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

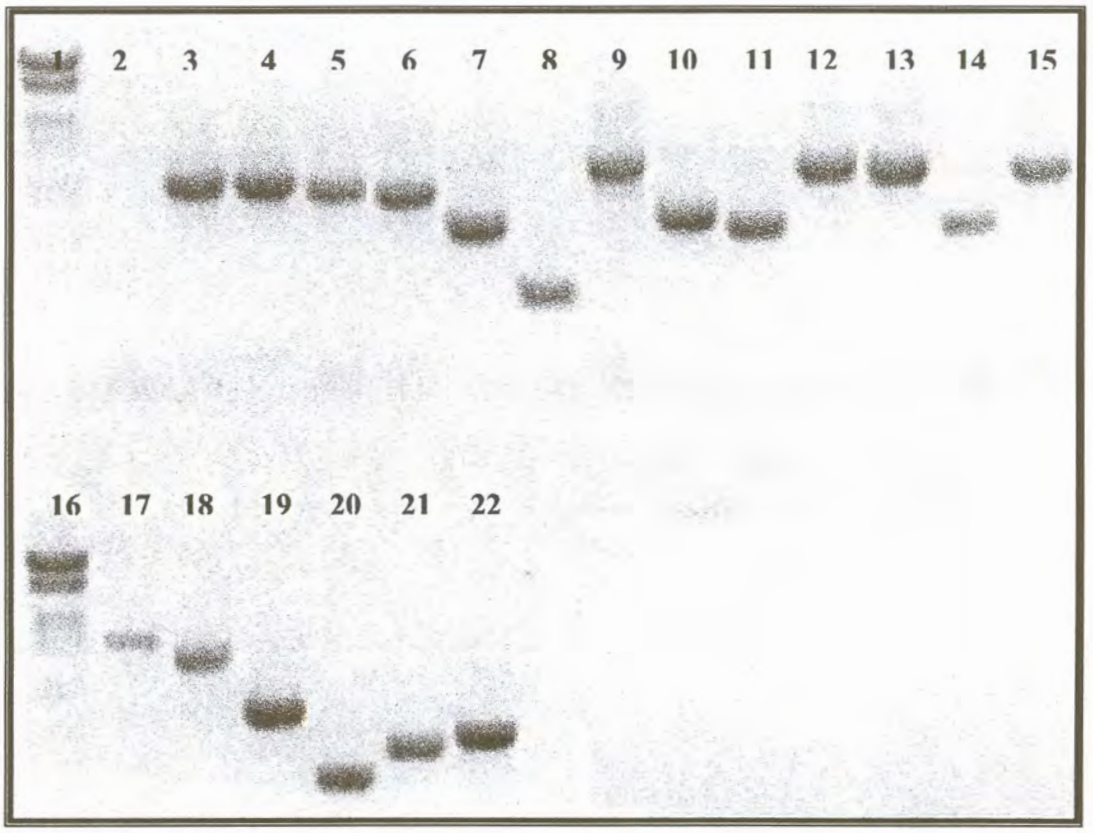

Figure 7.1 A colony PCR of cloned fragments originating from the primers HVH$(GTG)_5$ (lanes 3–7), DHB$(CGA)_5$ (lanes 8–14), DBD$(CAC)_5$ (lanes 15, 17 & 18), DBV$(CAT)_5$ (lanes 19–22). Lanes 1 and 16 are lambda III molecular weight markers. Lane 2 is the negative control of the PCR.

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).

[1] J.W. de Kock, Treasurer: *Clivia* Society and Northern *Clivia* Club, PO Box 38539, Garsfontein 0042, South Africa

[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

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

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

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]).

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

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.

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

This chapter has been presented in the format followed by the journal *Molecular Ecology*.

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

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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

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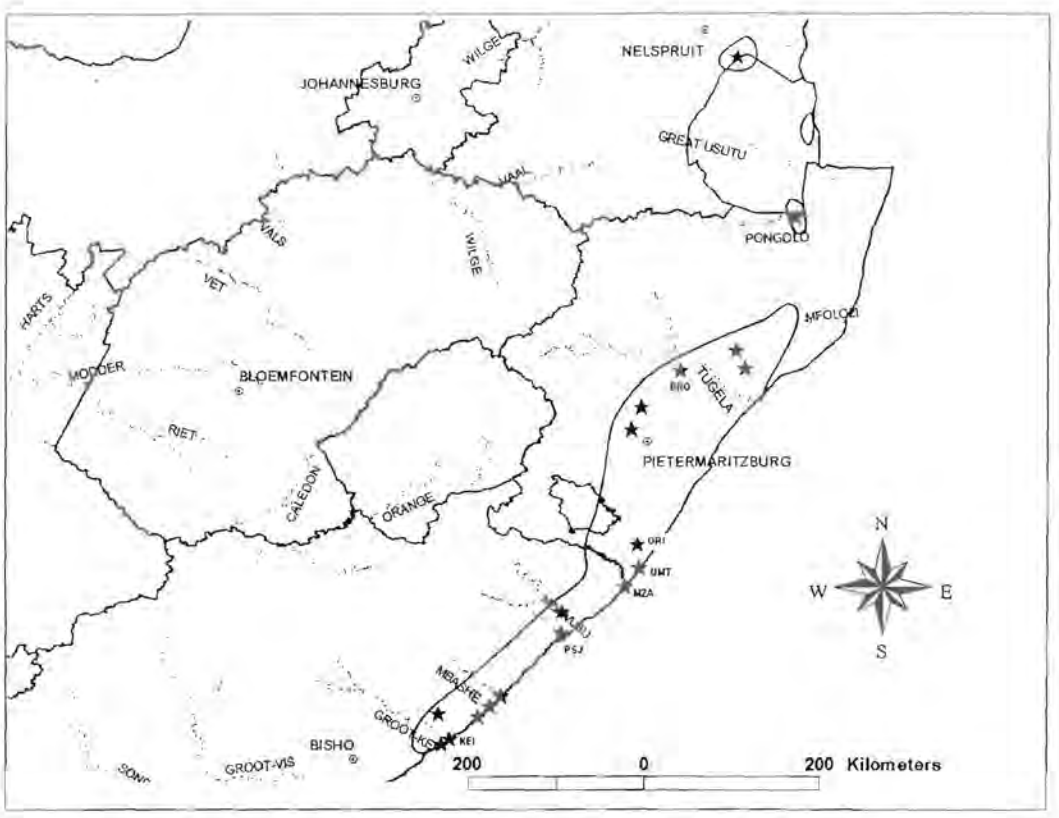

Figure 1 Map showing the distribution range of *Clivia miniata* (outline in black) and sample sites (indicated by a star). For locality abbreviations see *Table 1*.

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).

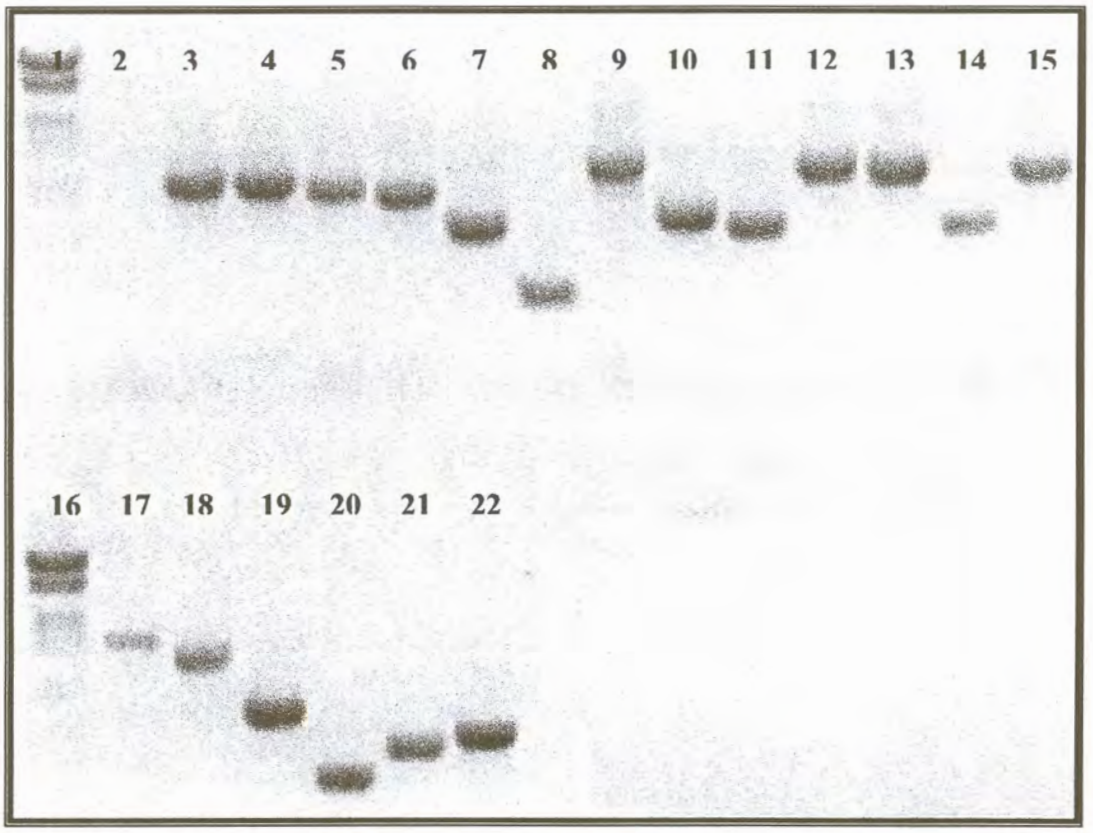

Figure 7.1 A colony PCR of cloned fragments originating from the primers HVH$(GTG)_5$ (lanes 3–7), DHB$(CGA)_5$ (lanes 8–14), DBD$(CAC)_5$ (lanes 15, 17 & 18), DBV$(CAT)_5$ (lanes 19–22). Lanes 1 and 16 are lambda III molecular weight markers. Lane 2 is the negative control of the PCR.

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.

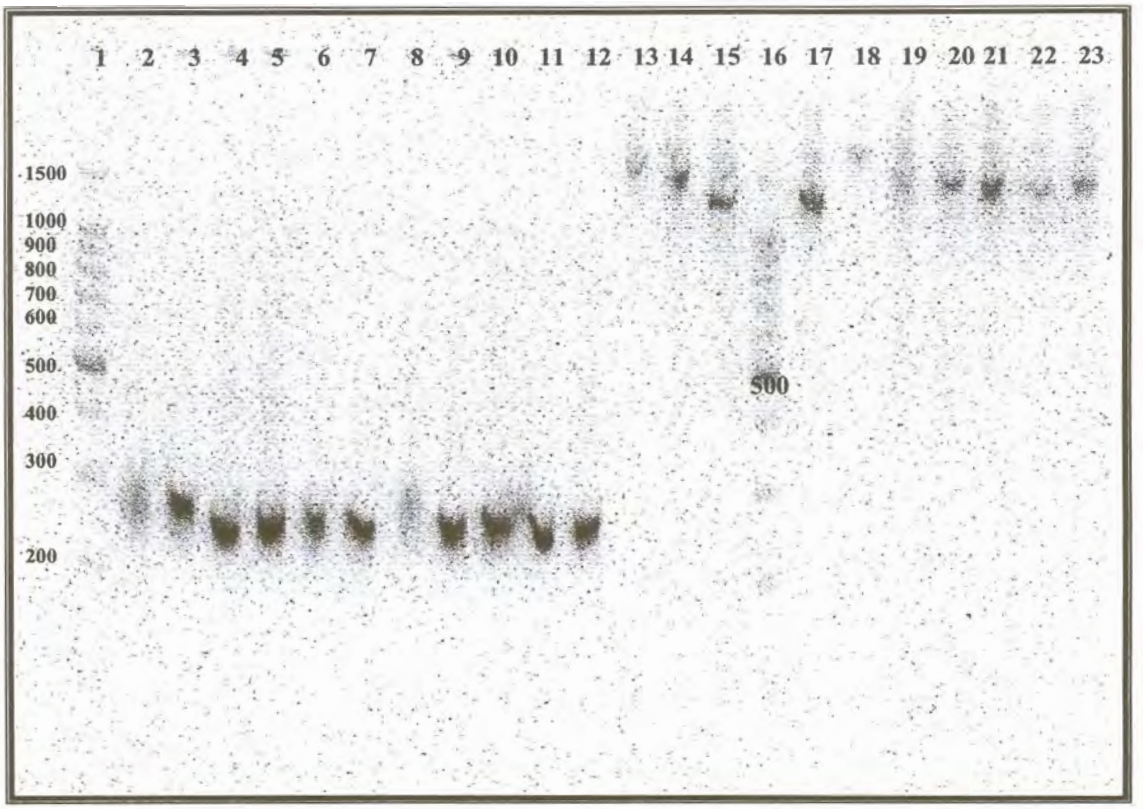

Figure 7.2 A 6% polyacrylamide gel indicating the different loci amplified using the primer sets CLV2 (lanes 2–12) and CLV4 (lanes 13–15, 17–23). Lanes 1 and 16 are lambda III molecular weight markers (Chapter 2). Lanes 2 and 13 Oribi Gorge; 3 and 14 Kentani area; 4,5, 15 and 17 Mzamba River; 6 and 18 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.

8.3 References

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