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Assessing *Clivia* taxonomy using the core DNA barcode regions, *matK* and *rbcLa*

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Background: *Clivia* is a genus of the family Amaryllidaceae endemic to South Africa and Swaziland. Six species and one natural hybrid have been described. Some morphological traits overlap between some species, thus causing taxonomic confusion.

Objectives: The discriminatory power of the core DNA barcodes (*matK* and *rbcLa*) was evaluated, and the current taxonomy of *Clivia* was assessed.

Method: Seventy-four two-locus DNA barcodes from 4 to 18 specimens per species were generated.

Results: The matK region had a higher mean intraspecific variation of 0.21 compared with the 0.02 of *rbcLa*. The two-locus barcodes have an aligned length of 1335 base pairs. Three species, *Clivia mirabilis*, *Clivia nobilis* and *Clivia caulescens*, are monophyletic in the Bayesian Inference (BI) cladogram. The remaining *Clivia* species (*Clivia miniata*, *Clivia gardenii*, *Clivia robusta* and their affinities) are paraphyletic. *Clivia* is divided into 17 haplogroups with those of *C. mirabilis* and *C. nobilis* being unique. *Clivia caulescens* has three haplotypes. The *Clivia* species from the north-eastern distribution range of the Eastern Cape and KwaZulu-Natal provinces have 11 haplogroups and no species-specific DNA barcodes. These groups have no correlation with the current taxonomy or geographical distribution.

Conclusions: Only 37.33% of the species can be correctly identified with the 'best match' option in SpeciesIdentifier. *Clivia mirabilis*, *C. nobilis* and *C. caulescens* have unique DNA barcodes to identify them. Specimens from the Ngome area in KwaZulu-Natal have a unique DNA barcode, separating them from the rest of *C. gardenii*. A taxonomic revision is suggested.

Introduction

Clivia Lindl., a shade-loving member of the family Amaryllidaceae J.St.-Hil., is endemic to South Africa and Swaziland and consists of six species, *C. mirabilis* Rourke, *C. nobilis* Lindl., *C. caulescens* R.A. Dyer, *C. miniata* (Lindl.) Regel, *C. gardenii* Hook., *C. robusta* B.G. Murray et al., and a natural hybrid *C.* ×*nimbicola* Z.H. Swanevelder et al. *Clivia miniata* is the only species with trumpet-like flowers, while the other species have pendulous flowers.

Fraud in the *Clivia*-trade industry (especially in the pot plant industry) occurs because of misidentification of species when investigating vegetative material. Natural variation in the shape and colouring of the leaves are the main contributing factors. Even identification based on the flowers can be problematic because most of the species with pendulous-shaped flowers can be morphologically very similar. Swanevelder and Fisher (2009) attempted to resolve the problem by creating a taxonomic key. However, this key is only helpful when identifying an adult plant in full bloom accompanied by additional information such as the collection site.

Undescribed species may exist, such as the *Clivia* populations along the Mzamba and Mtentu rivers in the Eastern Cape, the latter sometimes referred to as '*Clivia maxima*', although it has not yet been described as a separate species or even as a cultivar (Dixon 2005). This group of plants has either pendulous or trumpet-like flowers, and unlike the majority of species, it forms a stem at the base of the leaves. It is morphologically at the extreme spectrum of *C. robusta*. When self-fertilised in cultivation, it produces up to 10% albino plants in the progeny (Dixon 2005).

In addition, the natural yellow-coloured variations of *C. gardenii* and *C. robusta* found in nature have been split into *citrina* varieties (Swanevelder et al. 2006; Swanevelder, Van Wyk & Truter 2005; Watson 1899) based on the split of a yellow *C. miniata* specimen, *C. miniata* var. *citrina* S. Watson and *C. miniata* var. *miniata* (Watson 1899). No provision was made for other colour deviations such as peach, pink, blush or red.

There is a need for a reliable method to identify *Clivia* species based on any part of the plant. DNA barcoding has been successfully applied in identification of plant species (e.g. Bruni et al. 2015) as well as in re-evaluating taxonomic status and circumscriptions (e.g. Rastegar-Pouyani et al. 2014).

Two DNA barcoding regions have been selected as universal barcodes in plants, namely *rbcLa* and *matK* (CBOL Plant Working Group 2009). The *rbcLa* region is easy to amplify and sequenced over a broad spectrum of taxa (CBOL Plant Working Group 2009), and it has been suggested as a core barcoding region (Hollingsworth et al. 2009). Although *matK* is not useful in all plants (Lahaye et al. 2008), it is one of the most rapidly evolving coding sections of the plastid genome, and it is considered to be the equivalent of the animal barcode gene, CO1 (Hollingsworth, Graham & Little 2011).

This study assesses the current *Clivia* taxonomy and delimitations of the *Clivia* species with the core DNA barcodes (*matK* and *rbcLa*). In addition, the efficacy of these barcodes for *Clivia* identification is evaluated.

Materials and methods

A total of 110 specimens, representing six *Clivia* species and some atypical specimens, were obtained from legal collectors around the country.

DNA was extracted using the modified CTAB method of Rogstad (1992). The *matK* primer sets of Ford et al. (2009) and *rbcLa* primers of Levin et al. (2003) and Kress and Erickson (2007) were used for PCR amplification and sequencing amplification. DNA sequences were obtained using an Applied Biosystems 3130 Genetic Analyser. Sequence data are stored in the Barcode of Life Data System (BOLD: Ratnasingham & Hebert 2007) (Table 1).

TABLE 1: The list of material used for the two core DNA barcoding analyses.

Species	GENBANK Identified number		Voucher number	Country/province
	rbcLa	*matK*		
C. mirabilis	KX038949	KX038859	*Spies 8947**	Western Cape
	KX038947	KX038857	*Spies 8953**	Western Cape
	KX038956	KX038866	*Spies 8955**	Western Cape
	KX038953	KX038863	*Spies 8958**	Western Cape
C. nobilis	KX038889	KX038809	*Spies 8932**	Eastern Cape
	KX038901	KX038816	*Spies 8936**	Eastern Cape
	KX038946	KX038856	*Spies 8937**	Eastern Cape
	KX038926	KX038841	*Spies 8940**	Eastern Cape
	KX038895	KX038812	*Spies 8933*	Eastern Cape
C. caulescens	KX038902	KX038817	*Spies 8417**	Swaziland
	KX038908	KX038823	*Spies 8487**	Mpumalanga
	KX038927	KX038842	*Spies 8488**	Mpumalanga
	KX038945	KX038855	*Spies 8494**	Mpumalanga
	KX038950	KX038860	*Spies 8496**	Mpumalanga
	KX038918	KX038834	*Spies 8497**	Mpumalanga
	KX038881	KX038803	*Spies 8498**	Mpumalanga
	KX038894	KX038811	*Spies 8499**	Mpumalanga
	KX038952	KX038862	*Spies 8500**	Mpumalanga
	KX038920	KX038836	*Spies 8501**	Mpumalanga
	KX038928	KX038843	*Spies 8502**	Mpumalanga
	KX038931	KX038846	*Spies 8503**	Mpumalanga
	KX038962	KX038871	*Spies 8504**	Mpumalanga
	KX038903	KX038818	*Spies 8557**	Mpumalanga
	KX038925	KX038840	*Spies 8571**	Mpumalanga
	KX038916	KX038832	*Spies 8561**	Mpumalanga
	KX038909	KX038824	*Spies 8562**	Swaziland
	KX038923	KX038839	*Spies 8609**	Mpumalanga
C. miniata	KX038887	KX038807	*Spies 8327**	Eastern Cape
	KX038882	KX038804	*Spies 8391**	Eastern Cape
	KX038948	KX038858	*Spies 8394**	KwaZulu-Natal
	KX038936	KX038849	*Spies 8396**	Eastern Cape
	KX038934	KX038848	*Spies 8406**	KwaZulu-Natal
	KX038969	KX038878	*Spies 8408**	Eastern Cape
	KX038938	KX038850	*Spies 8410**	Eastern Cape
	KX038899	KX038815	*Spies 8419**	Eastern Cape
	KX038930	KX038845	*Spies 8469**	Eastern Cape
	KX038964	KX038873	*Spies 8470**	Eastern Cape
	KX038911	KX038827	*Spies 8558**	Mpumalanga
	KX038922	KX038838	*Spies 8616**	KwaZulu-Natal
	KX038963	KX038872	*Spies 8617*	KwaZulu-Natal
	KX038905	KX038820	*Spies 8637**	KwaZulu-Natal
	KX038921	KX038837	*Spies 8667**	Eastern Cape
	KX038924	-	*Spies 8686*	Eastern Cape
	KX038885	-	*Spies 8689*	Eastern Cape
C. aff. *miniata*	KX038921	KX038837	*Spies 8324*	Unknown
C. gardenii	KX038906	KX038821	*Spies 8367**	KwaZulu-Natal
	KX038883	KX038805	*Spies 8368**	KwaZulu-Natal
	KX038897	KX038814	*Spies 8369**	KwaZulu-Natal
	KX038965	KX038874	*Spies 8374**	KwaZulu-Natal
	KX038896	KX038813	*Spies 8376**	KwaZulu-Natal
	KX038951	KX038861	*Spies 8403**	KwaZulu-Natal
	KX038966	KX038875	*Spies 8405**	KwaZulu-Natal
	KX038933	KX038847	*Spies 8418**	KwaZulu-Natal
	KX038939	KX038851	*Spies 8444**	KwaZulu-Natal
	KX038961	KX038870	*Spies 8615**	KwaZulu-Natal
	KX038972	KX038880	*Spies 8780**	Eastern Cape
	KX038954	KX038864	*Spies 8884**	KwaZulu-Natal
	KX038914	KX038830	*Spies 8885**	KwaZulu-Natal
	KX038957	KX038867	*Spies 8887**	KwaZulu-Natal
	KX038955	KX038865	*Spies 8888**	KwaZulu-Natal
	KX038958	KX038868	*Spies 8889**	KwaZulu-Natal
	KX038915	KX038831	*Spies 8892**	KwaZulu-Natal
C. robusta	KX038917	KX038833	*Spies 8415**	KwaZulu-Natal
	KX038968	KX038877	*Spies 8440**	KwaZulu-Natal
	KX038967	KX038876	*Spies 8442**	Eastern Cape
	KX038940	KX038852	*Spies 8462**	KwaZulu-Natal

Table 1 continues

TABLE 1(Continues...): The list of material used for the two core DNA barcoding analyses.

Source: Authors' own work

*, *matK* and/or *rbcLa* sequences done by CCDB on DNA collected and extracted by authors.

The *matK* and *rbcLa* regions were separately analysed using the online tools in BOLD (Ratnasingham & Hebert 2007) and Geneious R6 software (http://www.geneious.com, Kearse et al. 2012). Sequences were aligned with the aid of Muscle (Edgar 2004). Only sequences with no missing data were analysed. The barcode gaps' (nearest neighbour, NN) intraspecific distances were determined for both regions separately using the Kimura-2-parameter distance model in BOLD Systems (Ratnasingham & Hebert 2007).

TABLE 2: The distance summaries of the *matK* and *rbcLa* regions of *Clivia* calculated with Barcode of Life Data System online tools.

Region	Relationship	*n*	Taxa	Comparisons	Min Dist (%)	Mean Dist (%)	Max Dist (%)	SE Dist (%)
matK	Within species	72	7	459	0	0.21	0.7	0
	Within genus	73	1	2169	0	0.46	0.9	0
rbcLa	Within species	86	7	732	0	0.02	0.19	0
	Within genus	87	1	3009	0	0.25	1.19	0

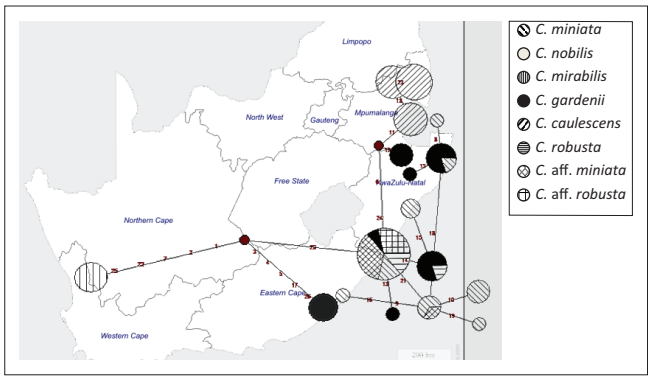
Source: Authors' own work

Mean Dist, mean distance; Min Dist, minimum distance; Max Dist, maximum distance; SE Dist, standard error distribution

TABLE 3: A comparison between the 'best match' and 'all species barcode' options in SpeciesIdentifier to determine the correct, ambiguous and incorrect identifications in percentages (%).

Variables	Best match	All species barcode
Correct identification (%)	37.33	12.00
Ambiguous (%)	52.00	86.66
Incorrect identification (%)	10.66	1.33
Sequence without match closer than 0.34%	0	0

Source: Authors' own work



Source: Authors' own work

FIGURE 1: The 17 haplogroups determined with Network 4.6.1.1 and compared with the distribution. The circled haplotypes have no correlation with either geographic distribution or species classification. The values on the branches indicate the number of mutations.

The *matK* and *rbcLa* sequences were combined using SequenceMatrix software (Vaidya, Lohman & Meier 2011). The MrBayes plugin Version 2.0.9 in Geneious R6 was used to construct a Bayesian Inference (BI) cladogram for phylogenetic analysis. The barcoding gap and species partitioning were determined with the online tool Automatic Barcode Gap Discovery (ABGD: Puillandre et al. 2011) with the default settings. A pairwise summary, best match and best close match were determined with the program SpeciesIdentifier (Meier et al. 2006). A threshold value of 0.349 was calculated from the pairwise summary (Meier et al. 2006). A median joining network was constructed in Network 4.6.1.1 to depict the number of mutational steps separating haplotypes of the six *Clivia* species.

Results and discussion

Molecular analyses

Bidirectional sequences for both *matK* and *rbcLa* were obtained for 74 specimens. Only specimens with both gene regions and no missing data were included in this study. The aligned *rbcLa* region was 552 base pairs and the aligned *matK* region was 783 base pairs, resulting in a combined aligned length of 1335 base pairs.

The genetic distances of the two core barcoding regions were separately analysed in BOLD (Table 2). The mean intraspecific variation of 0.02 in *rbcLa* is much lower than the 0.21 in *matK*. In contrast, the guanine-cytosine (GC) content of *rbcLa* was higher (mean 43.18% and range 42.70% – 45.54%, SE = 0.05) compared to the GC content in *matK* (mean 29.97% and range 23.30% – 32.31%, SE = 0.41). The *rbcLa* region demonstrated less genetic variation (1.44% variable sites) than *matK* (2.4% variable sites).

The ABGD algorithm makes use of pairwise distances to group sequences according to proposed species (Puillandre et al. 2011). Depending on the maximal distance (*P*) tested (*P* = 0.00100, 0.001668 or 0.002783), the six species are divided into one to three groups, respectively.

In the pairwise SpeciesIdentifier-based analysis (Table 3), the total overlap is 0.42% (from 0.0% to 0.42%, covering 80.03% of all intra- and interspecific sequences) at a 0.34% intraspecific cut-off. The majority (71.61%) of the intraspecific distances are between 0.0% and 5.0% with 28.38% of the species having an intraspecific distance of 0.0%. The interspecific distances in the genus are mainly (95.87%) between 0.0% and 5.0%. Only a small percentage (4.12%) of the species has an interspecific distance equal to 0.0% and has, therefore, no genetic differences between them.

According to the 'best match' and 'all species barcodes' option in SpeciesIdentifier, correct species identifications are 37.33% and 12.0%, respectively (Table 3); incorrect *Clivia* identification can be as high as 10.66% when using the 'best match' option. The non-variable sites were removed from the sequences, and a network was constructed in Network 4.6.1.1 (Figure 1) with 67 specimens clearly belonging to a species (in other words excluding specimens classified as *Clivia* 'aff.' specimens) in the data set resulting in 17 haplotypes.

Identification of *Clivia* species

Clivia mirabilis is the only species from the Western Cape Province, with several unique characteristics distinguishing it from the other species. Their seeds mature in 4–6 months compared to the 12–24 months of the other species. The root system of *C. mirabilis* is much thicker and more succulent than the other species. Their pedicels turn from red or orange during the flowering stage to green when bearing fruit. *Clivia mirabilis* is a sought-after plant, and seedlings of other species can be sold to inexperienced buyers under the name of *C. mirabilis*.

TABLE 4: The barcoding gap analyses for both the *matK* and *rbcLa* gene regions with the mean intraspecific, maximum intraspecific, nearest species and the distance to the nearest neighbour calculated in Barcode of Life Data System online tools for species of *Clivia*.

Species	Mean intraspecific	Max intraspecific	Nearest species	Distance to NN
matK				
C. caulescens	0.12	0.25	*C. ×nimbicola*	0.00
C. gardenii	0.36	0.70	*C. robusta*	0.00
C. miniata	0.24	0.62	*C. robusta*	0.00
C. mirabilis	0.00	0.00	*C. robusta*	0.37
C. ×nimbicola	0.00	0.00	*C. caulescens*	0.00
C. nobilis	0.00	0.00	*C. robusta*	0.37
C. robusta	0.12	0.25	*C. miniata*	0.00
rbcLa				
C. caulescens	0.00	0.00	*C. ×nimbicola*	0.00
C. gardenii	0.06	0.19	*C. robusta*	0.00
C. miniata	0.01	0.19	*C. robusta*	0.00
C. mirabilis	0.00	0.00	*C. gardenii*	0.56
C. ×nimbicola	0.00	0.00	*C. caulescens*	0.00
C. nobilis	0.00	0.00	*C. gardenii*	0.56
C. robusta	0.00	0.00	*C. gardenii*	0.00

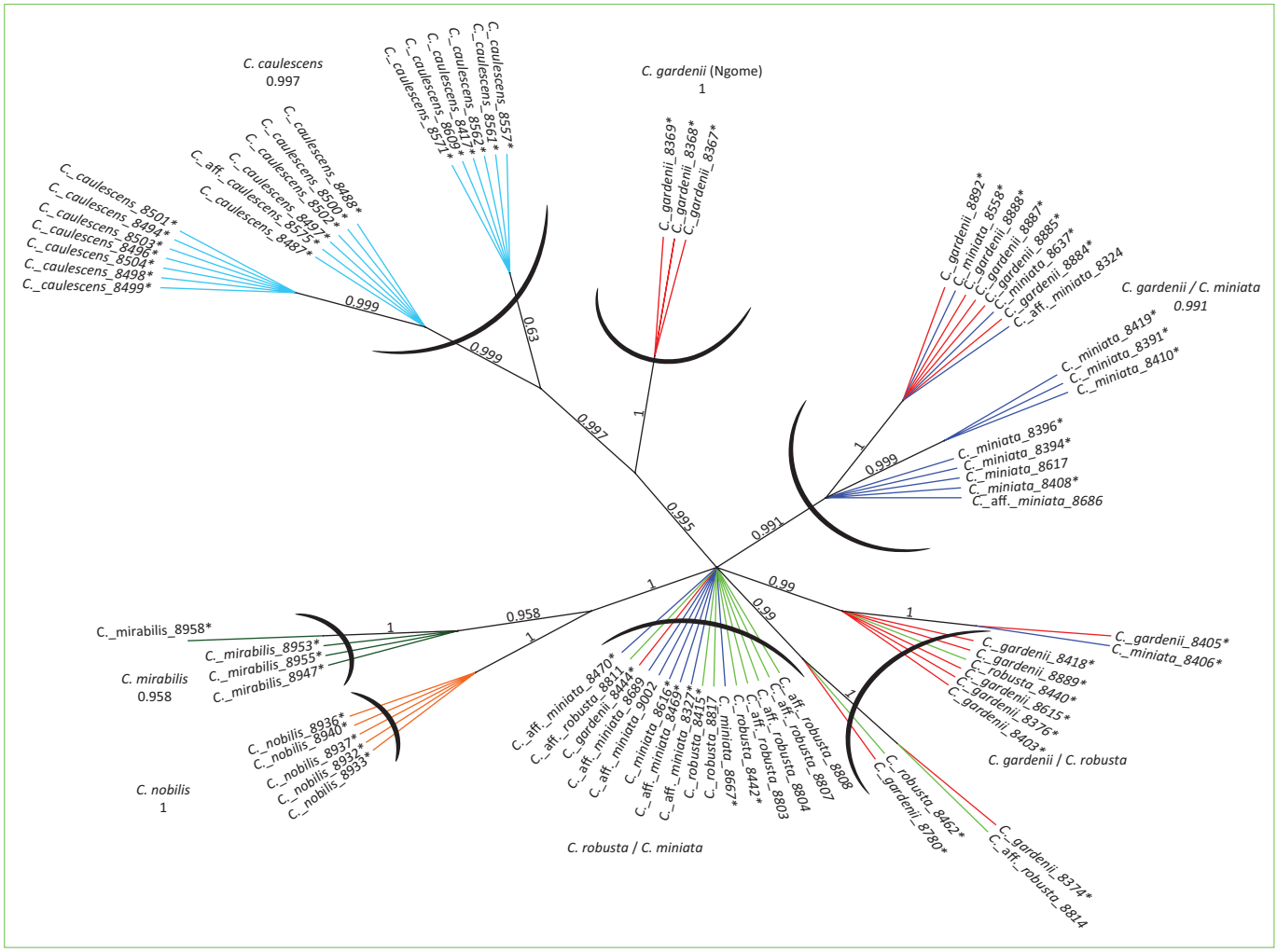
Source: Authors' own work

NN, nearest neighbour; ×, named interspecific hybrid.

The flowers of *C. nobilis* are similar to *C. mirabilis*, but are mainly distinguished by their compact umbel and the pedicels are shorter than in *C. mirabilis*.

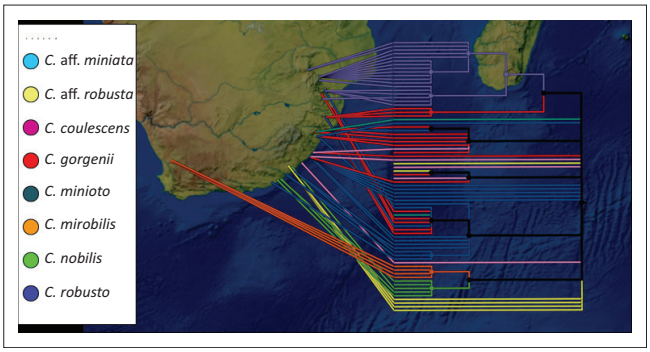
The nearest neighbours and barcoding gaps from BOLD are presented in Table 4. *Clivia nobilis* and *C. mirabilis* have distances greater than zero to their NNs. The remaining *Clivia* species do not have a barcoding gap or any significant genetic distances between them and their closest relatives. *Clivia nobilis* and *C. mirabilis* each have a single haplotype and demonstrated no gene flow with any other *Clivia* species. The haplotype network supports the monophyletic grouping of *C. nobilis* and *C. mirabilis* with five mutations separating each of these haplotypes from a common ancestor. Further support for separation is provided by a posterior probability of 1 for *C. nobilis* and 0.958 for *C. mirabilis* (Figure 2).

Clivia caulescens is distributed as isolated populations in the northern Limpopo and Mpumalanga provinces. The southern *C. caulescens* populations on Bearded Man Mountain are sympatric with and hybridise freely with



Source: Authors own work

FIGURE 2: Unrooted tree of the combined *matK* and *rbcLa* matrix. Each species is represented by different coloured branches: light blue, *C. caulescens*; dark blue, *C. miniata*; red, *C. gardenii*; light green, *C. robusta*; dark green, *C. mirabilis*; orange, *C. nobilis*. The thick black curved lines link similar species together based on their monophyletic groupings (such as *C. robusta* or *C. miniata*). The values on the branches are the posterior probabilities from the Bayesian analysis.



Source: Authors' own work

FIGURE 3: A phylogeographic comparison based on the Bayesian inference cladogram to indicate the low correlation between the phylogeny and geographic distribution in *Clivia*.

C. miniata resulting in the natural hybrid, *C.* ×*nimbicola* (Swanevelder et al. 2006). *Clivia caulescens* was divided into three haplotypes (Figure 1). The first haplotype is unique to Mariepskop Forest Reserve, but is not represented by all the plants from this population (three specimens from Mariepskop share their haplotype with the second haplogroup). The second haplogroup is widespread from the most northern (Wolkberg Mountains) to the most southern locality (Swaziland). The third haplogroup is mainly limited to the most southern distribution area (Bearded Man Mountains and Swaziland), but a single specimen from the central distribution area (God's Window) shares this haplotype. *Clivia caulescens* has three to five mutations corresponding to three haplogroups.

Phylogenetic results from the combined *matK* and *rbcLa* BI cladogram are presented in Figures 2 and 3. Three species (*C. mirabilis*, *C. nobilis* and *C. caulescens*) can be clearly identified based on monophyletic groupings in the BI analysis. The other species (*C. miniata*, *C. gardenii*, *C. robusta* and the *affinis* specimens) are paraphyletic. These results imply there is very little or no correlation between the phylogenetic cladogram and geographical distribution in *Clivia* (Figure 3).

Clivia miniata has a widespread distribution covering areas over three provinces and two countries, Eastern Cape, KwaZulu-Natal and Mpumalanga in South Africa and Swaziland. *Clivia miniata* has eight haplogroups, of which five are unique to the species. *Clivia mirabilis* is the only species in the western part of South Africa on the border between the Western and Northern Cape and has a unique haplogroup.

Clivia gardenii is a pendulous species from KwaZulu-Natal, of which the most southern distribution is Durban (Swanevelder & Fisher 2009). Felbert (2003) indicates that the distribution is as far south as Port Edward near the border between KwaZulu-Natal and the Eastern Cape. *Clivia gardenii* has six different haplogroups, of which three are unique to *C. gardenii*. The other three remaining haplogroups are shared with *C.* aff. *miniata*, *C.* aff. *robusta*, *C. miniata* and *C. robusta* (haplogroup 1); *C. robusta* (haplogroup 2) and *C. miniata* (haplogroup 3).

Clivia robusta and *C. nobilis* are species with pendulous flowers from the Eastern Cape Province and are separated from an area of the species *C. miniata* known for its upright-standing flowers. *Clivia nobilis* has a unique haplogroup, whereas *C. robusta* has two haplogroups that are not unique to the species.

A rare yellow flowering form has been observed in nature in all six *Clivia* species. Watson (1899) recognised this yellow form in *C. miniata* as a new variety (*C. miniata* var. *citrina* Watson) and since then new yellow varieties have been described for *C. gardenii* (*C. gardenii* var. *citrina* Swanevelder et al.) as well as *C. robusta* (*C. robusta* var. *citrina* Swanevelder et al.).

After the discovery and recognition of the yellow *C. miniata* var. *citrina* Watson, it was predicted in 1899 that more atypical colour variations (excluding the more common orange and yellow varieties observed in nurseries) will be found beyond the borders of KwaZulu-Natal (Watson 1899). Plants with unusual colour variants, such as 'versicolour' (contrasting inner and outer tepals), 'splash' (an unusual splash colouration), soft pink pastels, blushed yellows (plants that are more light sensitive) and ruby steward (deep yellow with pink flecking and spotting), have been collected and are preserved in a Heritage collection in KwaZulu-Natal (Chubb 2008). Examples of additional colour variants have been observed in numerous *Clivia* populations since the discovery of the first yellow plants including various pastel colours, such as peach and apricot. According to our results, the yellow colour variant does not deserve separate status and should be regarded as natural variation in the species.

Network for the complex

The largest haplogroup consists of 14 specimens representing the species groups, *C. miniata* (*Spies 8667*, *8616*), *C.* aff. *miniata* (*Spies 8327*, *8469*, *8689*, *8470*), *C. gardenii* (*Spies 8444*), *C. robusta* (*Spies 8442*, *8415*) and *C.* aff. *robusta* (*Spies 8808*, *8807*, *8804*, *8803*). These species have no significant structure or species-specific haplotype groupings.

Relationships in *Clivia*

Three species are molecularly distinct in the BI cladograms and network based on the combined *matK* and *rbcLa* data set. These are *C. mirabilis*, *C. nobilis* and *C. caulescens*. This finding supports previous findings of clear lineage formation involving these three species (Spies, Grobler & Spies 2011). An unknown sample falling in the same monophyletic groups as any of these species in a BI cladogram has a posterior probability of 1.0 of belonging to *C. mirabilis* or *C. nobilis* and a posterior probability of 0.997 of belonging to *C. caulescens*.

Although *C. caulescens* has three haplotypes in the network, the number of steps (3–5) to the separation of these haplogroups into separate species is small and gives therefore

weak support for the monophyly of these populations (haplogroups) as separate species. Furthermore, analyses relying on the intra- and interspecific genetic distances such as those performed by BOLD, ABGD and SpeciesIdentifier fail to recognise *C. caulescens* as monophyletic. This is mainly because of the degree of intraspecific variation (mean of 0.12).

Clivia mirabilis and *C. nobilis* are each geographically isolated with very little or no gene flow to other species. *Clivia mirabilis* grows in a few small isolated populations in the Northern and adjacent Western Cape provinces and has probably speciated because of isolation by distance. The geographic separation between *C. mirabilis* in the west and the other species in the east was primarily caused by climate change during the Cenozoic period leading to the extinction of tropical flora (Conrad, Reeves & Rourke 2003; Linder, Meadows & Cowling 1992; Meerow & Clayton 2004; Swanevelder & Fisher 2009). This is the only species in the Northern and Western Cape provinces; the geographic distance to the closest *Clivia* species (*C. nobilis*) is approximately 650 km east-southeast (ESE) (by direct measurement using Google Earth Pro). The isolation of *C. mirabilis* resulted in a lack of intraspecific variation. The nearest neighbours are *C. robusta* in *matK* and *C. gardenii* in *rbcLa* with genetic distances of 0.37 and 0.56, respectively, separating these species from *C. mirabilis* (Table 4). *Clivia gardenii* has the largest degree of intraspecific variation with a mean distance of 0.36 (and maximum 0.7) in *matK*, followed by *C. miniata* (mean distance of 0.24 and maximum distance of 0.62). *Clivia caulescens* and *C. robusta* both have a mean intraspecific distance of 0.12 (maximum of 0.25) in *matK*. The intraspecific distances in *rbcLa* are 0–0.19 (Table 4).

The geographic range of *C. nobilis* covers a 500 km stretch ranging from slightly north of Port Elizabeth to Coffee Bay in the Eastern Cape. *Clivia nobilis* is adapted to tolerate the salty, sandy and intense light conditions adjacent to the coastal areas of the Eastern Cape (Swanevelder & Fisher 2009). There are a few rare localities where *C. nobilis* and *C. miniata* overlap; the gene flow in these areas results in mutating colonies, containing morphologically unusual plants (Haselau 2010). Various mechanisms may have contributed to speciation of *C. nobilis*, of which the most dominant may have been adaptation to the harsh environmental conditions near the coast. *Clivia nobilis* and *C. mirabilis* share the same nearest species (*C. robusta* in *matK* and *C. gardenii* in *rbcLa*).

Clivia caulescens, the species occurring furthest north in Limpopo and Mpumalanga provinces, may have diverged from other *Clivia* species because of isolation by altitude. This species has a geographic distribution centred on high-lying areas in the Barberton Greenstone Belt region (elevation of 600 m–1800 m) (McCarthy & Rubidge 2006), Mariepskop Forest Reserve (elevation ~1026 m) and Wolkberg (elevation ~846 m). *Clivia caulescens* consists of three haplotype groups covering areas in Swaziland and in Mpumalanga. The first haplogroup correlates with populations in Swaziland (two specimens), and specimens in Mpumalanga at the God's Window tourist site (one specimen) and the Bearded Man Mountain (three specimens). Haplogroup 2 consists of three specimens from Mariepskop and two from the Wolkberg area. The last haplogroup consists only of specimens from Mariepskop.

Clivia miniata has the widest distribution of all *Clivia* species studied, occurring in patches from the most northerly point of the *C. nobilis* distribution area to the most southerly end of the *C. caulescens* range. Mutating colonies occur where *C. miniata* co-exists with other species (Haselau 2010). The first of these populations is located in the Songimvelo Nature Reserve on the Bearded Man Mountain in Mpumalanga. This is where the only described semi-pendulous hybrid, *C.* ×*nimbicola*, exists as a result of crosses between *C. miniata* and *C. caulescens*. The Ngome forest contains large numbers of yellow-flowered *C. gardenii* plants and is where the type specimen for *C. gardenii* var. *citrina* was originally collected (Swanevelder et al. 2005). These plants have a unique round thickening at the base of the stem, which is not a typical morphological trait of *C. gardenii*. Based on a distribution map of Felbert (2003), another overlapping area between *C. miniata* and *C. gardenii* is a 4946 km^2 area with a 630 km stretch from Port Edward to Eshowe and 73 km inland to Pietermaritzburg. The final overlapping distribution between these two species is a small area around Port St. Johns in the Eastern Cape (Felbert 2003).

Clivia miniata shares only a small fraction of a geographical area with *C. nobilis*, which is a 65 km stretch between two major rivers, the Great Kei River and the Mbashe River (measured in Google Earth Pro, according to the *Clivia* distribution map of Felbert [2003]).

A *Clivia* population in the area of the Mzamba River in the Eastern Cape grows in a dense and rocky habitat in a thicket biome. The leaves and flowers of these plants are unusual in that the leaves are greyish and hard and stems are present. The last is characteristic of *C. caulescens* in the most northern distribution range of *Clivia*. The flowers are upright, drooping or spider-like and have various colours such as pastel, orange or deep red (Forbes-Harding 2008). For the purpose of this article, these specimens are referred to as *C.* aff. *miniata*.

Clivia gardenii and *C. robusta* (previously known as swamp *gardenii*) are distinguished from each other by differences in their geographical range and morphology. *Clivia gardenii* grows in well-drained soil. The distribution is mainly in central and north-eastern parts of KwaZulu-Natal (north of Durban) (Swanevelder & Fisher 2009), and as far south as Port Edward (Felbert 2003). The distribution of *C. robusta* is confined to a small area in the Pondoland centre of endemism from Port Edward as the northern border to the most southerly distribution around Lusikisiki (Dixon 2005). This species prefers swampy conditions. The *C. robusta* plants in the north of the distribution area resemble *C. gardenii*. In the

southern distribution area, the plants have a greater resemblance to *C. nobilis* (Dixon 2005). The morphological differences between *C. robusta* and *C. gardenii* are that *C. robusta* is, as its name suggests, more robust with a prominent stem, it has more flowers (15–40) compared to the 14–20 of *C. gardenii* and the leaves differ slightly (Swanevelder & Fisher 2009).

Ran, Murray and Hammett (2001b) divided *C. miniata* into five groups based on a randomly amplified polymorphic DNA (RAPD) analysis. The network analysis in this study divides *C. miniata* into eight haplogroups. Three of these groups are shared with other species. *Clivia miniata* shares the largest of these three haplogroups with *C. gardenii*, *C.* aff. *robusta*, *C. robusta* and *C.* aff. *miniata*.

There are few areas where *C. gardenii* and *C. miniata* do not overlap. For *C. gardenii*, this is in the vicinity of Vryheid, and for *C. miniata*, it is an 85 km stretch from the vicinity of the Mbashe River to Port St. Johns in the Eastern Cape where it has no geographical overlap with other species.

From the network (Figure 1) and the BI cladogram (Figure 3), there is no clear differentiation between the species in the Eastern Cape (excluding *C. nobilis*) and KwaZulu-Natal. The species *C. miniata*, *C. gardenii*, *C. robusta* and the *C.* aff. *miniata* and *C.* aff. *robusta* specimens included in this study hybridise readily in cultivation and in nature. The lack of isolating mechanisms, such as isolation by distance or by geographical features, contributes to incomplete lineage sorting.

This study supports the findings of Ran, Hammett and Murray (2001a) that *C. miniata, C. gardenii* and *C. robusta* are closely related and we suggest that these species (including the yellow variants) need taxonomic revision. We also suggest that the term *C. gardenii* complex be used as a collective term for *C. gardenii*, *C. robusta*, *C.* aff. *robusta* and *C.* aff. *miniata*. These species contain many morphologic and genetic variations, and it may be difficult to delimit its members based on morphology and molecular data. The key reasons for this taxonomic overlap between species may lie in their geographic distributions or close proximity of different species, leading to hybridisation (ancient or recent) or other factors like adaptation to habitat conditions, for example, swampy areas leading to stem formation.

Conclusions

This article is the first report of the use of *matK* and *rbcLa* DNA barcodes to support the motivation for a taxonomic revision of *Clivia*.

Clivia mirabilis and *C. nobilis* have DNA barcodes that clearly distinguish them from the rest of the species. The two-locus barcode of *matK* and *rbcLa* can be used to identify both of these species.

Clivia caulescens consists of three haplogroups. The phylogenetic support for the monophyletic grouping is strong. The low number of mutations (3–5) separating *C. caulescens* from the *C. gardenii* complex is an indication that this species is closely related to the complex.

The *C. gardenii* complex consists of the species in the Eastern Cape (excluding *C. nobilis*) and KwaZulu-Natal. Although these species are distributed over a large geographic area, ancient and current gene flow results in morphologic and genetic overlap amongst them. This causes confusion with the identification and classification of many specimens.

This study not only provides us with DNA barcodes for *C. mirabilis*, *C. nobilis* and *C. caulescens* but also proves that DNA barcodes have insufficient discriminatory power to distinguish between *C. miniata*, *C. gardenii* and *C. robusta* as they are currently circumscribed morphologically. The latter three species have incomplete lineage sorting, and a taxonomic revision of these species is suggested.

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Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Authors' contributions

P.S. was responsible for conducting the research and writing the thesis. J.J.S. was responsible for contributing some ideas to the study and writing this manuscript.

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