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Microsatellite marker applications in *Cyclopia* (Fabaceae) species

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ABSTRACT

Cyclopia species, from which honeybush tea is made, are endemic to the Fynbos Biome of South Africa. Despite its commercial importance, no molecular resources are available to characterise this genus. The polyploid nature of the genus furthermore complicates the use of molecular markers. The Agricultural Research Council (ARC) maintains a field gene bank for several species. A set of six microsatellite markers was developed from *C. subternata*. These were used to characterise the accessions, thereby facilitating the management of the gene bank, such as keeping track of clones for seed orchards or commercial release and the avoidance of duplicates. In addition, the genetic diversity of three *C. subternata* wild populations was investigated and compared to the accessions. The *C. subternata* accessions were representative of the wild samples, excluding those from the genetically distinct Haarlem population. The microsatellite markers developed in this study can be used in the characterisation of wild populations of other species that could be included in the field gene bank and to detect, for example, gene flow between cultivated material and wild populations of *Cyclopia*. Further conservation strategies include the monitoring of wild harvesting as well as the inclusion of samples from the Haarlem population into the gene bank for the conservation of this genetically distinct population.

1. Introduction

Species of *Cyclopia* grow within the coastal and mountainous areas of the Cape, the southern tip of South Africa. Currently, 23 species are recognised, of which six are utilised to produce honeybush tea, a traditional South African herbal drink reported to have medicinal properties (Kamara et al., 2003, 2004; Marnewick, 2009; Joubert et al., 2011). These include a cure for respiratory irritations and digestive disorders as well as the stimulation of milk production in women. Wild populations of *Cyclopia* are not well protected and an increase in demand has led to unsustainable harvesting practices (Bester, 2013). The cultivation of *Cyclopia* is already underway to relieve pressure on wild populations. Many of the species, such as *C. genistoides*, *C. intermedia*, *C. longifolia*, *C. maculata*, *C. sessiliflora* and *C. subternata*, are of commercial importance and accessions of each species are maintained at the Agricultural Research Council (ARC), Stellenbosch, in field gene banks. The honeybush breeding and selection programme aims to develop cultivars with improved biomass yield and tea quality (Joubert et al., 2011; Bester et al., 2013, 2016).

Cyclopia subternata, more commonly known as vleitee, is one of the most important commercial honeybush tea species and can be classified as non-sprouters, which are dependent on seeds from the soil bank to germinate and grow (Schutte, 1997; Joubert et al., 2011). *Cyclopia* species have an endemic distribution range that spans the coastal and mountainous regions of the Eastern and Western Cape (Joubert et al., 2011). Specifically, wild populations of *C. subternata* are localised in the Tsitsikamma, Outeniqua and Langeberge mountains, stretching from Riversdal to Plettenberg Bay and Port Elizabeth.

Despite the commercial importance of *Cyclopia*, no molecular resources are available to characterise this genus. A recent study by Potts (2016) outlines the concerns for the honeybush industry in terms of the lack of knowledge on genetic diversity and the potential hybridisation between species. Genetic markers can provide tools for investigating the genetic diversity within and between wild species and between wild populations and cultivated accessions and for managing the gene bank, particularly as new accessions are incorporated, identifying accessions and detecting incorrect labelling (Fowler and Hodgkin, 2004; Rao, 2004; Acquaah, 2007). This could create space for the inclusion of plants representing a wider range of genetic variation in the field gene bank and prevents the waste of land resources (Ford-Lloyd, 2001; Rao and Hodgkin, 2002; Rao, 2004). The identification of clones for breeding applications or commercialisation can also be addressed using molecular markers.

Cyclopia species are polyploid with a basic chromosome number of $x = 9$. The only published data are for *C. maculata* ($2n = 4x = 36$), *C. subternata* ($2n = 6x = 54$) and *C. intermedia* and *C. meyeriana* ($2n = 14x = 126$) (Goldblatt, 1981; Schutte, 1997). Recently, it was established that *C. genistoides* is decaploid ($2n = 10x = 90$), and *C. longifolia* is hexaploid ($2n = 6x = 54$) (Motsa, 2016). The polyploid nature of the genus

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therefore limits the application of molecular markers in population genetic studies, which typically assume diploidy. Polyploid organisms have the added complexity of allele dosage ambiguity as the potential maximum number of alleles present in an individual will be equal to the ploidy level (Sampson and Byrne, 2012).

The primary aim of this study was to develop microsatellite markers that could be applied to the DNA fingerprinting of the accessions in the ARC gene bank and to investigate the genetic diversity among gene bank accessions and wild populations of *C. subternata,* taking into consideration the polyploid nature of the species.

2. Materials and methods

2.1. Plant material

Accessions of seven species currently growing in the ARC field gene bank at the Nietvoorbij and Elsenburg Research Farms near Stellenbosch were chosen for the study: *C. genistoides* (15), *C. intermedia* (4), *C. longifolia* (30), *C. maculata* (10), *C. pubescens* (10), *C. sessiliflora* (10) and *C. subternata* (22). The origin of the samples includes Kanetberg, Tolbos and Haarlem from the Western Cape and Groendal from the Eastern Cape. Approximately 0.05–0.15 g of healthy, green, young leaves were picked with gloved hands and placed in 1.5- ml Eppendorf tubes.

In addition, three wild *C. subternata* populations were selected on the basis of their accessibility and their use by local farmers for vleitee production. Sites included one area from the Eastern Cape - Guava Juice (population 1 with 30 individuals sampled), and two locations from the Western Cape - Haarlem (population 2 with 30 individuals sampled) and George (population 3 with 24 individuals sampled) (Fig. 1). Prior to extraction, samples were preserved in a CTAB/NaCl preservation buffer (Rogstad, 1992).

2.2. DNA extraction

DNA was extracted from young leaves with the following optimised method. A stainless-steel bead was added to each tube and, using the TissueLyser (Qiagen), the leaf material was pulsed for 1 min at 30 Hz. A prewarmed (60 °C) 2% (w/v) CTAB buffer (100 mM Tris-hydroxymethyl-aminomethane hydrochloride [Tris–HCL], pH 8.0; 20 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0; 1.4 M sodium chloride [NaCl]; 2% (w/v) hexadecyltrimethylammonium bromide [CTAB]; 2% (m/v) polyvinylpyrrolidone [PVP]) was added to the lysed tissue and thoroughly mixed and ground in the TissueLyser for 1 min at 30 Hz. After the addition of 2- μl β-mercaptoethanol and 2-μl protein kinase K (10 mg/ml), the tubes were incubated at 60 °C overnight. Chloroform:isoamylalcohol (24:1) was added in equal volumes after which the solution was centrifuged at a relative centrifugal force (RCF) of 16,100 for 10 min at 4 °C. The aqueous phase was removed and the DNA was precipitated with 2/3 volume ice-cold isopropanol for 1 h at −80 °C. The precipitated DNA was spun down at a RCF of 16,100 for 20 min at room temperature and the supernatant discarded. The DNA pellet was cleaned using 200 μl of 70% (v/v) ethanol after which the tubes were spun down at a RCF of 16,100 for 10 min at room temperature before the ethanol was decanted. This step was repeated and the pellet was dried completely before being re-suspended in 30- μl ddH_2O. DNA concentrations were measured using the NanoDrop ND-1000 spectrophotometer (Thermo-Fisher).

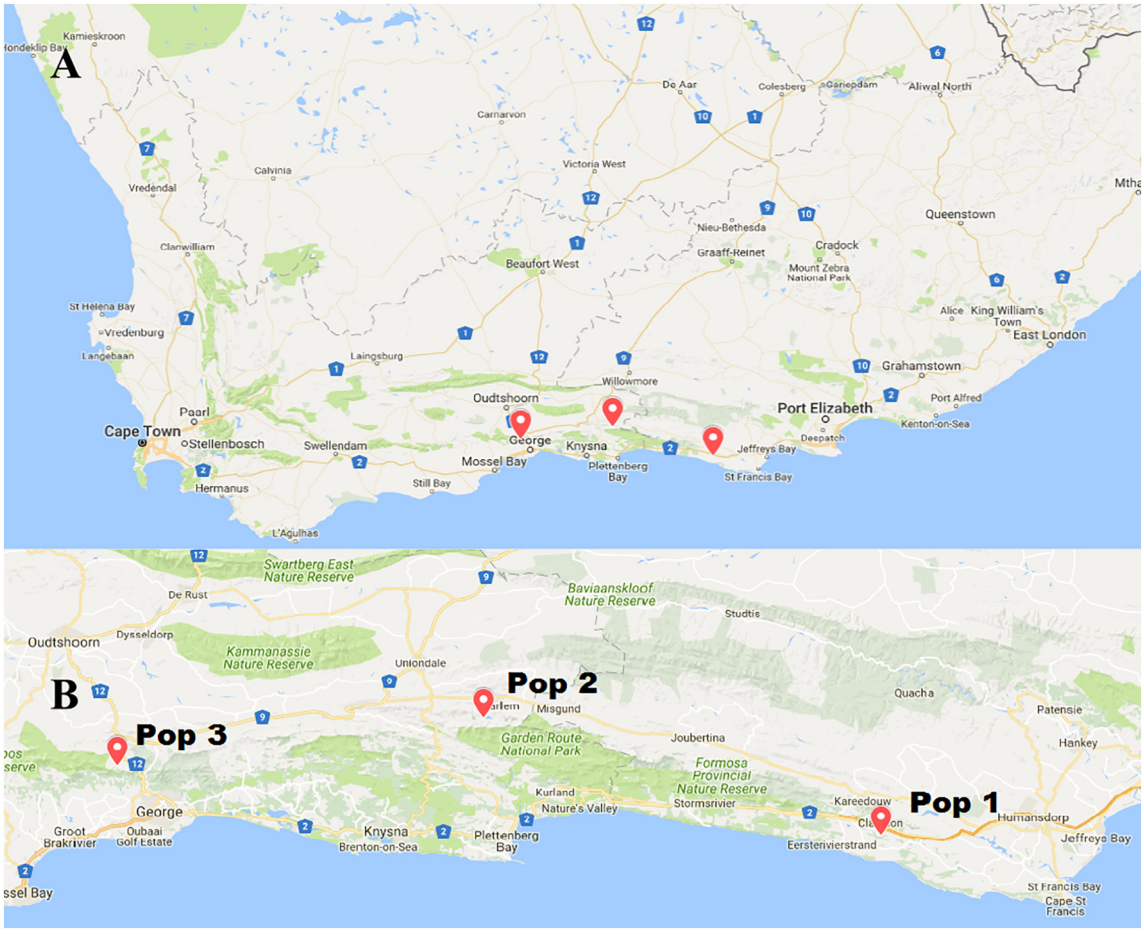

Fig. 1. Locations of the three wild C. *subternata* populations. Map A indicates the locations of the populations in South Africa, while map B focuses on the exact areas. Population 1 = Guava Juice (S 34.04595; E 024.34648) in the Eastern Cape, population 2 = Haarlem (S 33.777688; E 023.304393) and population 3 = George (S 33.88580; E 022.34306) in the Western Cape.