





Assessment of the genetic diversity of the critically endangered *Aloe peglerae* Schönland (Asphodelaceae) by means of Inter-Simple Sequence Repeat (ISSR) markers

Xander Schmidt , Nigel P. Barker *, Arnold Frisby

Department of Plant & Soil Sciences, University of Pretoria, P. Bag X 20, Hatfield, 0028, Pretoria, South Africa

ARTICLE INFO

Edited by: Dr N. Makunga

Keywords:

Aloe peglerae
Inter-simple sequence repeats
Genetic diversity
Conservation genetics
Critically endangered plant

ABSTRACT

Aloe peglerae Schönland, commonly known as the Red-hot Poker *Aloe* or Magaliesberg *Aloe*, is listed as Critically Endangered on the Red List of endangered plant species of South Africa. It is a slow growing succulent, endemic to the Magaliesberg, with a disjunct population near Krugersdorp, within the Gauteng and North West provinces of South Africa. Sharp population declines due to poaching and habitat loss have prompted both reintroduction programmes and associated studies on the genetic variability of the species across its distribution range, all in an attempt to better inform future conservation efforts. This study sampled 12 *A. peglerae* localities from which the genetic diversity was assessed using Inter Simple Sequence Repeat (ISSR) DNA “fingerprinting”, coupled with automated fragment detection. Three fluorescently labelled ISSR primers were used to amplify ISSR profiles from the 174 samples obtained from the 12 localities. Following rigorous data quality assessment, a final data set of 145 samples was obtained. The use of automated detection of ISSR fragments was extremely sensitive and the data set comprised over 2500 bands obtained from just three primers. Analysis of genetic variation was conducted using four different methods: STRUCTURE analysis, Networks, Phenetic analyses (using both UPGMA and Neighbour Joining (NJ)), and AMOVA. Results from these analyses indicate a gradual genetic transition between eastern and western localities along the Magaliesberg mountain range, representing one large population, as well as a completely distinct population from the Krugersdorp area. While samples from different localities usually possess enough differentiation to be generally identified as separate groups, those in close geographical proximity share sufficient similarities to suggest that conservation translocations can utilise propagules from neighbouring localities to augment and/or reintroduce depleted populations. Both *in-situ* and *ex-situ* conservation of the Krugersdorp population is urgently required, and translocations into this habitat should be done using propagules from within this population alone. This study highlights the advantages of using automated detection of ISSR fragments to provide many more data points than conventional agarose gel visualisation. As such, this approach is cost-effective and is recommended for studies assessing the genetic diversity of rare and endangered plant taxa.

1. Introduction

Home to over 20 400 plant species, South Africa is known for its floristic diversity (SANBI 2019). However, according to the SANBI Red List (<https://redlist.sanbi.org/stats.php#National%20statistics>), there are 3267 taxa that are on the Red List (excluding those that are data deficient). The protection and conservation of these species thus represents a massive challenge, especially for those species known to be in population decline. One such species is *Aloe peglerae* Schönland, a succulent endemic to South Africa which primarily grows on the quartzitic

ridges of the Magaliesberg and Witwatersberg mountain ranges in the Gauteng and North West provinces. It is a stemless *Aloe*, restricted to north-facing rocky ridges in “fire-safe” sites (Arena et al., 2015) (Fig. 1).

Previous studies on the population trends of *A. peglerae* showed significant declines in its population densities, of up to 43 % between 1999 and 2010 alone (Phama et al., 2014). Based on these population trends, without intervention, it is predicted that the total population of the species will decline by 80 % within three generations (an estimated 30 years per generation; Pfab et al., 2016). The observed rate of decline is due mainly to illegal harvesting and trade (Pfab and Scholes, 2004).

* Correspondence author.

E-mail address: nigel.barker@up.ac.za (N.P. Barker).

<https://doi.org/10.1016/j.sajb.2025.08.042>

Received 10 March 2025; Received in revised form 16 July 2025; Accepted 31 August 2025

Available online 7 September 2025

0254-6299/© 2025 The Authors. Published by Elsevier B.V. on behalf of SAAB. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

To sustainably harvest *A. peglerae*, Pfab and Scholes (2004) concluded that no $>0.12\%$ of a population can be removed per year. As many populations of the species are usually too small to support this rate of collection, this species cannot currently be sustainably harvested from the wild. Other anthropogenic activities, such as habitat destruction and fragmentation, mining activities, and livestock overgrazing have also added to the species' drastic population declines (Arena, 2013). With a limited area of occupancy (AOO) of $\sim 3445\text{ km}^2$ (Arena, 2013) and the threats faced by this species and the associated rate of population decline, it has been listed as Critically Endangered (CR A4 abd); in South Africa (the only country in which it occurs; IUCN 2024; SANBI, 2024).

Habitat fragmentation and the associated decreasing population sizes of *A. peglerae* may also have negative effects on the abundance of their pollinators, which will have subsequent negative effects on the species' seed production and recruitment (see for example Donaldson et al., 2002). This could, in turn, lead to further reduction in gene flow in the species. *Aloe peglerae* is predominantly pollinated by generalist and opportunistic avian nectarivores, notably the Cape Rock Thrush (Arena et al., 2015), contributes $\sim 60\%$ of probing visits and resulting pollination of *A. peglerae*. Payne et al. (2019) further found that small nocturnal mammals are also pollinators of the species. During a "good" flowering year in 2013, Payne et al. (2019) recorded 266 visits by diurnal bird pollinators of which 80% were by the Cape Rock Thrush. Similarly, the Cape Rock Thrush visits made up 80% of the 70 diurnal pollinator visits in the following "bad" flowering year of 2014. As *A. peglerae* populations become smaller and more widely spread out, the various pollinator abundance may become reduced, in which case higher levels of inbreeding among *A. peglerae* individuals can be expected, potentially resulting in inbreeding depression and leading to a loss of fitness (Ottewell et al., 2016; Brook et al., 2002)

Conservation translocations are an increasingly widespread practice used to address the threatened statuses of various taxa (IUCN 2013). This practice seeks to introduce individuals of a threatened species into

suitable habitats to reduce the species' risk of extinction. Conservation translocations have been used as part of conservation efforts around the world, but assessing the success of these is difficult (e.g. Zimmer et al., 2019; Godefroid et al., 2025). A review of translocations of 376 species in Australia indicated that success was highly variable, and the general number of propagules required to convey success is large, set at least at 500 (Silcock et al. 2019). Zimmer et al. (2019) note that the costs of translocations is comparable to other options and that a careful extinction risk analysis is required. In South Africa, this approach has not been widely implemented (Milton et al., 1999) but there have been some successes (van Jaarsveld, 1999; Harris et al., 2014). One possible reason why this approach has not been adopted is that it is costly, and requires years of follow-up monitoring and adaptive management planning (Maschinski et al., 2023; Bellis et al., 2024). As noted by Pfab and Victor (2002), while this approach is commonly suggested to mitigate impacts of developments, translocation is not without its challenges, including possible negative ecological and genetic impacts. However, according to Novak et al. (2021) negative or unintended consequences of translocations are rare and only occur in the absence of adequate conservation practices and legislation.

Prior to undertaking large-scale conservation translocations, knowledge of the *in-situ* genetic diversity of the target species is crucial, particularly given the risks of genetic contamination, inbreeding depression, and local adaptation etc. (Menges, 2008; Marsden et al., 2013). However, a study by Schäfer et al. (2020) suggests that translocations using propagules from a mix of gene pools are more likely to have short term success. With relevance to *A. peglerae*, a recent study has outlined a protocol that can be used for large-scale conservation translocations (Frisby et al., 2025). However, prior to undertaking large scale re-introductions and translocations, the lack of knowledge of the genetic diversity of *A. peglerae* must first be addressed. This knowledge will inform future *in-situ* and *ex-situ* conservation actions for this species.

There are numerous DNA-based methods to assess genetic diversity

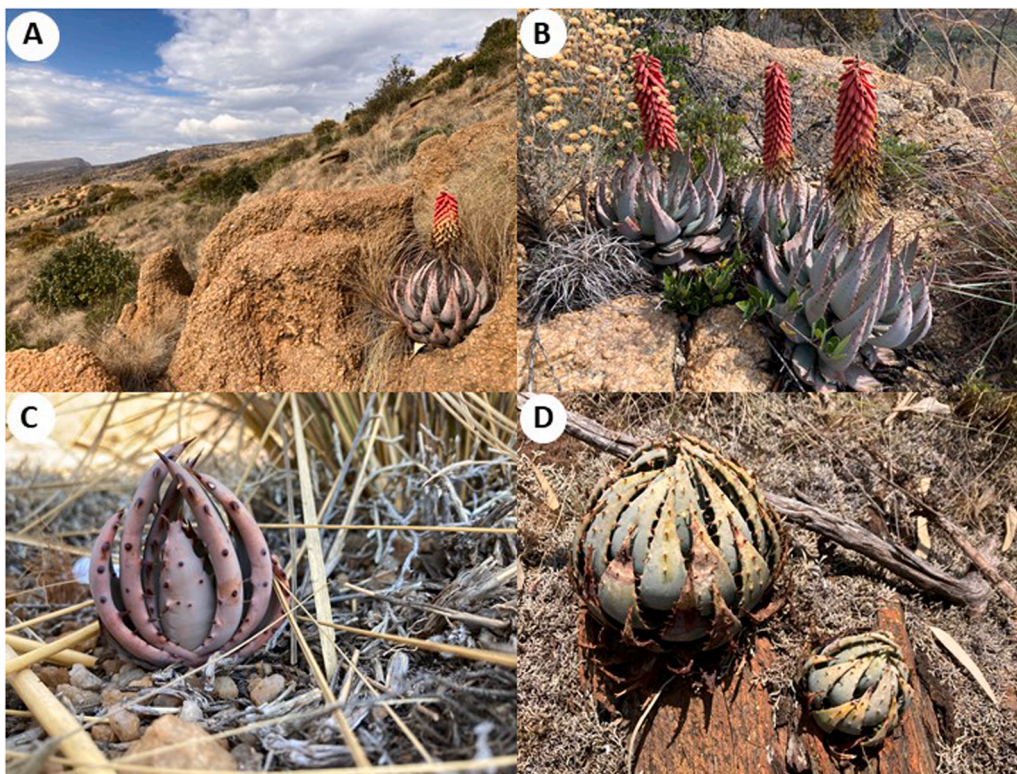


Fig. 1. Photographs of *Aloe peglerae* in situ. A: Flowering individual in the Magaliesberg, showing the general nature of the terrain. B: Three individuals growing in rock crevices on quartzite geology in the Magaliesberg. C: Juvenile plant growing near sheltering grass tussock. D: Two mature plants growing in shale rocks at the Krugersdorp locality. Photos: A. Frisby.

of plant species (e.g. Mondini et al., 2009; Loera-Sánchez et al., 2019), each with a range of advantages and disadvantages relating to reproducibility, technological requirements, and cost. Not all these methods are financially or technologically accessible to researchers in developing countries, many of which are biodiverse and where such studies are urgently required (Murphey et al., 2024). Because it is a PCR-based marker system, the assessment of genetic diversity using Inter-Simple Sequence Repeats (ISSR's) is in widespread use, but does not always provide a great deal of loci on which genetic analyses can be based, as it almost always relies on an agarose gel system for fragment separation and profiling (Nybom, 2004). However, as indicated by Fang and Roose (1997), the method is repeatable and thus more reliable than earlier methods such as RAPDs. As a consequence, ISSRs have been used for a wide range of applications, including the study of genetic diversity of small isolated populations of rare and endangered species (Xiao et al., 2004; Ge et al., 2005; Hao et al., 2006; Taylor and Barker, 2012; Bentley et al., 2015).

The ISSR “DNA fingerprinting” method can also be adapted for automated detection of bands using DNA-sequencing technology, as summarized by Murphy et al. (2024) who demonstrated automated ISSR fingerprinting as a suitable, rapid, and cost-effective method, providing far more useable bands than conventional methods to investigate genetic diversity. This method has been previously used in studies on rare plants (Murphey et al., 2024, Bentley et al., 2015). Here we report an assessment of the genetic diversity of *A. peglerae* across its distribution range using automated detection of ISSR fragment profiles.

2. Materials and methods

2.1. Sampling localities and sample collection

We note that the term “population” has many meanings (Waples and Gaggiotti, 2006), and we thus will only use this term in a genetic sense of a community of interbreeding individuals. Unless the data indicates that these communities can be detected, we refer to sampling localities.

The Global Biodiversity Information Facility (GBIF) database (GBIF.

org, 2022) was used to identify localities of *A. peglerae*. Using this information, potential sampling localities were identified that encompassed the entire known historical distribution of the species. From this distribution range, a number of localities were identified as being accessible for sample collection.

It is important to note that in this study there is a sampling gap in the Hartbeespoort area (Fig. 2). No sampling was conducted in this area despite historical records of *A. peglerae* indicating their existence. This was because we were aware of translocation efforts undertaken by a conservation organization in this area using propagules of unknown genetic origin. As a result of this, sampling this area may have compromised our results, and was thus not included in this study.

Where *A. peglerae* individuals could be located in-situ at each of the sampling localities, a small part of the epidermis (~2,5 cm by 2,5 cm) was cut from the underside of the leaves using a sterile scalpel blade. The sample was then placed into an airtight container with enough silica gel to dry the sample out completely. After each sample collection, the scalpel was cleaned with 70 % alcohol and flamed to prevent contamination. This method was repeated until ideally 15 samples from each locality were collected, with a minimum number of 10 samples considered suitable. More samples were collected from a locality if enough individuals were present and if time allowed.

2.2. DNA extraction, ISSR PCR amplification and automated detection

Once dry, the leaf material was sent to the Forest Molecular Genetics Programme Laboratory (Department of Biochemistry, Genetics and Microbiology, University of Pretoria) for DNA extraction. The dried material was ground with metal beads using the Geno/Grinder 2012 and DNA was isolated from the tissues using the Sbeadex Maxi Plant kit and the Oktopure robot (LGC Biosearch Technologies, Hoddesdon, United Kingdom). DNA concentrations ranged from 4,1 ng/μL to 796,13 ng/μL, and where possible all samples were diluted down to 60 ng/μL, as this was approximately the mean concentration of the samples, and falls within the recommended template DNA concentrations for ISSR (e.g. Subedee et al., 2020; Mohamad et al., 2017)

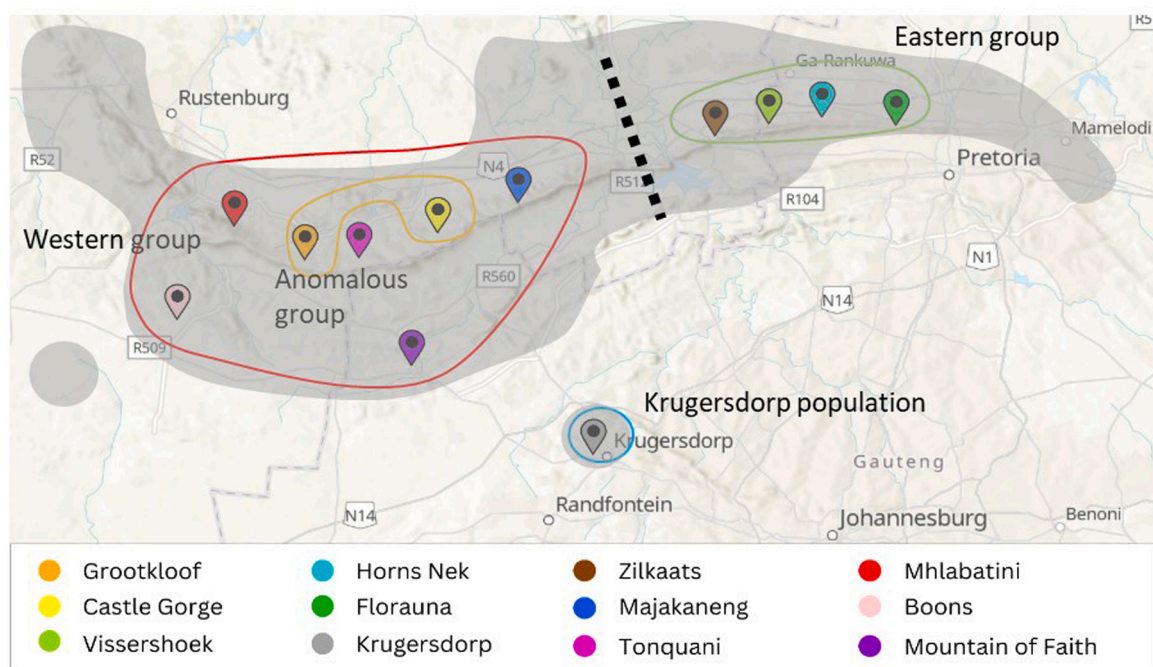


Fig. 2. Map of the sampling localities for *A. peglerae* used in this study. The colours used in this map correspond to the colours used in the Network and clustering analyses. The larger groupings of the populations are indicated by the colour of the surrounding border. The western group is surrounded by a red border, the eastern group by a green border, the Krugersdorp group by a blue border and the anomalous group by an orange border. The historic range of *A. peglerae*, as inferred from GBIF records, is indicated by grey shading. The dashed line indicates the gap in sampling in the Hartbeespoort area.

A substantial array of possible ISSR primers are available, and many were designed and developed by the University of British Columbia over three decades ago. Eight fluorescently labelled primers (Table 1) were tested at various annealing temperatures, and visualized using agarose gel electrophoresis. The gel was made using 0,45 g agarose dissolved in 50 ml TAE buffer. In order to visualize the DNA 0,3uL ethidium bromide was added to the gel mix prior to its cooling. Five microliters of PCR product were added per lane, and each gel was also loaded with 5uL of a DNA ladder. Following electrophoresis, the gel was visualized using the BIO RAD Molecular Imager Gel Doc XR+ with Image Lab Software.

A primer was deemed successful if it was able to consistently produce clear bands that were polymorphic between samples from different sampling localities. In order to determine the ideal PCR parameters, various annealing temperatures (Ta) and number of cycles were tested for optimal amplification. The final parameters used were a Ta of 59°C and 30 cycles.

While the annealing temperature (Ta) of a primer used in PCR is typically 5 °C lower than the melting temperature (Tm) of the primers, a higher annealing temperature was used as it increases PCR specificity, reducing the amount of spurious PCR products. A higher Ta than Tm will, however, reduce the yield of PCR products, but since this study uses a very sensitive automated detection system, this was not a concern (Innis et al., 1990; Murphy et al., 2024).

The PCR cycling parameters comprised an initial denaturation phase at 95 °C for 3 min, followed by 30 cycles of 95 °C denaturation for 30 s, 59 °C annealing for 30 s, 72 °C extension for 2 min, and ending with a 5-min extension at 72 °C. The PCR reaction mixture consisted of the following: 25 µL of reaction mixture consisting of 4 µL of DNA, 1 µL of 25 µM ISSR primer, 12.5 µL of 2 × Ampliqon Master mix (Ampliqon Taq DNA polymerase, 0.4 mM each of dNTP, Tris-HCL pH 8.5, (NH₄)₂SO₄, 3 mM MgCl₂, 0.2 % TweenTM 20, inert red dye, and stabilizer) and 4 µL of distilled water.

Following the screening of the eight primers, three primers that provided the best amplification were selected, and a bulk ISSR PCR amplification was done with every sample (Fig. 3). PCR success was assessed using gel electrophoresis, and samples which did not amplify could be identified visually and were excluded from further analysis. The samples which passed this screening were then sent to the Central Analytical Facility at Stellenbosch University for automated fragment profiling using an ABI 2500XL sequencer equipped with capillary electrophoresis and fluorescence detection paired with fragment profiling software. This process made use of the 1200LIZ size standard, allowing for fragment sizes between 20 and 1200 base pairs.

Following the screening of the eight primers, three primers that provided the best amplification were selected, and a bulk ISSR PCR amplification was done with every sample. PCR success was assessed using gel electrophoresis, and samples which did not amplify could be identified visually and were excluded from further analysis. The samples which passed this screening were then sent to the Central Analytical Facility at Stellenbosch University for automated fragment profiling. Using an ABI 2500XL sequencer equipped with fragment profiling software, capillary electrophoresis and automated detection was

Table 1

Primers screened for use in this study. ** Indicates the primers that were selected on the basis of their informativeness for the bulk amplification step.

ISSR primer name	Sequence
Manny**	CACCACCACACRC 3'
Mao**	CTCCTCCTCCTCRC 3'
UBC 811**	GAGAGAGAGAGAGAC 3'
Omar	GAGGAGGAGGAGRC 3'
812	GAGAGAGAGAGAGAA 3'
UBC 830	TGTGTGTGTGTGG 3'
UBC 868	GAAGAAGAAGAAGAA 3'
856	ACACACACACACACYA 3'

performed. This process made use of the 1200LIZ size standard, allowing for fragment size estimation between 20 and 1200 base pairs.

2.3. Data manipulation and data set creation

Following automated detection, Genemapper6 was used to convert the raw data into a series of tables which presented the ISSR bands as being present (1) or absent (0). We adopted the data assessment protocols published by Murphey et al. (2024), and used multiple user-defined fluorescent cut-off values (25, 100, and 150 relative fluorescence units (rfu)) in order to create data sets comprising bands of different intensities. It must be noted that the default rfu setting in GeneMapper6 is 50 rfu, so the use of 25 rfu was selected to further enhance the sensitivity of the detection levels. These cut-off values are a little lower than used by Murphey et al. (2024), and were chosen to assess the sensitivity and presence of PCR products (bands) that are weakly amplified.

The separate data sets for each of the three primers used were then combined, leaving out any samples that did not amplify. In an attempt to minimize the noise from poorly amplified samples, 25 % of samples with the lowest number of amplified and detected bands were then removed from each rfu data set, as it was found that this improved the quality and completeness of the data (also reported by Murphey et al. (2024)).

2.4. Analysis of genetic diversity

The ISSR data is amenable to a range of methods of analysis to indicate genetic diversity and possible genetic structure. The methods used in this study are STRUCTURE analysis (Pritchard et al., 2000), haplotype network analysis (Bandelt et al., 1999), cluster analysis (Sokal and Sneath, 1973) and Analysis of Molecular Variance (AMOVA) (Fitzpatrick, 2009).

2.4.1. Structure analysis (Bayesian MCMC)

STRUCTURE analysis uses a methodical Bayesian clustering algorithm through Markov Chain Monte Carlo (MCMC) estimation to categorise individuals into clusters that suggest potential populations (Porrás-Hurtado et al., 2013; Pritchard et al., 2000). This analysis was conducted using the CIPRES portal (Miller et al., 2010). A burnin of 10, 000 and an MCMC chain of 50,000 generations was used for this analysis as suggested by Porrás-Hurtado et al. (2013). These run lengths were determined through experimentation with a burnin value up to 500,000 and MCMC up to 100,000 to determine the optimal value as it is not possible to determine these parameters theoretically (Pritchard et al., 2010). Values higher than the ones used in this study yielded the same results but took much longer in terms of computing to finish. Each k-value was run for 20 iterations, as recommended by Porrás-Hurtado et al. (2013) for reliable results, with bands treated as haploid and band frequencies set to be correlated with the admixture protocol using location data. Alternative settings without location data were also tested, however this yielded the same results without context for where each individual is from and were instead disregarded for the locprior results.

The results were then analysed using the StructureSelector website (Li and Liu, 2018) to determine the best k-value using the Evanno method (Evanno et al., 2005) and to generate bar plot representations for the data. The Delta-K values were tested ranging from $k = 1$ to $k = 15$ as it is stated by Porrás-Hurtado et al., (2009) to use a k-value up to 3 more than the expected number of populations and it was not assumed that there would be multiple populations at any given sampling location.

2.4.2. Haplotype network analysis

A haplotype network analysis is used to visualize the genealogical relationships at the intraspecific level within a population (Leigh and Bryant, 2015). While this type of analysis may not be useful for inference of phylogenetic or evolutionary relationships of taxa, it is useful for

detecting patterns in the data (Leigh and Bryant, 2015; Murphy et al., 2024).

A Minimum Spanning Network analysis was done using a Nexus file format in PopART (Population Analysis with Reticulate Trees) version 1.7 (Leigh and Bryant, 2015) with epsilon set to zero (Bandelt et al., 1999).

2.4.3. Clustering (phenetic) analysis

Genetic distance matrices using a variety of similarity coefficients were computed using NTSYSpC-2.02k (Rohlf et al., 2009). The Unweighted Pair Group Method with Arithmetic Averages (UPGMA) and Neighbor Joining (NJ) method was used to create phenograms. The ability of the phenogram to reflect the similarity matrix was tested using cophenetic correlation analysis. The use of the cophenetic correlation assists in the selection of the phenogram which best represents the signal in the triangular matrix of (dis)similarity (Sokal and Sneath, 1973). This is thus a mathematical selection criterion, and not one based on the biological informativeness of the result based on the assessment of the clusters obtained and shown in the phenogram. The range of coefficients was thus assessed using the cophenetic correlation values as well as their reflection of biological “reality” in grouping samples from each locality.

2.4.4. Statistical analysis (AMOVA)

Analysis of Molecular Variance (AMOVA) was done to assess the distribution of observed genotype variation using PopART version 1.7. In doing this, nested groups based on the clusters retrieved by both the Network and Cluster analyses, were created. A second AMOVA analysis was done making use of groupings according to the best-k value of each data set obtained from the STRUCTURE analysis. The Φ_{ST} values were calculated for 1000 permutations of ISSR haplotypes among localities in order to determine if the STRUCTURE analysis results are reliable.

3. Results

3.1. Sampling

In total, samples were collected from twelve localities across the

Table 2
Details of localities where *A. peglerae* was sampled.

Location	Sample size	Sample ID	Latitude	Longitude
Grootkloof, Gauteng	15	TF001-TF005; TF021-TF030	25,84,555°S	27,40,435°E
Castle Gorge, Gauteng	15	TF006-TF020	25,81,202°S	27,57,291°E
Visser's hoek, Gauteng	15	TF031-TF045	25,688,565°S	27,984,639°E
Horn's Neck, Gauteng	15	TF046-TF060	25,683,681°S	28,055883°E
Florauna, Gauteng	15	TF061-TF075	25,6,897,690°S	28,1,481,360°E
Krugersdorp, Gauteng	26	TF076-TF101	26,07144°S	27,77,775°E
Zilkaats, North West	15	TF102-TF116	25,69,450°S	27,91,798°E
Majakaneng, North West	8	TF117-TF124	25,77,537°S	27,67,431°E
Tonquani, North West	15	TF125-TF139	25,84,224°S	27,47,558°E
Mhlabatini, North West	15	TF140-TF154	25,81,091°S	27,31,726°E
Boons, North West	10	TF155-TF164	25,92,318°S	27,24,656°E
Mountain of Faith, Gauteng	10	TF165-TF174	25,97,081°S	27,54,262°E

species' extant distribution range (Fig. 2; Table 2). The historical distribution range of *A. peglerae* proved to be larger than the range that we were able to sample. As an example, this species used to occur in Kgaswane Nature Reserve (near Rustenburg at the western end of the distribution range), but upon visiting this locality, no *A. peglerae* could be located by either ourselves or by Kgaswane Nature Reserve staff, leading to the assumption that this specific locality has been extirpated by poaching. Similarly, no individuals could be located east of Florauna (in Pretoria). All but one of the sample localities were in the Magaliesberg, which have quartzite-derived substrates, but one disjunct locality near the town of Krugersdorp in the Witwatersberg was also sampled, where the substrate was predominantly derived from shale. This sampling range covers much of the species' distribution range as based on historical herbarium records (and indicated in Fig. 2).

3.2. ISSR amplification and data set creation

The primers “Manny”, “Mao” and “UBC 811” yielded successful PCR amplification results, while the rest of the primers yielded either unsuccessful results or results with no clear polymorphisms. Many ISSR studies use considerably more primers, but as these studies use agarose gel detection methods, far fewer bands are recorded. The use of only three primers with automated detection produced many more bands than conventional methods. A summary of the amplification results for the selected primers from the GeneMapper 6 analysis is indicated in Table 3.

Removing 25 % of the samples which did not amplify well reduced the number of samples but resulted in data sets where the samples had many ISSR markers. However, this had the unintended consequence for two localities (Castle Gorge and Grootkloof), which had a substantial reduction in the number of samples in the final data set (Table 4). These two localities are referred to here as the “anomalous” localities.

3.3. Analysis of ISSR data set

3.3.1. Network analysis

The Minimum Spanning Network analysis (Fig. 4), shows the eastern group linking to the western group. The anomalous localities of Castle Gorge and Grootkloof form two smaller clusters which connect to the eastern and western groups. Three samples from Grootkloof TF028, TF029, and TF030 connect to the eastern group at TF047 from the Horns Nek locality, while all other remaining samples from the two anomalous localities connect to the western group at TF172 from the Mountain of Faith locality. TF129 and TF130 from Tonquani connect to individuals from the Krugersdorp and Horns Nek localities. This suggests that despite the geographical distance, these individuals may contain a genetic signal that suggests there is (or has recently been) geneflow between these localities. The Krugersdorp locality is split by a single sample from Boons, TF155, which is also the point of connection with the western group. Samples from Mountain of Faith (TF174), Mhlabatini (TF141), and Majakaneng (TF118, TF124) are found in the eastern

Table 3

The success rate of the various primers screened based on the number of successful amplifications obtained from the full data set.

Primer	Successful sample amplifications out of 174 samples	Average no bands per sample	Total number of alleles in data set
Manny	148	251,99	984
Mao	146	215,90	807
UBC 811	149	327,87	858
Combined data set	144	747,58	2567
Combined data set with 25 % of poorly amplified samples removed	108	774,39	2567

Table 4

The number of samples from each population before and after the removal of 25 % of the samples that were considered to have not amplified well and thus had a reduced number of bands.

Sample locality	Combined data set	Combined data set with 25 % samples removed
Grootkloof	15	12
Castle Gorge	15	2
Vissershoeck	14	10
Horns Nek	15	14
Florauna	14	6
Krugersdorp	25	24
Zilkaats	9	8
Majakaneng	4	4
Tonquani	9	7
Mhlabatini	11	11
Boons	6	5
Mountain of Faith	7	5
Total	144	108

group rather than the western group where these localities were found, also suggesting geneflow between the larger groups.

3.3.2. NTSYS clustering analysis

The UPGMA tree building algorithm based on similarities calculated using the Russel and Rao coefficient had the highest cophenetic correlation r -value and the lowest t -value for the data sets and thus mathematically this combination produces the phenogram best represented the similarity matrix. However, this dendrogram did not group samples according to geographic origin (results not shown). Even though some similarity coefficients had high cophenetic correlation values, the DICE coefficient was selected to create a matrix of similarity for the UPGMA and NJ analyses, as this coefficient produced the most biologically informative results. Murphey et al. (2024) also found the DICE coefficient to be the best at recovering biologically relevant groupings of samples. The results from the NJ analysis using the DICE coefficient are shown in Fig. 5. In this figure, the Castle Gorge and Grootkloof localities

form an anomalous group with only four samples which group separately from the rest of the Grootkloof samples. The western localities all group together, with only one exception where Majakaneng grouped with the eastern localities instead. However, the Zilkaats locality does not group with the rest of the eastern localities and is found in the western group. One sample from Horns Nek is also found in the western group while the Krugersdorp locality is a distinct group.

3.3.3. AMOVA

The first AMOVA used nested groups based on the general groupings observed in the Network and UPGMA analyses and yielded a Φ_{ST} value of 0.15905, a value which, according to Ng and Tan (2015) indicates that the data set for a STRUCTURE analysis requires >200 bands in order to be deemed reliable. As the data set far exceeded this number of bands (Table 3), this requirement is met, and the results of the STRUCTURE analysis is discussed below. The levels of variation among localities is low (5.37 %) and the within locality variation is high (81.48 %), and among the larger groups there is also limited variation (10.53 %; Table 5).

The second AMOVA used nested groups which were informed by the results of the STRUCTURE analysis in which the sampling localities were divided into only three groups. Group 1 contains samples from Grootkloof, Castle Gorge, Vissershoeck, Horns Nek, and Florauna, Group 2 contains the Krugersdorp samples, and Group 3 contains samples from Zilkaats, Boons, Majakaneng, Tonquani, Mhlabatini, and Mountain of Faith. This analysis yielded similar results (Table 5). The variation among localities is low (6.36 %), and variation among the larger groups is also low (10.15 %) whereas the variation among localities is high (79.28 %).

Regardless of the different ways in which the samples localities are grouped, the results consistently show low variation among sampling localities and among the larger groups, and high variation within the localities. All of the results produced significant differences of genetic variation ($p < 0.001$).

3.3.4. STRUCTURE analysis

The Evanno method, as indicated by StructureSelector (Li and Liu,

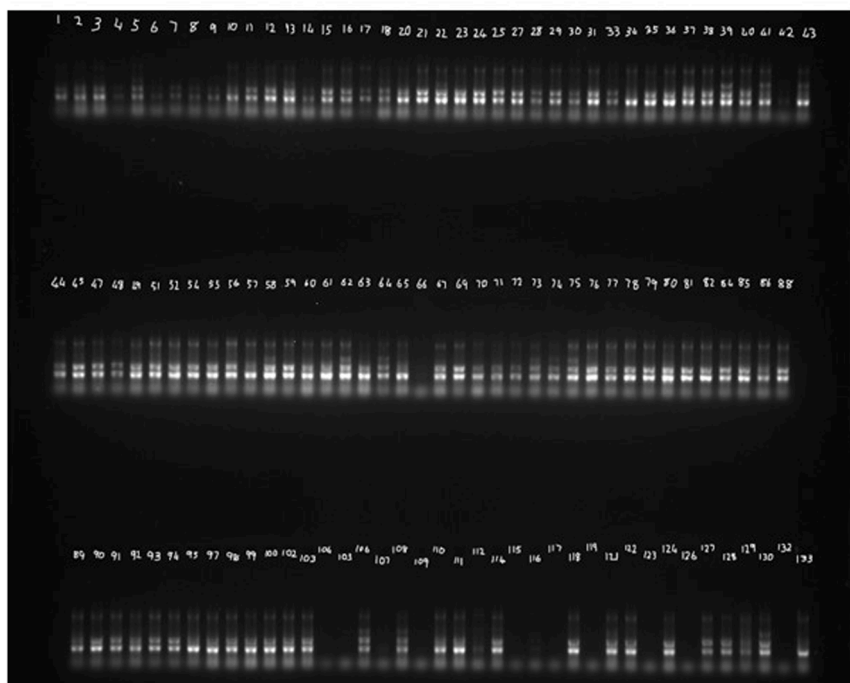


Fig. 3. Agarose gel showing the pre-screening of samples amplified with the Mao primer to determine which samples amplified well enough to be sent for automated detection. Each number directly corresponds to the sample code, e.g. 107 is TF107, and correlates to the locality and sample numbers provided in Table 2.

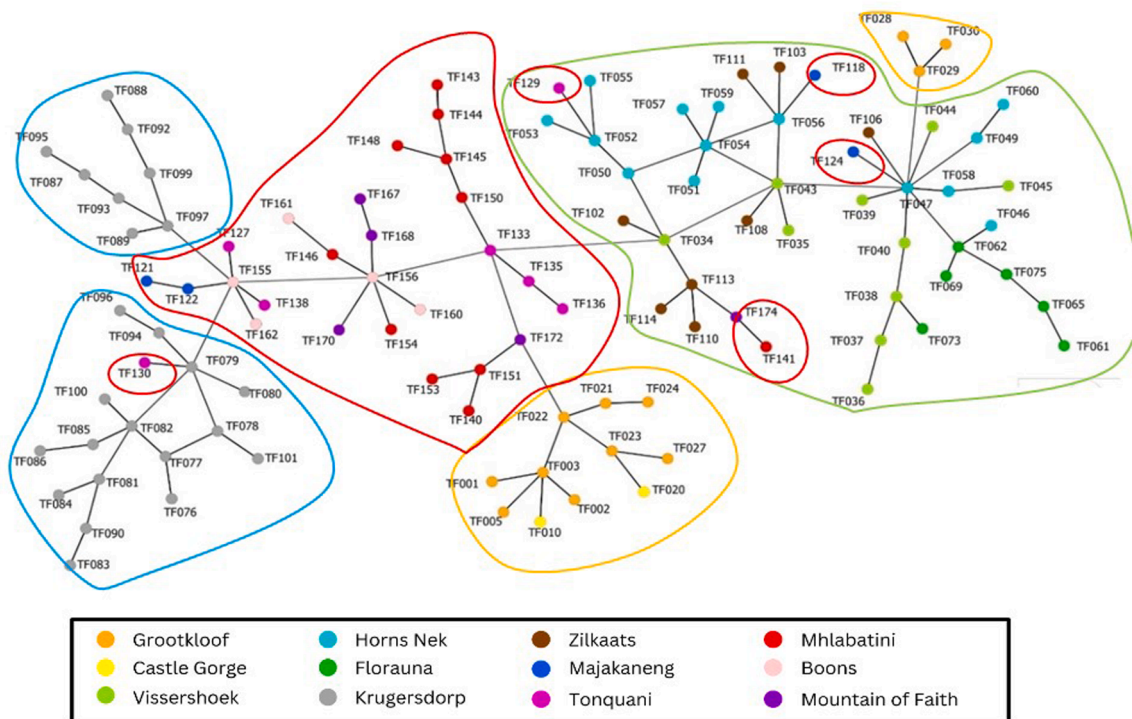


Fig. 4. Network analysis obtained using PopART. Colours of individual samples (labelled according to Table 2) and outlines of sample groupings correspond to localities and outlines indicated in Fig. 2.

Table 5

Results from the AMOVA analyses using the groups derived from different analytical methods.

Groups determined by geographical proximity and general groupings in the Network and UPGMA analyses.	
Variation among populations (%)	5.37185
Variation within populations (%)	84.09535
Variation among larger groups (%)	10.53279
Fixation index (1000 permutations) (ΦST)	0.15905
Significance (1000 permutations) (all indices)	< 0.001
Groups determined using the best-k value and the groups obtained from the STRUCTURE analysis.	
Variation among populations (%)	8.58834
Variation within populations (%)	84.06082
Variation among larger groups (%)	7.35084
Fixation index (1000 permutations) (ΦST)	0.15939
Significance (1000 permutations) (all indices)	< 0.001

2018) indicates a k-value of 3 (Fig. 6). There is a clear grouping of the Vissershhoek, Florauna and Horns Nek samples. These localities are the eastern most locations in the distribution range. This analysis does not distinguish the Grootkloof and Castle Gorge localities as completely anomalous, and indicates these two localities as having an affinity with larger eastern group. There is also a grouping of the more western localities of Majakaneng, Tonquani, Mountain of Faith, and Boons. In the case of Zilkaats, which is geographically close to the eastern group, the genetic affinity lies with the western group. The samples from the Krugersdorp locality are completely distinct.

4. Discussion

4.1. Samples, localities and groupings

In general, samples from each locality tended to group together, but with some mixing between adjacent localities, and the indication that these localities can be grouped into a bigger regional metapopulation

comprising the western and eastern groups along the Magaliesberg. The few samples which clustered in unexpected positions were generally found to have much higher band numbers than the minimum required to avoid the 25 % cut-off level, suggesting that insufficient data was not responsible for their unexpected placement.

A common, yet unexpected result across all of the data sets and analyses is that the Castle Gorge and Grootkloof localities are somewhat distinct. As noted above, numerous samples from the Castle Gorge were excluded when the 25 % of most poorly were removed. One possible reason for this poor amplification may be a consequence of the sampling and subsequent storage. The samples from these localities were gathered some months before the other samples, and were used for the PCR optimisation process. After this testing phase was over, DNA was then extracted from the remaining leaf material, which had by then been left in the dry silica for a few months. It is thus possible that the DNA of the Castle Gorge and Grootkloof samples degraded over time. In contrast, all of the other localities had their DNA extracted directly after they were collected and therefore did not spend extended amounts of time in the silica gel.

Investigating any other differences between samples such as the purity of the DNA did not suggest any anomalies, as the purity of the extracts from these two localities were in the same ranges as the DNA from all other locations (Data not shown but available upon request). This may simply indicate that these two localities are genetically distinct and do not share as much genetic material with neighbouring localities as a result of a lack of pollinator visitations or seed dispersal between these areas and the ones nearby. However, the possibility of DNA degradation due to a longer storage time remains the most logical explanation for the anomalous results obtained here.

4.2. How many populations of *Aloe peglerae* are there?

All of the analyses suggest an east-west linear grouping of the sample localities along the Magaliesberg distribution range. The localities on the western side of the distribution range grouped together, with some intermixing of samples from the constituent locality. The localities on

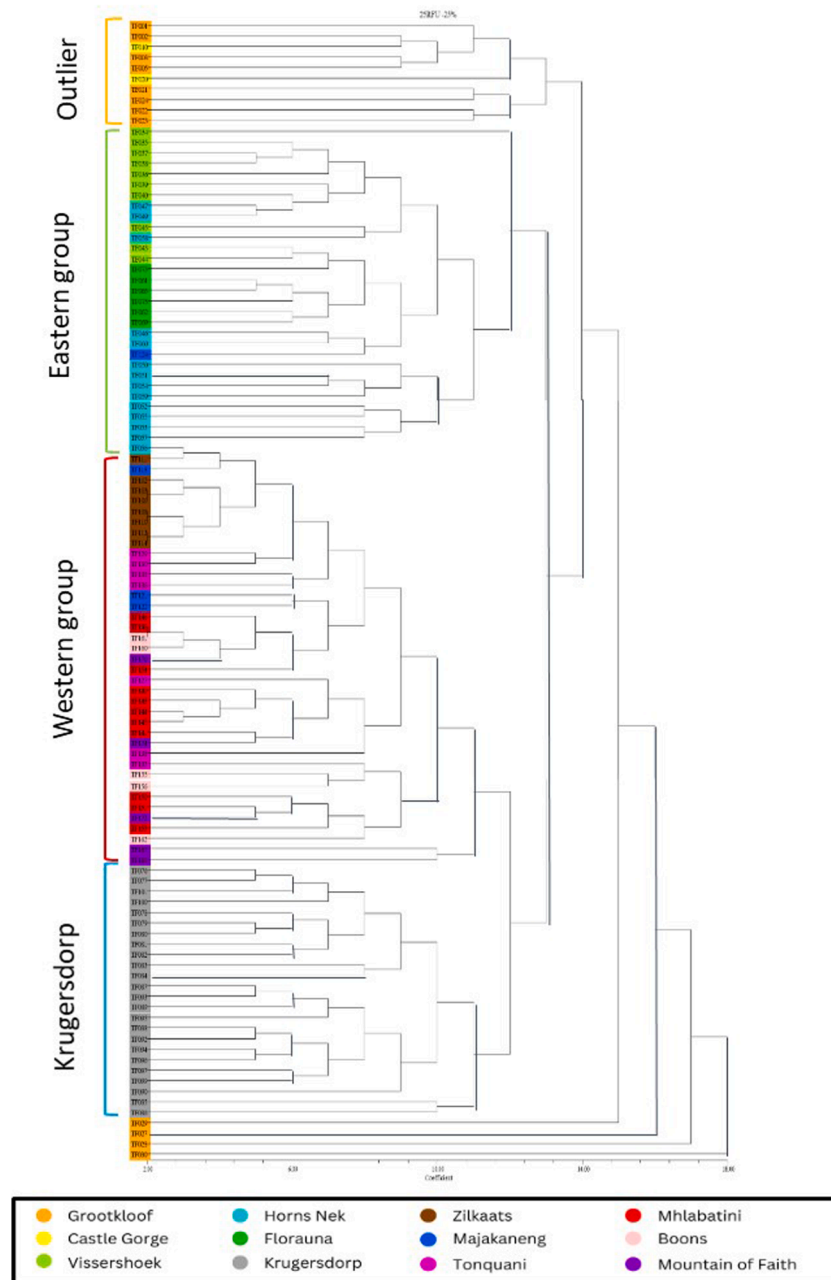


Fig. 5. the NJ clustering phenogram based on the DICE similarity coefficient obtained using NTSYS. The larger groupings of the populations are indicated by coloured lines, and individual sample colours correspond to the colours shown in Fig. 1.

the eastern side of the distribution range show a similar trend. Interestingly, in the results from most analyses, samples from Zilkaats do not cluster entirely with the other eastern localities, but rather have an intermediate similarity to both the eastern and western groups. Had we sampled a locality in the Hartbeespoort area, it is conceivable that the linking of the east and west localities would become continuous.

Even though the samples from each sampling locality were generally grouped together irrespective of the method of analysis, these cannot be considered as discrete populations, as the AMOVA results suggest that geneflow between the eastern and western sampling localities is occurring or has recently occurred. Furthermore, had the Hartbeespoort area been sampled, the distinction between the western and eastern groupings may well fall away, and the entire Magaliesberg area of occupancy be viewed as a single panmictic population.

In contrast, the Krugersdorp locality was consistently distinct in all our results, and forms its own separate cluster with minimal mixing from

the other sampling localities from the Magaliesberg, and can thus be considered a second distinct and smaller population. Thus, we propose that *A. peglerae* exists in two populations, each of which are genetically distinct, with the Magaliesberg population much larger and more genetically variable than the Krugersdorp population.

4.3. Implications for the conservation of *Aloe peglerae*

The threats faced by *A. peglerae* are still prevalent, and indeed increasing. It is clear that even when many of the localities are located within protected areas (The Magaliesberg Biosphere Reserve and private conservation areas within this), they are not immune to the threat of poaching, as was evident by the possible extirpation of all *A. peglerae* in Kgawane Nature Reserve. This raises concerns that in-situ conservation alone will not be enough to safeguard populations of *A. peglerae* against long term extinction. Conservation efforts of *A. peglerae* must consider

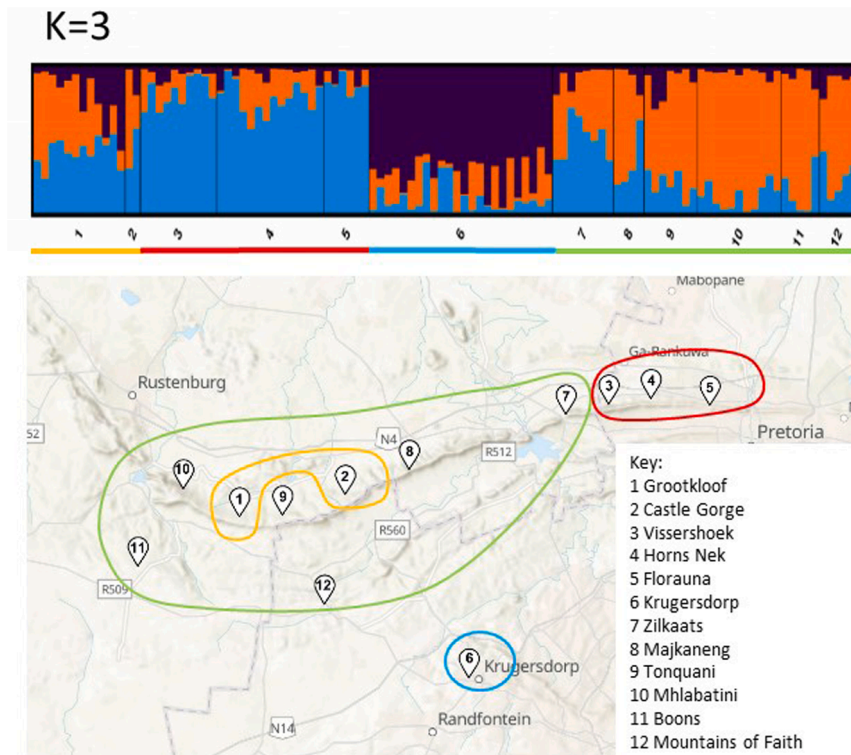


Fig. 6. Result from the STRUCTURE analysis. Each vertical bar represents a single plant ($n = 108$). The overall genetic similarity groupings obtained from the STRUCTURE analysis are indicated by the coloured bars and these correlate to the polygons on the map. Numbered locality groupings in the STRUCTURE analysis are also indicated on the map.

the ongoing threats faced by these populations in order to overcome them and successfully reach conservation targets. The results of this genetic diversity study will contribute to these future conservation efforts.

The finding that this species can be recognized as comprising of two populations, one of which is small and localized, and the other widespread but more genetically diverse, has implications for the conservation of the species. The consideration of genetic diversity has only recently been emphasized in translocations (Schäfer et al., 2020). This is likely due to the cost and time constraints associated with analyses of genetic diversity. However, as shown by this study, technology has advanced sufficiently that this is no longer the case, and genetic diversity can be readily considered when setting up a genetic conservation area (GCA; Grادل et al., 2022). Depending on the plant species studied, various levels of conservation have been identified as required to meet genetic conservation thresholds (Neel and Cummings 2003; Whitlock et al. 2016; Hoban et al., 2021). Grادل et al. (2022) concluded that there needs to be a minimum conservation of two to three populations to preserve 95 %, and six to nine populations to preserve 99 % of genetic diversity. In other studies, it was found that >35 % of populations will need to be conserved in order to reach the Convention on Biological Diversity's recommendation to conserve 70 % of genetic diversity (Brown and Briggs, 1991; Whitlock et al., 2016; Grادل et al., 2022). The discrete Krugersdorp population is small, genetically distinct, and under substantial threat from various anthropogenic activities including mining and urbanization. This population is thus in urgent need of in-situ and ex-situ conservation measures. Ex-situ conservation of the Krugersdorp population should thus be done using propagules sourced from within this population, with an emphasis on utilising propagules from as many source individuals as possible (Schäfer et al., 2020).

The geneflow in the Magaliesberg population makes it possible to augment shrinking localities within this range with seeds or seedlings derived from other nearby localities without risk of outbreeding

depression (Frankham et al., 2011; Marsden et al., 2013), and it is suggested that practises such as those outlined by Bragg et al. (2020) are adopted. The translocation efforts in the Hartbeespoort Dam area mentioned above are thus not going to negatively impact this species. However, translocating material from more distant localities must be avoided.

4.4. Methodological considerations

As ISSR has the challenge of reproducibility (Ng and Tan, 2015; Amiteye, 2021), it is essential to be consistent with the treatment of samples during sampling, storage, DNA-extraction, and PCR optimization. The inconsistency of sample storage potentially affected the results from the Castle Gorge and Grootkloof localities used in this study, highlighting the importance of a rigorous and consistent treatment of samples. The hypotheses that storage condition may have resulted in DNA degradation could be assessed by means of gel electrophoretic or other methods, and should be considered in any similar future studies.

The use of the Oktopure DNA-extraction robot to provide bulk DNA extractions likely helped to minimize variability in the DNA-extraction process. It is also imperative to maintain DNA concentration within certain thresholds to increase the rates of successful amplification, and it is also recommended that the same thermal cyclers are used (Prince, 2015).

The efficacy of the methods of DNA extraction and PCR amplification could also be better assessed if duplicate samples were included, as one of the main concerns with ISSR analysis relates to a lack of reproducibility. Including replicates of the samples would allow for the comparison of amplified products from the same but replicated samples, and bands common to both samples included in analyses. However, the inclusion of too many duplicates would increase the costs of analysis (Prince, 2015; Murphy et al., 2024).

5. Conclusion

Given the disparity in the distribution of biodiversity between the global North and global South versus the location of, and investment in, conservation activities (Hobohm and Barker, 2023), the biodiversity-rich nations and regions which are generally from the developing world need an accurate and rapid means to assess genetic diversity of species of conservation concern. Modern molecular methods have enabled these studies, but, as indicated by Carvalho et al. (2019), the adoption of certain methods shows a bias towards a country or region. Furthermore, there appears to be no preferential application of the method to rare species (Carvalho et al., 2019).

Despite the richness of South Africa's flora and the high proportion of rare taxa, studies that document the genetic diversity of South African rare plants are few and far between. Those that have been conducted include studies using allozymes (Matolweni et al., 2000), RAPD (Tansley and Brown, 2000; Barker et al., 2012), AFLP (Da Silva et al., 2012) and ISSR's (Bentley et al., 2015; Murphy et al., 2024).

Here we demonstrate the value of automated detection of ISSR PCR profiles for assessing the genetic diversity of the Critically Endangered *Aloe peglerae*. Our results show the value of the automated ISSR fingerprinting as a viable method for determining the genetic diversity across this species' distribution range, providing large amounts of informative data which would not be possible using conventional gel-based scoring methods. With the application of the rigorous sampling, DNA extraction, and PCR amplification methods, we recommend this method as a cost-effective means of rapidly assessing genetic diversity of rare plant species and populations.

Funding

This research was funded by the University of Pretoria awarded to NP Barker. X. Schmidt acknowledges the Haylett Trust Fund for financial support.

CRediT authorship contribution statement

Xander Schmidt: Writing – original draft, Software, Investigation, Formal analysis. **Nigel P. Barker:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Arnold Frisby:** Writing – review & editing, Supervision, Investigation.

Declaration of competing interest

I hereby declare there are no conflicts of interest relating to the submission titled

Acknowledgements

We are grateful to Renate Zipfel and Deanne Murphy for their help and guidance in using the GeneMapper 6 software, and some of the analytical methods used in this study. We thank all of the landowners that provided access to their properties and, in some cases, taking us straight to localities of *A. peglerae*.

References

- Amiteye, S., 2021. Basic concepts and methodologies of DNA marker systems in plant molecular breeding. *Heliyon* 7.
- Arena, G., 2013. The Reproductive Biology and Habitat Requirements of *Aloe peglerae*, a Montane Endemic Aloe of the Magaliesberg Mountain Range, South Africa. MSc thesis, University of the Witwatersrand, Johannesburg, South Africa.
- Arena, G., Witkowski, E.T.F., Symes, C.T., 2015. Growing on rocky ground: microhabitat predictors for locality-occupancy of *Aloe peglerae*, an endangered endemic species with a restricted range. *South African J. Bot.* 100, 174–182.
- Bandelt, H.J., Forster, P., Rohl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* 16, 37–48.
- Bellis, J., Osazuwa-Peters, O., Maschinski, J., Keir, M.J., Parsons, E.W., Kaye, T.N., Kunz, M., Possley, J., Menges, E., Smith, S.A., Roth, D., 2024. Identifying predictors of translocation success in rare plant species. *Conserv. Biol.* 38 (2), e14190.
- Bentley, L., Barker, N.P., Dold, A.P., 2015. Genetic diversity of the endangered *Faucaria tigrina* (Aizoaceae) through ISSR “fingerprinting” using automated fragment detection. *Biochem. Syst. Ecol.* 58, 156–161.
- Bragg, J.G., Cuneo, P., Sherieff, A., Rossetto, M., 2020. Optimizing the genetic composition of a translocation population: incorporating constraints and conflicting objectives. *Mol. Ecol. Resour.* 20, 54–65.
- Brook, B.W., Tonkyn, D.W., O'Grady, J.J., Frankham, R., 2002. Contribution of inbreeding to extinction risk in threatened species. *Conserv. Ecol.* 6 (1).
- Brown, H.D., Briggs, J.D., 1991. Sampling strategies for genetic variation in ex situ collections of endangered plant species. In: Falk, D.A., Holsinger, K.E. (Eds.), *Genetics and Conservation of Rare Plants*. Oxford University Press New York, NY, pp. 99–120.
- Carvalho, Y.G., Vitorino, L.C., Souza, U.J.D., Bessa, L.A., 2019. Recent trends in research on the genetic diversity of plants: implications for conservation. *Diversity (Basel)* 11 (4), 62.
- Da Silva, J.M., Donaldson, J.S., Reeves, G., Hedderson, T.A., 2012. Population genetics and conservation of critically small cycad populations: a case study of the Albany Cycad, *Encephalartos latifrons* (Lehmann). *Biolog. J. Linnean Society* 105, 293–308.
- Donaldson, J., Nänni, I., Zachariades, C., Kemper, J., 2002. Effects of habitat fragmentation on pollinator diversity and plant reproductive success in renosterveld shrublands of South Africa. *Conserv. Biol.* 16 (5), 1267–1276.
- Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. *Mol. Ecol.* 14, 2611–2620.
- Fang, D.Q., Roose, M.L., 1997. Identification of closely related citrus cultivars with inter-simple sequence repeat markers. *Theoret. Appl. Genet.* 95, 408–417.
- Fitzpatrick, B.M., 2009. Power and sample size for nested analysis of molecular variance. *Mol. Ecol.* 18, 3961–3966.
- Frisby, A.W., Momberg, M., le Roux, P.C., 2025. Improving success rates of remote conservation translocations by mitigating harsh in-situ environmental conditions: a case study on a critically endangered succulent. *J. Nat. Conserv.*, 126851.
- GBIF.org, 2022. GBIF Occurrence download [WWW Document]. URL <https://doi.org/10.15468/dl.ubp9nq> (accessed 6.13.24).
- Ge, X.J., Zhang, L.B., Yuan, Y.M., Hao, G., Chiang, T.Y., 2005. Strong genetic differentiation of the East-Himalayan *megacodon stylophorus* (Gentianaceae) detected by Inter-simple sequence repeats (ISSR). *Biodivers. Conserv.* 14, 849–861.
- Godefroid, S., Lacquaye, S., Ensslin, A., Dalrymple, S., Abeli, T., Branwood, H., Ferrando Pardo, I., Ferrer Gallego, P.P., Zippel, E., Gouveia, L., Lobo, C.A., 2025. Current state of plant conservation translocations across Europe: motivations, challenges and outcomes. *Biodivers. Conserv.* 34, 769–792.
- Gradi, E., Lehman, T.A., Poschlod, P., Reisch, C., 2022. Building up a network of genetic conservation areas – A comprehensive approach to select target localities for the preservation of genetic variation in wild plant species. *Biolog. Conserv.* 274, 109748.
- Harris, E., Siebert, S.J., Smit, J.H.L., Van den Berg, J., 2014. Translocation of an endangered succulent plant species from sandstone outcrops earmarked for coal mining. *J. South Afr. Inst. Min. Metall.* 114, 904–912.
- Hao, B., Li, W., Linchun, M., Li, Y., Rui, Z., Mingxia, T., Weikai, B., 2006. A study of conservation genetics in *Cupressus chengiana*, an endangered endemic of China, using ISSR markers. *Biochem. Genet.* 44, 31–45.
- Hoban, S., Bruford, M.W., Funk, W.C., Galbusera, P., Griffith, M.P., Grueber, C.E., Heuert, M., Hunter, M.E., Hvilson, C., Stroil, B.K., Kershaw, F., 2021. Global commitments to conserving and monitoring genetic diversity are now necessary and feasible. *Bioscience* 71, 964–976.
- Hobohm, C., Barker, N., 2023. Centers of endemism and the potential of zoos and botanical gardens in conservation of endemics. *J. Zool. Bot. Gard.* 4, 527–548.
- IUCN/SSC, 2013. Guidelines for Reintroductions and Other Conservation Translocations. Version 1.0. IUCN Species Survival Commission, Gland, Switzerland. www.iucnsscrs.org.
- IUCN, 2024. Magaliesberg Aloe [WWW Document]. IUCN Red List Threatened Spec. URL <https://www.iucnredlist.org/species/39586/10242961> accessed 6.27.24.
- Leigh, J.W., Bryant, D., 2015. POPART: full-feature software for haplotype network construction. *Methods Ecol. Evol.* 6, 1110–1116.
- Li, Y.L., Liu, J.X., 2018. StructureSelector: a web-based software to select and visualize the optimal number of clusters using multiple methods. *Mol. Ecol. Resour.* 18, 176–177.
- Loera-Sánchez, M., Studer, B., Kölliker, R., 2019. DNA-based assessment of genetic diversity in grassland plant species: challenges, approaches, and applications. *Agronomy* 9, 881.
- Marsden, B.W., Engelhardt, K.A.M., Neel, M.C., 2013. Genetic rescue versus outbreeding depression in *Vallisneria americana*: implications for mixing seed sources for restoration. *Biol. Conserv.* 167, 203–214.
- Maschinski, J., Coates, D., Monks, L., Dillon, R., Barrett, S., Possley, J., Lange, J., Duquesnel, J., Goodman, J., Hermanutz, L., Copp, C., 2023. Rare and threatened plant conservation translocations: lessons learned and future directions. *Ecological Restoration: Moving Forward Using Lessons Learned*. Springer International Publishing, Cham, pp. 287–322.
- Matolweni, L.O., Balkwill, K., McLellan, T., 2000. Genetic diversity and gene flow in the morphologically variable, rare endemics *Begonia dregei* and *Begonia homonyma* (Begoniaceae). *Am. J. Bot.* 87, 431–439.
- Menges, E.S., 2008. Restoration demography and genetics of plants: when is a translocation successful? *Aust. J. Bot.* 56, 187–196.
- Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. 2010 Gateway Computing Environments Workshop (GCE). IEEE, pp. 1–8.

- Milton, S.J., Bond, W.J., Du Plessis, M.A., Gibbs, D., Hilton-Taylor, C., Linder, H.P., Raitt, L., Wood, J., Donaldson, J.S., 1999. A protocol for plant conservation by translocation in threatened lowland fynbos. *Conserv. Biol.* 13, 735–743.
- Mohamad, A., Alhasnawi, A.N., Kadhim, A.A., Isahak, A., Yusoff, W.M.W., Che Radziah, Z., 2017. DNA isolation and optimization of ISSR-PCR reaction system in *Oryza sativa* L. *Int. J. Adv. Sci., Eng. Inform. Technol.* 7 (6), 2264.
- Mondini, L., Noorani, A., Pagnotta, M.A., 2009. Assessing plant genetic diversity by molecular tools. *Diversity (Basel)* 1, 19–35.
- Murphy, D., Barker, N.P., Frisby, A., 2024. Automated ISSR fingerprinting is a cost-effective way to assess genetic diversity and taxonomic differentiation—A case study on the *Encephalartos eugene-maraisii* species complex. *Diversity (Basel)* 6, 507.
- Neel, M.C., Cummings, M.P., 2003. Effectiveness of conservation targets in capturing genetic diversity. *Conserv. Biol.* 17, 219–229.
- Ng, W.L., Tan, S.G., 2015. Inter-simple sequence repeat (ISSR) markers: are we doing it right. *Akademi Sains. Malaysia Sci. J.* 9, 30–39.
- Novak, B.J., Phelan, R., Weber, M., 2021. US conservation translocations: over a century of intended consequences. *Conserv. Sci. Pract.* 3 (4), e394.
- Nybom, H., 2004. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Mol. Ecol.* 13, 1143–1155.
- Ottewell, K.M., Bickerton, D.C., Byrne, M., Lowe, A.J., 2016. Bridging the gap: a genetic assessment framework for population-level threatened plant conservation prioritization and decision-making. *Divers. Distribut.* 22, 174–188.
- Payne, S.L., Witkowski, E.T.F., Symes, C.T., 2019. Good times, bad times: inter-annual reproductive output in a montane endemic succulent (*Aloe peglerae* Asphodelaceae) driven by contrasting visitor responses of small mammals and birds. *Aust. J. Bot.* 67, 116–127.
- Pfab, M.F., Victor, J.E., 2002. Threatened plants of Gauteng. *South Afr. J. Bot.* 68, 370–375.
- Pfab, M.F., Scholes, M.A., 2004. Is the collection of *Aloe peglerae* from the wild sustainable? An evaluation using stochastic population modelling. *Biol. Conserv.* 118, 695–701.
- Pfab, M.F., von Staden, L., Hahn, N., 2016. *Aloe peglerae* Schönland. *Natl. Assessm.: Red List of South Afr. Plants [WWW Document]*.
- Phama, J.O., Panagos, M.D., Myburgh, W.J., Pfab, M.F., 2014. The population status of the endangered endemic plant *aloe peglerae*: area of occupancy, population structure, and past population trends. *South Afr. J. Bot.* 93, 247–251.
- Porras-Hurtado, L., Ruiz, Y., Santos, C., Phillips, C., Carracedo, Á., Lareu, M.V., 2013. An overview of STRUCTURE: applications, parameter settings, and supporting software. *Front Genet* 4, 98.
- Prince, L.M., 2015. Plant genotyping using fluorescently tagged inter-simple sequence repeats (ISSRs): basic principles and methodology. *Methods Mol. Biol.* 1245, 63–75.
- Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. *Genetics* 155, 945–959.
- Pritchard, J.K., Wen, X. and Falush, D., 2010. Documentation for structure software: version 2.3. University of Chicago, Chicago, IL, 1, p. 37.
- Rohlf, F.J., 2009. Applied Biostatistics, Inc., Exeter Software (Firm). NTSYS-pc : Numerical Taxonomy and Multivariate Analysis System. Applied Biostatistics, Inc.
- SANBI (South African National Biodiversity Institute), 2019. National Biodiversity Assessment 2018: The Status of South Africa's Ecosystems and Biodiversity. Part 1: South Africa's Biodiversity Profile. Synthesis Report. South African National Biodiversity Institute, an entity of the Department of Environment, Forestry and Fisheries, Pretoria, pp. 31–38.
- SANBI (South African National Biodiversity Institute), 2024. Magaliesberg Aloe [WWW Document]. Red List of South African Plants. URL: <http://redlist.sanbi.org/species.php?species=2206-198>. accessed 6.27.24.
- Schäfer, D., Vincent, H., Fischer, M., Kempel, A., 2020. The importance of genetic diversity for the translocation of eight threatened plant species into the wild. *Glob. Ecol. Conserv.* 24, e01240.
- Silcock, J.L., Simmons, C.L., Monks, L., Dillon, R., Reiter, N., Jusaitis, M., Vesk, P.A., Byrne, M., Coates, D.J., 2019. Threatened plant translocation in Australia: a review. *Biolog. Conserv.*, 236, 211–222.
- Sokal, R.R., Sneath, P.H.A., 1973. Principles of Numerical Taxonomy. W. H. Freeman, San Francisco, CA, USA.
- Subedee, B.R., Tripathi, G.R., Chaudhary, R.P., 2020. DNA isolation and optimization of PCR protocol for ISSR analysis of *girardinia diversifolia*: a medicinal and economic plant species from Nepal Himalaya. *Afr. J. Biotechnol.* 19, 747–753.
- Tansley, S.A., Brown, C.R., 2000. RAPD variation in the rare and endangered *Leucadendron elimense* (Proteaceae): implications for their conservation. *Biol. Conserv.* 95, 39–48.
- Taylor, C.L., Barker, N.P., 2012. Species limits in *Vachellia (Acacia) karroo* (Mimosoideae: leguminosae): evidence from automated ISSR DNA “fingerprinting”. *South Afr. J. Bot.* 83, 36–43.
- van Jaarsveld, E., 1999. The reintroduction of *Gasteria baylissiana*—A check up. *Brit. Cactus Succulent J.* 17, 119–121.
- Waples, R.S., Gaggiotti, O., 2006. An empirical evaluation of some genetic methods for identifying the number of gene pools and their degree of connectivity. *Mol. Ecol.* 15, 1419–1439.
- Whitlock, R., Hipperson, H., Thompson, D.B.A., Butlin, R.K., Burke, T., 2016. Consequences of in-situ strategies for the conservation of plant genetic diversity. *Biol. Conserv.* 203, 134–142.
- Xiao, L.Q., Ge, X.J., Gong, X., Hao, G., Zheng, S.X., 2004. ISSR variation in the endemic and endangered plant *Cycas guizhouensis* (Cycadaceae). *Ann. Bot.* 94, 133–138.
- Zimmer, H.C., Auld, T.D., Cuneo, P., Offord, C.A., Commander, L.E., 2019. Conservation translocation - an increasingly viable option for managing threatened plant species. *Aust. J. Bot.* 67, 501–509.