispersed? How genetically diverse are the different species? Is there gene flow between and within populations? How extensive is gene flow?) much more research still needs to be done on this fascinating southern African group. Hopefully this dissertation will serve to facilitate such studies.

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CURRICULUM VITAE

Zacharias Hendrik Swanevelder was born in Klerksdorp, North-West Province, on 1 June 1978. In 1996 he matriculated at the Hoërskool Verwoerdburg, Gauteng, with five distinctions.

In 1997 he started his tertiary education with an enrolment at the University of South Africa (UNISA). He obtained the degree Baccalaureus Scientiae (*cum laude)* in 1999 (Botany and Biochemistry as majors). The degree Baccalaureus Scientiae Honores, specialising in Plant Physiology, was completed at the University of Pretoria (UP) in 2000 and awarded *cum laude*. During this period he received the Margaretha Mes-Medal for the best honours student in Plant Physiology. In 2000 the University of Pretoria awarded him academic colours for the honours degree.

In 2001 he enrolled for the degree Magister Scientiae in the Department of Botany, University of Pretoria. The following year results obtained from research for this degree were presented as a poster at the SAAB 2002 meeting ("The development of microsatellite markers for population studies of *Clivia miniata* (Amalyllidaceae)"). In the same year he published a paper in the *Clivia* Society's annual publication ("*Clivia* pollen: function and structure", *Clivia* 3: 13–15). A paper on the reseach for his master's degree was presented at SAAB 2003 ("Chloroplast DNA variation in *Clivia miniata* (Amaryllidaceae)"). This presentation resulted in a third place for the Van Staden Prize for best presentation by a master's student.

APPENDIX I

RECORDS USED IN THE CONSTRUCTION OF *CLIVIA* DISTRIBUTION MAPS

This appendix contains the records used in the construction of the various distribution maps used in Chapter 4. Only the reference/collection numbers of the herbaria are given in an attempt to prevent illegal collecting from these sites. Where neither the reference or collection number is known, the collector and collection date are used. The records are divided according to species and records believed to be incorrect are indicated.

Clivia caulescens R.A.Dyer

Reference/collection number, collector and collection date	Herbarium
22301, F.A. Rogers, 11/1917	C.E. Moss Herbarium
K. Balkwill, 30/04/1980	C.E. Moss Herbarium
K. Balkwill , 28/01/1981	C.E. Moss Herbarium
70, J.A. Heymans, 13/03/1993	C.E. Moss Herbarium
994, D.A. McCallum, K. Balkwill & R.A. Reddy, 28/09/2000	C.E. Moss Herbarium
2165, J.P. Rourke, 8/1999	Compton Herbarium
433/99, Live collection	Kirstenbosch NBG
ZH6, PRU92692, Z.H. Swanevelder, 10/2001	H.G.W.J. Schweickerdt Herbarium
ZH7, PRU92691, Z.H. Swanevelder, 10/2001	H.G.W.J. Schweickerdt Herbarium
ZH8, PRU92690, Z.H. Swanevelder, 10/2001	H.G.W.J. Schweickerdt Herbarium
ZH9, PRU92694, Z.H. Swanevelder, 10/2001	H.G.W.J. Schweickerdt Herbarium
ZH11, PRU92673, Z.H. Swanevelder, 10/2002	H.G.W.J. Schweickerdt Herbarium
PRU81136, F. Venter, 8/4/1986	H.G.W.J. Schweickerdt Herbarium
PRU13117, J.C. Scheepers, 2/7/1958	H.G.W.J. Schweickerdt Herbarium
PRU12123, J.C. Scheepers, 8/7/1958	H.G.W.J. Schweickerdt Herbarium
PRU45994, N. Grobbelaar, 2/12/1981	H.G.W.J. Schweickerdt Herbarium
PRU7151, G.B. Gouws, 12/1946	H.G.W.J. Schweickerdt Herbarium
3347, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3589, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3590, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3706, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3707, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3708, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
247/1970, J.P. Nel	Lowveld Herbarium
333/1980, J.P. Kluge	Lowveld Herbarium
NH16538.0, F.A. Rogers, 11/1918	Natal Herbarium
NH122108.0, N. Crouch, 7/2/1998	Natal Herbarium
PRE26511, P. van der Merwe, 10/1941	Pretoria Herbarium
1102, E.E. Galpin, 10/1890	Pretoria Herbarium
3937, G.W. Reynolds, 11/1941	Pretoria Herbarium

3937, G.W. Reynolds, 9/1942	Pretoria Herbarium
311, J.E. Repton, 9/10/1929	Pretoria Herbarium
3937, G.W. Reynolds, 25/11/1941	Pretoria Herbarium
4401, R.A. Dyer, 9/10/1941	Pretoria Herbarium
315, Van der Merwe, 7/1/1936	Pretoria Herbarium
446, M. Barnard, 29/12/1944	Pretoria Herbarium
2539, S. Killick. 19/11/1958	Pretoria Herbarium
9977, A.D.J. Meeuse, 3/3/1957	Pretoria Herbarium
3309, D.W. Codd, 12/11/1947	Pretoria Herbarium
7870, L.E.W. Codd, 24/4/1953	Pretoria Herbarium
SKF1476, J.C. Scheepers, 3/1960	Pretoria Herbarium
4862, Van Der Schijf, 12/1959	Pretoria Herbarium
87, P. Germishuizen, 9/1961	Pretoria Herbarium
407, J.C. Scheepers, 2/7/1958	Pretoria Herbarium
407, J.C. Scheepers, 2/7/1958	Pretoria Herbarium
413, J.C. Scheepers, 8/7/1958	Pretoria Herbarium
1639, D.R.J. van Vuuren, 8/11/1963	Pretoria Herbarium
1483, N.H.G. Jacobsen, 5/12/1970	Pretoria Herbarium
200, S. Muller, 24/4/1971	Pretoria Herbarium
2122, P.J. Muller, 9/12/1971	Pretoria Herbarium
TM28287, V.A. Fitzsimons, 11/1925	Pretoria Herbarium
TM13398, Pott-Leendertz, 11/1913	Pretoria Herbarium
224, N.J. van Warmelo, 11/1941	Pretoria Herbarium
1850, O.B. Werderman, 15/1/1959	Pretoria Herbarium
771, L.E. Taylor, 6/8/1935	Pretoria Herbarium
1222, S. Venter, 15/11/1967	Pretoria Herbarium
1230, S. Venter, 15/11/1967	Pretoria Herbarium
31715, R.H. Compton, 26/10/1963	Pretoria Herbarium
1813, J.P. Kluge, 26/3/1979	Pretoria Herbarium
7124, S. Venter, 16/10/1981	Pretoria Herbarium
741, M. Stalmans, 4/11/1985	Pretoria Herbarium
1230, S. Venter, 15/11/1976	Pretoria Herbarium
1222, S. Venter, 15/11/1976	Pretoria Herbarium
11147, S. Venter, 23/10/1985	Pretoria Herbarium
30687, R.H. Compton, 31/8/1961	Pretoria Herbarium
PRE37030, B. Nicholson, 5/9/1938	Pretoria Herbarium
PRE15229, R. Farquharson	Pretoria Herbarium
1102, E. Galpin, 10/1890	Selmar Schonland Herbarium
4183, H.H. Burrows, 27/11/1994	Selmar Schonland Herbarium

Clivia miniata Lindl.

Reference/collection number, collector and collection date	**Herbarium**
3354, K. Balkwill & M.J. Cadman, 04/10/1986	C.E. Moss Herbarium
814, J. Munday, 04/09/1974	C.E. Moss Herbarium
NBG 176775, J.P. Rourke, Sept. 2000	Compton Herbarium
NBG 176776, J.P Rourke, Sept 2000	Compton Herbarium
NBG167133, J.P Rourke, Aug 1999	Compton Herbarium
NBG167029, J.P Rourke, Sept 1998	Compton Herbarium
J.P. Rourke, Aug 1999	Compton Herbarium
NBG167026, J. Winter, Sept 1996	Compton Herbarium
2143, J.P. Rourke, Sept 1998	Compton Herbarium
NBG 167034, J.P. Rourke, Sept. 1998	Compton Herbarium
431/99, Kirstenbosch live collection	Kirstenbosch NBG

327/00, Kirstenbosch live collection	Kirstenbosch NBG
524/98, Kirstenbosch live collection	Kirstenbosch NBG
520/98, Kirstenbosch live collection	Kirstenbosch NBG
435/99, Kirstenbosch live collection	Kirstenbosch NBG
720/96, Kirstenbosch live collection	Kirstenbosch NBG
436/99, Kirstenbosch live collection	Kirstenbosch NBG
515/98, Kirstenbosch live collection	Kirstenbosch NBG
PSJ729/96, Kirstenbosch live collection	Kirstenbosch NBG
PRU49515, A. Abbott, 18/9/1983	H.G.W.J. Schweickerdt Herbarium
2096, PRU92682, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
ZH3, PRU92698, Z.H. Swanevelder, 10/2001	H.G.W.J. Schweickerdt Herbarium
3014, PRU92687, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3107, PRU92688, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3108, PRU92684, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3284, PRU92685, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3611, PRU92683, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3213, PRU92689, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3619, PRU92686, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
ZH1, PRU92697, Z.H. Swanevelder, 10/2001	H.G.W.J. Schweickerdt Herbarium
ZH4, PRU92699, Z.H. Swanevelder, 10/2001	H.G.W.J. Schweickerdt Herbarium
ZH5, PRU92693, Z.H. Swanevelder, 10/2001	H.G.W.J. Schweickerdt Herbarium
ZH12, PRU92676, Z.H. Swanevelder, 10/2002	H.G.W.J. Schweickerdt Herbarium
PRU 91194, A. Hardinge	H.G.W.J. Schweickerdt Herbarium
PRU 91195, A. Hardinge	H.G.W.J. Schweickerdt Herbarium
201/1971, J.P. Nel	Lowveld Herbarium
104/1999, P.J.H. Hurter	Lowveld Herbarium
PRE8724, C.R. Saunders	Pretoria Herbarium
1176, G.T. Thorncroft, 10/1922	Pretoria Herbarium
1192, G.T. Thorncroft, 6/1922	Pretoria Herbarium
2143, H.J.T. Venter, 9/1965	Pretoria Herbarium
1153, E.J. Moll, 9/1964	Pretoria Herbarium
95, P.M. Hitchins, 10/1961	Pretoria Herbarium
A1266, Thode, 9/1927	Pretoria Herbarium
24662R, W.J. Haygarth, 10/1921	Pretoria Herbarium
PRE 37084, J. Admiraal, 8/1960	Pretoria Herbarium
295, H.G. Flanagan, 9/1889	Pretoria Herbarium
8122, R.H. Marloth	Pretoria Herbarium
5746, E.E. Galpin, 9/1900	Pretoria Herbarium
2104, A. Pegler, 9/1914	Pretoria Herbarium
28678, F.A. Rogers	Pretoria Herbarium
6025, R.G. Strey, 9/1965	Pretoria Herbarium
3332(48), N.H.G. Jacobsen, 8/1967	Pretoria Herbarium
465, E.S. Kemp, 11/1976	Pretoria Herbarium
385, C. Reid, 9/1980	Pretoria Herbarium
394, J. Admiraal, 9/1960	Pretoria Herbarium
NH4413.0, J. Medley-Wood, 0/10/0000	Natal Herbarium
NH10104 .1, J. Medley-Wood, 26/9/1904	Natal Herbarium
NH10104 .2, J. Medley-Wood, 26/9/1904	Natal Herbarium
NH11354 .0, Ritchie, 0/10/1906	Natal Herbarium
NH21338 .1,13/9/1931	Natal Herbarium
NH21338 .2, H.M.L Forbes, 13/9/1931	Natal Herbarium
NH24691 .0, H.J. Thode, 0/9/1927	Natal Herbarium
NH38959 .0, J.G. Lawn, 4/9/1949	Natal Herbarium
NH53214.0, R.G. Strey, 14/9/1965	Natal Herbarium
NH54786.0, E.J. Moll, 30/9/1964	Natal Herbarium
NH66717.1, R.G. Strey, 5/1/1965	Natal Herbarium

NH66717.2, R.G. Strey, 5/1/1965	Natal Herbarium
NH77792.0, A.T.D. Abbott, 18/9/1983	Natal Herbarium
NH87091 .0, M.C. Ward, 11/9/1986	Natal Herbarium
NH103226.0, W.J. Haygarth, 0/0/1921	Natal Herbarium
NH108807.0, H.H. Rudatis, 31/8/1913	Natal Herbarium
NH108808.1, H.H. Rudatis, 31/8/1913	Natal Herbarium
NH108808.2, H.H. Rudatis, 31/8/1913	Natal Herbarium
NH108808.3, H.H. Rudatis, 31/8/1913	Natal Herbarium
NH108816.0, R.W. Adlam, 0/11/1885	Natal Herbarium
NH108850.0, W.J. Haygarth, 0/0/1921	Natal Herbarium
NH108852.1, H.J. Thode, 0/9/1913	Natal Herbarium
NH108852.2, H.J. Thode, 0/9/1913	Natal Herbarium
NH108855.2, H.J. Thode, 0/9/1895	Natal Herbarium
NH108855.1, H.J. Thode, 0/9/1895	Natal Herbarium
NH112693.0, H.J. Thode, 0/0/1913	Natal Herbarium
NH118517.0, E. Cloete, 13/9/1992	Natal Herbarium
NH118765.1, T.B. Sikhakhane, 29/9/1992	Natal Herbarium
NH118765.2, T.B. Sikhakhane, 29/9/1992	Natal Herbarium
168, H.Hutton, 1901	Selmar Schonland Herbarium
E.Bohling, 1958	Selmar Schonland Herbarium
1099, E. Cloete, 25/11/1991	Selmar Schonland Herbarium

Clivia nobilis Lindl.

Reference/collection number, collector and collection date	**Herbarium**
NBG117000	Compton Herbarium
Kirstenbosch NBI, records with population information	Compton Herbarium
Kirstenbosch NBI, records with population information	Compton Herbarium
J.P Rourke, Sept 2000	Kirstenbosch NBI
3102, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3449, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3484, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3524, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
ZH2, Z.H. Swanevelder, 10/2001	H.G.W.J. Schweickerdt Herbarium
235, E.E. Galpin, 11/1888	Pretoria Herbarium
12781, J.H.P. Acocks (Given as C. gardenii believed to be *C. nobilis*)	Pretoria Herbarium
6585, Charles, 9/1965	Pretoria Herbarium
E. Galpin, 235, 1888	Selmar Schonland Herbarium
Daly & Sole 323, Oct. 1902	Selmar Schonland Herbarium
Dr Becker, Dec. 1907	Selmar Schonland Herbarium
H. & K. Grant, 32, Sept. 1918	Selmar Schonland Herbarium
G.V. Britten, 731	Selmar Schonland Herbarium
L. Britten, Sept 1947	Selmar Schonland Herbarium
G.V. Britten	Selmar Schonland Herbarium
E. Archibald, 3691, 28/09/1951	Selmar Schonland Herbarium
G. Blackbeard, May 1957	Selmar Schonland Herbarium
A. Martin, 9047, 29/05/1956	Selmar Schonland Herbarium
A. Jacot-Guillarmod, 3507, 05/10/1958	Selmar Schonland Herbarium
L. Starke, 22/12/1966	Selmar Schonland Herbarium
R. Jibb, 1972	Selmar Schonland Herbarium
H. Burrows, 3756, 22/8/1992	Selmar Schonland Herbarium
T. Dold, 1137, 1994	Selmar Schonland Herbarium
E. Cloete, 2602, 6/03/1994	Selmar Schonland Herbarium

H. Burrows, 4115, 17/09/1994	Selmar Schonland Herbarium
K. Hammett, 17/09/1998	Selmar Schonland Herbarium
70/1987, J.P. Kluge	Lowveld Herbarium

Clivia mirabilis Rourke

Reference/collection number, collector and collection date	Herbarium
2220, J.P. Rourke (Type)	Compton Herbarium

Clivia gardenii Hook.

Reference/collection number, collector and collection date	Herbarium
NBG 167016, J.P. Rourke, 6/1999	Compton Herbarium
NBG 167017, JP Rourke, June 1999	Compton Herbarium
NBG 167018, JP Rourke, June 1999	Compton Herbarium
NBG167019, JP Rourke, June 1999	Compton Herbarium
124/99, Live collection	Kirstenbosch NBG
160/99, Live collection	Kirstenbosch NBG
437/99, Live collection	Kirstenbosch NBG
432/99, Live collection	Kirstenbosch NBG
430/99, Live collection	Kirstenbosch NBG
ZH10, PRU92672, Z.H. Swanevelder, 6/2002	H.G.W.J. Schweickerdt Herbarium
3047, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3094, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3095, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
3169, J.T. Truter	H.G.W.J. Schweickerdt Herbarium
52/2000, W.C. Froneman	Lowveld Herbarium
NH 38355 .0, J.G. Lawn, 20/3/1949	Natal Herbarium
NH 38535 .0, J.G. Lawn, 15/5/1949	Natal Herbarium
NH 38537 .0, J.G. Lawn, 2/6/1949	Natal Herbarium
NH 55179.0, R.G. Strey, 8/5/1966	Natal Herbarium
NH 79601.0, I.G. Gordon, 15/6/1982	Natal Herbarium
NH 87481.0, H. Heilgendorff, 10/7/1986	Natal Herbarium
NH 108861.3, H.J. Thode, 1915	Natal Herbarium
NH 108861.1, H.J. Thode, 1915	Natal Herbarium
NH 108861.2, H.J. Thode, 1915	Natal Herbarium
NH 112694.0, H.J. Thode, 1915	Natal Herbarium
NH 121708.0, D.J.B. Killick, 28/5/1949	Natal Herbarium
NH 123140.0, N. Crouch, 27/5/1999	Natal Herbarium
NH 2272.0, J. Medley-Wood, 0/5/1884	Natal Herbarium
NH 11002 .0, J. Medley-Wood, 0/5/1884	Natal Herbarium
9572, L.E.W. Codd, 9/1965	Pretoria Herbarium
9572, L.E.W. Codd, 10/1964	Pretoria Herbarium
2051, J.J. Gerstner, 2/6/1937	Pretoria Herbarium
5824, R.G. Strey, 5/1/1965	Pretoria Herbarium
PRE 37035, A.H. Crundall, 26/6/1959	Pretoria Herbarium
PRE 37037, I.B. Pole-Evans, 10/1953	Pretoria Herbarium
PRE 37039, S. Culverwell, 1/8/1938	Pretoria Herbarium
6628, R.G. Strey, 8/5/1966	Pretoria Herbarium
2951, E.J. Moll, 19/1/1966	Pretoria Herbarium
1989, D.J.B. Killick, 23/5/1953	Pretoria Herbarium
466, D.J.B. Killick, 28/5/1949	Pretoria Herbarium

Records of *Clivia* 'Robust' *gardenii* currently identified as *C. gardenii* or erroneously as *C. nobilis*

Reference/collection number, collector and collection date	Herbarium
NBG 170521, J.P. Rourke (*C. gardenii*)	Compton Herbarium
NBG 170519, J.P. Rourke (*C. gardenii*)	Compton Herbarium
517/98, Live collection (*C. gardenii*)	Kirstenbosch NBG
313/00, Live collection (*C. gardenii*)	Kirstenbosch NBG
314/00, Live collection (*C. gardenii*)	Kirstenbosch NBG
319/00, Live collection (*C. gardenii*)	Kirstenbosch NBG
PRU50750, A. Abbott, 29/4/1984 (*C. gardenii*)	H.G.W.J. Schweickerdt Herbarium
PRU91172, A Abbott, 5/6/2001 (*C. gardenii*)	H.G.W.J. Schweickerdt Herbarium
PRU91174, A Abbott, 5/6/2001 (*C. gardenii*)	H.G.W.J. Schweickerdt Herbarium
PRU91178, A Abbott, 5/6/2001 (*C. gardenii*)	H.G.W.J. Schweickerdt Herbarium
PRU91168, A Abbott, 5/6/2001 (*C. gardenii*)	H.G.W.J. Schweickerdt Herbarium
PRU92677, A. Hardinge, (*C. gardenii*)	H.G.W.J. Schweickerdt Herbarium
PRU92678, A. Hardinge, (*C. gardenii*)	H.G.W.J. Schweickerdt Herbarium
173/1990, P.J.H. Hurter (*C. gardenii*)	Lowveld Herbarium
NH 118513.0, E. Cloete, 25/8/1992 (*C. nobilis*)	Natal Herbarium
NH 29656 .0, F.W. Stewart, 0/6/1938, (*C. gardenii*)	Natal Herbarium
NH 29668 .0, P.S. Ngcobo, 0/6/1938 (*C. gardenii*)	Natal Herbarium
NH 80082.0, A.T.D. Abbott, 29/4/1984 (*C. gardenii*)	Natal Herbarium
NH 115950.0, B. Tarr, 14/8/1992 (*C. gardenii*)	Natal Herbarium
NH 124782.0, A.T.D. Abbott, 18/5/1995 (*C. gardenii*)	Natal Herbarium
PRE 37056, W Chiazzari, (*C. gardenii*)	Pretoria Herbarium
PRE 37058, W Chiazzari (*C. gardenii*)	Pretoria Herbarium
PRE 37025, Abernethy, 1/1930 (*C. gardenii*)	Pretoria Herbarium
13407, W. Tyson, 8/1916 (*C. nobilis*)	Pretoria Herbarium
TM 171 24, W. Tyson, 9/1916 (*C. nobilis*)	Pretoria Herbarium
UM/1/A, G Frazer, (*C. gardenii*)	Pretoria Herbarium
1639, A.E. van Wyk, 7/1976 (*C. gardenii*)	Pretoria Herbarium
885, S. Venter, 7/1976 (*C. gardenii*)	Pretoria Herbarium
3864, S. Venter, 7/1976 (*C. gardenii*)	Pretoria Herbarium
124, Vorste Venter, 8/1976 (*C. gardenii*)	Pretoria Herbarium

We would like to thank the following people and herbaria for participating:

C.E. Moss Herbarium (J), University of the Witwatersrand – R. Reddy
Compton Herbarium (NBG), National Botanical Insitute - visited by Z.H. Swanevelder
Herbarium (GLOW), Lowveld National Botanical Garden – J. Hurter
H.G.W.J. Schweickerdt Herbarium (PRU), Univeristy of Pretoria – M. Nel and E. van Wyk
Natal Herbarium (NH), National Botanical Institute - H. Aboobaker, PRECIS and printout
National Herbarium (PRE), National Botanical Institute, Pretoria - R. Archer and PRECIS printout
Selmar Schonland Herbarium (GRA), Rhodes University Botany Dept. – T. Dold

APPENDIX II

RFLP DATA MATRIX

Table AII contains all those fragments that were polymorphic after digestion with different restriction enzymes. PCR products and restriction enzymes with no visible polymorphisms are not given here, but in Table 2, Chapter 6.

Table A RFLP data matrix: chloroplast fragments analysed that showed polymorphisms after digestion with various restriction enzymes.

Sample code	PsbA–trn2Kr			rpl2–trnK			rbcL – trnV	Haplotype	Locality[1]
	AluI[2]	*MspI*[3]	*MspI*[3]	*Sau*3AI[3]	*Tru*9I[3]	*Tru*9I[2]	*AluI*[2]		
	300 bp insertion (1)	400 bp (1)	210 bp (1)	Fragmented 510+300 bp (1)	Fragmented 200+150 bp (1)	200 bp (1) 150 bp (2)	950 bp (1)		
M6	1	1	1	1	0	0	0	A	BMM
M44	1	1	1	1	0	0	0	A	BMM
M45	1	1	1	1	0	0	0	A	BMM
M46	1	1	1	1	0	0	0	A	BMM
M47	1	1	1	1	0	0	0	A	BMM
M146	1	1	1	0	1	2	0	F	BRO
M147	1	1	1	0	1	2	0	F	BRO
M148	1	1	1	0	1	2	0	F	BRO
M149	1	1	1	0	1	2	0	F	BRO
M150	1	1	1	0	1	2	0	F	BRO
M151	1	1	1	0	1	2	0	F	BRO
M152	1	1	1	0	1	2	0	F	BRO
M153	1	1	1	0	1	2	0	F	BRO
M154	1	1	1	0	1	2	0	F	BRO
M92	1	1	1	1	0	0	0	A	DON
M93	1	1	1	1	0	0	0	A	DON
M94	1	1	1	1	0	0	0	A	DON
M95	1	1	1	1	0	0	0	A	DON
M96	1	1	1	1	0	0	0	A	DON
M97	1	1	1	1	0	0	0	A	DON
M98	1	1	1	1	0	0	0	A	DON
M99	1	1	1	1	0	0	0	A	DON
M100	1	1	1	1	0	0	0	A	DON

Sample code	PsbA–trn2Kr			rpl2–trnK			rbcL – trnV	Haplotype	Locality[1]
	*Alu*I[2] 300 bp insertion (1)	*Msp*I[3] 400 bp (1)	*Msp*I[3] 210 bp (1)	*Sau*3AI[3] Fragmented 510+300 bp (1)	*Tru*9I[3] Fragmented 200+150 bp (1)	*Tru*9I[2] 200 bp (1) 150 bp (2)	*Alu*I[2] 950 bp (1)		
M35	1	1	1	1	0	0	0	A	KAR
M37	1	1	1	1	0	0	0	A	KAR[4]
M38	1	1	1	1	0	0	0	A	KAR
M39	1	1	1	1	0	0	0	A	KAR
M40	1	1	1	1	0	0	0	A	KAR
M8	1	1	1	1	0	0	0	A	KAR
M1	1	1	1	1	0	0	0	A	KOE
M2	1	1	1	1	0	0	0	A	KEI
M3	0	0	0	1	1	1	0	B	ORI
M4	1	1	1	1	0	0	0	A	KEN
M5	1	1	1	1	0	0	0	A	KOE
M7	1	1	1	1	0	0	0	A	LEB
M121	1	1	1	1	0	0	0	A	MBA
M122	1	1	1	1	0	0	0	A	MBA
M123	1	1	1	1	0	0	0	A	MBA
M124	1	1	1	1	0	0	0	A	MBA
M125	1	1	1	1	0	0	0	A	MBA
M126	1	1	1	1	0	0	0	A	MBA
M127	1	1	1	1	0	0	0	A	MBA
M128	1	1	1	1	0	0	0	A	MBA
M129	1	1	1	1	0	0	0	A	MBA
M130	1	1	1	1	0	0	0	A	MBA
M131	1	1	1	1	0	0	0	A	MBA
M132	1	1	1	1	0	0	0	A	MBA
M133	1	1	1	1	0	0	0	A	MBA
M134	1	1	1	1	0	0	0	A	MBA
M111	1	1	1	1	0	0	0	A	NQO
M112	1	1	1	1	0	0	0	A	NQO
M113	1	1	1	1	0	0	0	A	NQO
M114	1	1	1	1	0	0	0	A	NQO
M115	1	1	1	1	0	0	0	A	NQO
M116	1	1	1	1	0	0	0	A	NQO
M117	1	1	1	1	0	0	0	A	NQO
M118	1	1	1	1	0	0	0	A	NQO
M119	1	1	1	1	0	0	0	A	NQO
M120	1	1	1	1	0	0	0	A	NQO
M135	1	1	1	1	0	0	0	A	NTO
M136	1	1	1	1	0	0	0	A	NTO
M137	1	1	1	1	0	0	0	A	NTO
M138	1	1	1	1	0	0	0	A	NTO
M139	1	1	1	1	0	0	0	A	NTO

Sample code	PsbA–trn2Kr			rpl2–trnK			rbcL – trnV	Haplotype	Locality[1]
	*Alu*I[2]	*Msp*I[3]	*Msp*I[3]	*Sau*3AI[3]	*Tru*9I[3]	*Tru*9I[2]	*Alu*I[2]		
	300 bp insertion (1)	400 bp (1)	210 bp (1)	Fragmented 510+300 bp (1)	Fragmented 200+150 bp (1)	200 bp (1) 150 bp (2)	950 bp (1)		
M140	1	1	1	1	0	0	0	A	NTO
M141	1	1	1	1	0	0	0	A	NTO
M142	1	1	1	1	0	0	0	A	NTO
M143	1	1	1	1	0	0	0	A	NTO
M144	1	1	1	1	0	0	0	A	NTO
M145	1	1	1	1	0	0	0	A	NTO
M14	0	0	0	1	1	1	1	C	MZA
M15	0	0	0	1	1	1	1	C	MZA
M16	0	0	0	1	1	1	1	C	MZA
M17	0	0	0	1	1	1	1	C	MZA
M18	0	0	0	1	1	1	1	C	MZA
M19	0	0	0	1	1	1	1	C	MZA
M20	1	1	1	1	0	0	0	A	PSJ
M21	1	1	1	1	0	0	0	A	PSJ
M22	1	1	1	1	0	0	0	A	PSJ
M23	1	1	1	1	0	0	0	A	PSJ
M24	1	1	1	1	0	0	0	A	PSJ
M25	1	1	1	1	0	0	0	A	PSJ
M26	1	1	1	1	0	0	0	A	PSJ
M27	1	1	1	1	0	0	0	A	PSJ
M28	1	1	1	1	0	0	0	A	PSJ
M29	1	1	1	1	0	0	0	A	PSJ
M30	1	1	1	1	0	0	0	A	PSJ
M31	1	1	1	1	0	0	0	A	PSJ
M32	1	1	1	1	0	0	0	A	PSJ
M34	1	1	1	1	0	0	0	A	PSJ
M36	1	0	1	1	0	0	0	D	PSJ
M41	1	1	1	1	0	0	0	A	PSJ
M42	1	1	1	1	0	0	0	A	PSJ
M43	1	1	1	1	0	0	0	A	PSJ
M48	1	1	1	1	0	0	0	A	PSJ
M49	1	1	1	1	0	0	0	A	PSJ
M50	1	1	1	1	0	0	0	A	PSJ
M51	1	1	1	1	0	0	0	A	PSJ
M52	1	1	1	1	0	0	0	A	PSJ
M53	1	1	1	1	0	0	0	A	PSJ
M54	1	1	1	1	0	0	0	A	PSJ
M55	1	1	1	1	0	0	0	A	PSJ
M56	1	1	1	1	0	0	0	A	PSJ
M57	1	1	1	1	0	0	0	A	PSJ
M58	1	1	1	1	0	0	0	A	PSJ

Sample code	PsbA–trn2Kr			rpl2–trnK			rbcL – trnV	Haplotype	Locality[1]
	*Alu*I[2] 300 bp insertion (1)	*Msp*I[3] 400 bp (1)	*Msp*I[3] 210 bp (1)	*Sau*3AI[3] Fragmented 510+300 bp (1)	*Tru*9I[3] Fragmented 200+150 bp (1)	*Tru*9I[2] 200 bp (1) 150 bp (2)	*Alu*I[2] 950 bp (1)		
M59	1	1	1	1	0	0	0	A	PSJ
M60	1	1	1	1	0	0	0	A	PSJ
M101	1	1	1	1	0	0	0	A	QOR
M102	1	1	1	1	0	0	0	A	QOR
M103	1	1	1	1	0	0	0	A	QOR
M104	1	1	1	1	0	0	0	A	QOR
M105	1	1	1	1	0	0	0	A	QOR
M106	1	1	1	1	0	0	0	A	QOR
M107	1	1	1	1	0	0	0	A	QOR
M108	1	1	1	1	0	0	0	A	QOR
M109	1	1	1	1	0	0	0	A	QOR
M110	1	1	1	1	0	0	0	A	QOR
M61	1	1	1	1	0	0	0	A	NGO
M62	1	1	1	1	0	0	0	A	NGO
M63	1	1	1	1	0	0	0	A	NGO
M64	1	1	1	1	0	0	0	A	NGO
M65	1	1	1	1	0	0	0	A	NGO
M66	1	1	1	1	0	0	0	A	NGO
M67	1	1	1	1	0	0	0	A	NGO
M68	1	1	1	1	0	0	0	A	NGO
M69	1	1	1	1	0	0	0	A	NGO
M70	1	1	1	1	0	0	0	A	NGO
M71	1	1	1	1	0	0	0	A	UMT
M72	1	1	1	1	0	0	0	A	UMT
M73	0	0	0	1	1	1	1	C	UMT
M74	0	0	0	1	1	1	1	C	UMT
M75	0	0	0	1	1	1	1	C	UMT
M76	1	1	1	1	0	0	0	A	UMT
M77	1	1	1	1	0	0	0	A	UMT
M78	0	0	0	1	1	1	1	C	UMT
M79	1	1	1	1	1	1	0	E	UMT
M80	0	0	0	1	1	1	1	C	UMT
M81	1	1	1	1	0	0	0	A	UMT
M82	0	0	0	1	1	1	1	C	UMT
M83	1	1	1	1	0	0	0	A	UMT
M84	0	0	0	1	1	1	1	C	UMT
M85	1	1	1	1	0	0	0	A	UMT
M86	1	1	1	1	0	0	0	A	UMT
M87	0	0	0	1	1	1	1	C	UMT
M88	0	0	0	1	1	1	1	C	UMT
M89	0	0	0	1	1	1	1	C	UMT

Sample code	PsbA–trn2Kr				rpl2–trnK		rbcL – trnV	Haplotype	Locality[1]
	*Alu*I[2]	*Msp*I[3]	*Msp*I[3]	*Sau*3AI[3]	*Tru*9I[3]	*Tru*9I[2]	*Alu*I[2]		
	300 bp insertion (1)	400 bp (1)	210 bp (1)	Fragmented 510+300 bp (1)	Fragmented 200+150 bp (1)	200 bp (1) 150 bp (2)	950 bp (1)		
M90	0	0	0	1	1	1	1	C	UMT
M91	1	1	1	1	0	0	0	A	UMT
M9	0	0	0	1	1	1	1	C	UMT
M10	0	0	0	1	1	1	1	C	UMT
M11	0	0	0	1	1	1	1	C	UMT
M12	0	0	0	1	1	1	1	C	UMT
M13	0	0	0	1	1	1	1	C	UMT

[1] Locality abbreviations: BMM–Bearded Man Mountain, BRO–Broedershoek Farm, DON–Donkeni, HOW–Howick Falls, KAR–Karkloof, KEI–Kei River Mouth, KOE–Koek-Koek River, KEN–Kentani Area, LEB–Lebombo Mountains, MBA–Mbashe River, NQO–Nqobara River, NTO–Ntomeni Forest, MZA–Mzamba River, ORI–Oribi Gorge, PSJ–Port St Johns, QOR–Qora, NGO–Ngoye Forest, UMT–Umtamvuna Nature Reserve.

[2] Insertion/deletion mutation.

[3] Point mutation.

[4] Sample from Howick falls near Karkloof.

APPENDIX III

MICROSATELLITE SEQUENCE DATA

1) Sequences used for the development of microsatellites markers

Microsatellite CLV1 isolated with primer DBV(CAT)$_5$

NNCCACGTCCCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGGG
CATCATCTTCATCATCATCATCATNTTCTTCTTCTTCTTCGATTTACTATTCTA
ATGAGTTTTTTCGCTCGAATTACGATCGAATGGTGAAGGATTTCAAGGCTTTC
ATTTACCCAGATGGGGATCCGAATACTTATTACCAGACGCCGAGGAAATTGA
CGGGGAAGTACTCGAGCGAGGGCTACTTCTTCCAGAACATTCGAGAAAGCGG
GTTTCCGACCCAGGATCCGGATCAGGCCGATCTCTTTTTTGTACCGATATCGT
GCCATAAGATGAGAGGAAAGGTGAGATTTTGTTGGGTTCTGTCTGTTGATATT
TGATCCGATGGTAATAATGTGAATTTACTCATTTACTATAAGAATGTGCGTAT
ACTGAAAGCTGTTTGTCTATAAAATGATGTGTAGATCTAAATGTTATTGTTCA
TTTA***CAATAATGTGGCTAATGGGTTG***TTTAAGTAGCATATTAGTCAGATAAAA
GCCTTGGAAATGTGATTTTTTTATGTTTGGTTGAATACTTTCTGTTGATATTGG
ATTTTTATTTTTTGAAATTTGAATCACTAGTGAATTCGCCGGCCGCCTGCAGG
TCGACCATATGGGAGAGCTCCCAACG***CCGTTGGATGCATAGCTTGAG***TATTCT
ATAGTGTCCCCCCCAAAA

Primers are indicated by arrows and microsatellite by the box.

Microsatellite CLV4 isolated with primer DBV(CAT)$_5$

CCTTTAAGCCTTTCNACGTCCCATGCTCCCGGCCGCCATGGCGGCCGCGGGAA
TTCGATTGGCCATCATCATCATCATCATCATCCTTNATCTTCACGAGACA***TGC***
ATCCCTTGCTCCTCTACTCCTTCCAACCCCCCATAGGCAGCAATCCTCGAGTC
CCCTACTTCAGCCTCCTTCTCCTCCTCGCTTTTTTAATGTATATTACCTTCTC
TTTGTTCCTTACTCAGGACTCATCAATCACTAGTGAATTCGCGGCCGCCTGCA
GGTCGACCATATGGGAGAGCTCCCAACG***CGTTGGATGCATAGCTTGAG***TATTC
TATAGTGTCACCTAAATAANCNCCC

Primers are indicated by arrows and microsatellite by the box.

Microsatellites CLV2 and CLV3 isolated with primer HBD(GACA)$_4$

CTAAGTCCCTTTNCCNACGNCCCATGCTCCNTGCCGCCATGGCGGCCGCGGGA
ATTCGATTCTTGGACAGACAGACAGACAGAGANATGTNGATCGCGATGGGACC
TGCTAATATAGTTTAAATTTTTGTTT***TTGCAAAAACTTGTTGTAGCTTGT***AATAG
CTAGAATAATTTA**GTGGTGTGTGTGTGTGTGTGCGT**ATGCCCCTCTGGCTCC
CTCCACAATGGAAGCTACGAGGTCAGTCGTTTTTGCTTTTCTTTCTGTTTCCTAT
TTTTGTTCTGTGTGTGAGAAAGGTCCAAAATGACAGCTTAGCTGTCGTTCTTAN
ACTCTCTCTTGATGAGAACGA***CAACTCCTCTGCCGTTCAGA***GAACAAATTCTTT
TTTCAGGAAAAATTCTCTGAGCGACAGGTTGCCTGCCGCTCAGAGAGCTTTCTT
AGACAAAGCAACACCCTGCATGCCGCTCTAAAGATTTGAGCTCTTTGCACCAA
AACTCTCATTTTC**AAAAAAAAAAAT**T***GCTCTTTGTTAGGCGCTCTTT***ACGNCCT
ATATNCTACATCTGGAAGATACACCTCCACCTTGCACTAGTGCACTGCACCCAT
ATGGACATACNCTTAATCATTTGCNCCNCCATCTCATAGATCTAGGAAACCAAT
CCTTTATTAAAATTGGCGCTTGGGGGGTTCTGCTTATCAGTTAACCCCACCTCC
GAGTTCGGGTTAAAAATACCCTAAAGCGGNGGNCG

Primers are indicated by arrows and microsatellites by the boxes.

Microsatellites identified, but no primers designed

Sequence produced by primer DHB(CGA)$_5$

CGACGACGACGATTGGGATCTCAATGACGACAACGACGACGAAAATTGGGA
TCTTGATGACGACAATTCGAAAACCCAAGAAAATTTGGAGGATGATGAGGT
GGACAAAGAGCCGCAGCGGGCCTTACCACCTCGCAGCGCCCGAGGCCGTCG
ATTTGTGATAAACGAAGATAGTGATTTTGAGAACAATTCATTTGGGATCGAA
GAAGAGGACGATGATGAAGAAGAGGAATTTGGGATTGAAGAGAATAAGGGG
AATGGTAATGGGGGATATGAGATTGATGTTGTTGGGAAGGCGTTGAGGAAAT
GTGCAAAGATATCAGCAGAGTTGAGGAAGGAGCTATATGGGTCGGCTACGGT
TTTGGAAGATAGGTACGCTGAAGTGGAGG**TCTCGTCGTCGTCGTCGTCG**AA
A

AATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCC
CAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCCT

The box indicates a microsatellite.

Sequence produced by primer HBD(GACA)$_4$

CTAAGTCCCTTTNCCNACGNCCCATGCTCCNTGCCGCCATGGCGGCCGCGGG
AATTCGATTCTTGGACAGACAGACAGACAGAGANATGTNGATCGCGATGGGA
CCTGCTAATATAGTTTAAA**TTTTTGTTTTT**GCAAAAACTTGTTGTAGCTTGTA
ATAGCTAGAATAATTTA*GTGGTGTGTGTGTGTGTGTGCGT*ATGCCCCTCTGGCT
CCCTCCACAATGGAAGCTACGAGGTCAGTCG**TTTTTGCTTTTCTTT**CTGTTT
CCTATTTTTGTTCTGTGTGTGAGAAAGGTCCAAAATGACAGCTTAGCTGTCGT
TCTTANACTCTCTCTTGATGAGAACGACAACTCCTCTGCCGTTCAGAGAACAA
ATTCTTTTTTCAGGAAAAATTCTCTGAGCGACAGGTTGCCTGCCGCTCAGAGA
GCTTTCTTAGACAAAGCAACACCCTGCATGCCGCTCTAAAGATTTGAGCTCTT
TGCACCAAAACTCTCATTTTC**AAAAAAAAAAA**TTGCTCTTTGTTAGGCGCTCT
TTACGNCCTATATNCTACATCTGGAAGATACACCTCCACCTTGCACTAGTGCA
CTGCACCCATATGGACATACNCTTAATCATTTGCNCCNCCATCTCATAGATCT
AGGAAACCAATCCTTTATTAAAATTGGCGCTTGGGGGGTTCTGCTTATCAGTT
AACCCCACCTCCGAGTTCGGGTTAAAAATACCCTAAAGCGGNGGNCG

The boxes indicate Microsatellites.

Sequence produced by primer DBD(CAC)$_5$

TTNCGTTTGTTCCCNTTCGCATGCTCCCGGCCGCCTGGNNGNNCACNATATGG
GANNGGTGCCNNCGCGTNGGNTGCCTNNCTATGAGTATTCTATAGNGTCACC
TNAATATCCCAAGCTTGCTTCAACTCAGGCAACCTAAATGACCTCTGCACCTG
GAAAATGAACTCCTGAATCCTCACACCTAGTTGTGTACCCCCTTTCTAGCAAC
ATGGAATGTAGACACAGTTGTACATACAAGCTTGTCATGGCCAGGGGAACAT
GTACTCAGTGCCAGAGTCTGAAATTGAACCCCCATATGATAAAGAATCCAAT
CTGAGCAAAAAAACACCCCAAACCTCCAAGCGAATCCCAAACCCCAATAAA

GCATGTAACTCCTATTTAGAATTTAGGAATGAGCTTGCAGCCATTTGCCTGCC
TTCTCATTTGTTCCTCAACATTTCATTGCCAAAAGGTAATTGATAACTTTTCTA
ATAATAAAATCCTAAAGCAAACCCACAAAATTCATAGAAGAATTTGCTCAAA
CACTATCTAGGCAACTCATTTATTTATCAAATTATTCCCAAAAAACAAAACCN
ATTGGGTTTCTTCTACATAATCAAAACAAACCCATCAAGTCTCTCATAGAAAT
NTACTCAAGCCTAATAAACCAACTCATTTCTTCATCAAATTAGACAACAATTT
TTTACTCAAGAACTAATAAACCATCTCATTTTCNAATACATACCTTT

The box indicates a microsatellite.

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