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Disentangling genetic variation in the Critically Endangered tangle wattle (Acacia volubilis) to guide seed production area development and conservation management

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Abstract

Threatened plant species with consistently low or absent seed set are challenging for conservation because seed typically form the primary source of regenerative material used in recovery programs. For such species, Seed Production Areas (SPAs), artificial populations maintained for seed production, offer the potential to produce and harvest larger volumes of seed, which can then be utilised for restoration and conservation activities. Genomic data can inform how founding plants should be sourced and arranged in SPAs to enhance the production of genetically diverse seed. Here, we use range-wide sampling and genome-wide single nucleotide polymorphisms to assess clonality, population structuring, and genetic diversity to inform the conservation management of a Critically Endangered species, *Acacia volubilis*, which sets little to no seed in wild sites. Clonality was found to be common in *A. volubilis*, although the patterns of clonality varied among the sites sampled. Ordination, sNMF, and AMOVA analyses detected moderate population structuring, with three genetic clusters that aligned with northern and southern disjunctions across the species' range. Southern genetic clusters showed higher estimates of heterozygosity and lower estimates of inbreeding compared to the more disturbed northern cluster. Genetic diversity in an existing translocation was comparable to the wild source site, with no indications of clonality. We recommend sourcing material from all three genetic clusters to establish a SPA for *A. volubilis* to maximise opportunities for outcrossing and promote a genetically diverse seed supply. Our results highlight the value of collecting baseline genetic data to inform SPA design and assist in managing remaining genetic diversity.

INTRODUCTION

Ex situ seed banking is a widely applied approach for maintaining genetically representative seed collections of threatened plant species for use in conservation management activities, including translocation. Ideally, these seed are harvested from multiple wild populations across a species' range to capture interpopulation neutral and adaptive genetic diversity (Martyn Yenson et al. 2023). However, some species, termed exceptional species, can be challenging to conserve as they: i) may produce little or no seed, ii) seeds are produced but they cannot survive drying, iii) seeds are produced and can survive partial drying, but cannot be stored long-term, or iv) seeds are produced, but they exhibit complex dormancies that are difficult to break (Pence et al. 2022, White et al. 2023; Martyn Yenson et al. 2023). As such, seed for these species may not be available in sufficient quantities, or be able to produce adequate seedlings, to support conservation management activities (Pence et al. 2022; Martyn Yenson et al. 2023). These species require unique resources, skills and technologies to develop scientifically informed species conservation strategies that improve their recovery.

For exceptional species that produce limited seed in the wild, Seed Production Areas (SPAs) offer a means of increasing seed availability. Typically used to generate large amounts of seed for broad-scale restoration (Nevill et al. 2016; Broadhurst et al. 2021; Gibson-Roy et al. 2021), SPAs are increasingly being considered to facilitate the recovery of rare species (St. Clair et al. 2020; Barrett et al. 2024; Hardstaff et al. 2025). These purpose-designed areas have adjustable external inputs (e.g., water, fertiliser, and light) that aim to increase seed production (Pierson et al. 2016, Broadhurst et al. 2021; Zinnen et al. 2021). The source material and their arrangement within the SPA can also be managed to maintain or improve genetic diversity in the resulting seed produced. For instance, plants can be arranged to either mimic wild population structuring (e.g., Broadhurst et al. 2021) or allow for mixtures of multiple populations that may otherwise be fragmented in the wild (St. Clair et al. 2020). Mimicking wild population structure may preserve natural mating patterns, while admixing may overcome mate-limitation, and potentially inbreeding, by facilitating greater access to unrelated mates (St. Clair et al. 2020; Broadhurst et al. 2021). Ultimately, enhancing the genetic diversity of SPA-derived seed may strengthen adaptive potential for exceptional species with low seed set,

thereby buffering translocated populations established using this seed against founder effects and inbreeding depression, both of which are commonly associated with small populations (Ellstrand and Elam 1993; Allendorf et al. 2013; St. Clair et al. 2020). Outcrossing between locally adapted source populations within SPAs may, however, unintentionally erode progeny fitness through outbreeding depression (Pierson et al. 2016). SPAs therefore require appropriate planning (e.g., tailored mixing or separation of source populations) and active management (e.g., assessing genetic diversity, inbreeding, and relatedness) to balance both the genetic advantages and potential risks (Pedrini et al. 2020; St. Clair et al. 2020; Leger et al. 2021). To avoid unintended negative genetic legacies, such as inbreeding or outbreeding depression that can undermine conservation efforts (Dolan et al. 2008; Dyer et al. 2016), genetic assessments of the target species are recommended (Broadhurst et al. 2017). These assessments help ensure that key factors, including clonality, population structuring and genetic diversity, are considered when planning SPA establishment.

The Southwest Australian Floristic Region (SWAFR) is a globally recognised biodiversity hotspot containing over 9,000 plant taxa, around half of which are considered endemic to the region (Myers et al. 2000; Gioia and Hopper, 2017; Brundrett et al. 2021). This rich diversity of both relictual and recently evolved taxa is due to both the persistence of many species since the Pleistocene and limited historic extinctions (Myers et al. 2000; Hopper and Gioia, 2004; Byrne, 2007). The SWAFR has, however, undergone significant land transformation since European settlement, with almost 70% of the region's primary vegetation removed and, like other global biodiversity hotspots, is at significant conservation risk from habitat fragmentation and degradation (Myers et al. 2000; Mittermeier et al. 2011; Monks et al. 2019). The SWAFR has over 399 state-listed Threatened species and more than 2000 poorly known or rare state-listed Priority species within its boundaries (Monks et al. 2019). Though these patterns of diversity and land transformation are characteristic of the entire SWAFR, they are most pronounced in the Transitional Rainfall Zone (300–800 mm annual rainfall zone) in Western Australia's primary agricultural region, the Wheatbelt (Maslin and Striton, 1997; Beard et al. 2000; Buist et al. 2002). Here, the significant clearing of native vegetation has resulted in fragmented and often small natural remnants (Beeston et al. 1996). These circumstances often correlate with increased levels of inbreeding depression and may result in low seed production and reduced fitness for the remaining native vegetation (Coates and Atkins, 2001). These factors compound the conservation challenge to restore genetic diversity and increase population sizes for any remaining threatened species.

Acacia, Australia's largest plant genus, shows both the highest levels of species diversity and an overrepresentation of threatened species in the Western Australian Wheatbelt region (ca. 30% of all threatened *Acacia* in W.A.; see Hopper 1979; Hnatiuk and Maslin 1988; Buist et al. 2002). *Acacia volubilis* Mueller is one such threatened Wheatbelt species, historically known from just 19 sites, and currently known from fewer than 240 individuals, distributed among 16 small and fragmented sites that are largely restricted to highly degraded road verges (Harris and Brown 2003) (Figs. 1 and 2). These road verge populations are threatened by a combination of weed competition, herbicide drift, modified fire regimes, road verge grading, and changes in hydrology due to agricultural intensification (Harris and Brown 2003). Additionally, seed production of the species is low, with seed not observed at most sites. A combination of low plant numbers, low recruitment and high degree of threat has resulted in *A. volubilis* being listed as Critically Endangered both under state (*Western Australian Biodiversity Conservation Act 2016)* and Commonwealth (*Environment Protection and Conservation Act 1999*) legislation.

Two factors have constrained conservation efforts for *A. volubilis* to date. The first is limited germplasm availability for recovery actions and the second is lack of knowledge regarding levels of clonality in the species. In 2004, a declining site was augmented using both seedlings and cuttings. However, limited seed availability for the species meant that seed was largely sourced from a single, small site and cutting propagation methods generally had a low success rate. Repeated attempts to collect seed for ex situ banking have largely been unsuccessful for most sites

(Subroy et al. 2021), resulting in the species being categorised as an exceptional species (EF1b; Martyn Yenson et al. 2023). Further translocation planting after 2004 has therefore only involved small numbers of seedlings. To increase seed availability for this species, a SPA was proposed, which would facilitate an expanded translocation program (Subroy et al. 2021). The second issue is that clonal regeneration via rhizomes has been observed for the species, however the extent of clonality was unknown, such that the number of genetically distinct plants in each of the remaining sites may be lower than census counts indicate (Harris and Brown, 2003), meaning overall genotypic diversity may be much reduced. Baseline genetic information is needed regarding the levels of clonality within sites, and the genetic differentiation among sites, to guide the management of diversity within the SPA.

Here, we undertook range-wide sampling and high-resolution population genomic analysis to assess spatial patterns of genetic variation within and among sites of *A. volubilis*. Specifically, we aimed to address the following questions to improve understanding of the wild and translocated sites to guide management of the proposed SPA and existing translocation, with the aim of maintaining, and where possible enhancing, the genetic resilience of this Critically Endangered exceptional species:

1). What is the extent of clonality within wild sites?

2). Does the previous translocation capture the genetic diversity of the source sites?

3). What is the genetic diversity of the remaining wild sites and how differentiated are they across the species' range?

METHODS

Study species

Acacia volubilis, commonly known as tangle wattle, is a small dome-shaped shrub that grows to 0.4 m high and up to 1 m wide (Mueller, 1877; Maslin, 1995). Large numbers of golden-coloured globular inflorescences are produced predominantly between June and August (Harris and Brown 2003). The reproductive biology of *A. volubilis* has not been studied, although insect-mediated pollination is assumed based on studies of pollination syndromes within the genus (Bernhardt 1987; Stone et al. 2003). *Acacia volubilis* can exhibit a range of growth habits (Fig. 1). When co-occurring with dense vegetation, including weeds, *A. volubilis* shows a prostrate morphology, while in more open habitats, it forms dome shaped clumps, either discrete or intermingled. The 19 historically known occurrences of *A. volubilis* have a disjunct geographical distribution (Fig. 2), with a large group of northern sites (sites A-L) separated by ca. 50 km from a smaller number of southern sites (sites M-P). The translocation is situated in the northern group and is an augmentation of a declining wild population (site H). Five cohorts of plants were established in the translocation across five non-consecutive planting sessions between 2004 and 2018. These five cohorts of plants were propagated from both seed (*N* = 270 plants) and cuttings (*N* = 19 plants), with material largely sourced from site C (94% of plants) but also sites G, O and P (6%). All plants had been tagged using aluminium tags with a unique plant number to support long term monitoring. At the time of sampling for this study (2024), 56 translocated plants had survived, most of which were sourced from site C, with only a single living plant from each of sites G and O (see Fig. 2). None of the cutting derived plants survived.

Sampling

Green stem tissue and phyllodes were sampled across the distribution of *A. volubilis* in 2024. To attain a baseline of clonality within each site, tissue samples were collected from all visually distinct plants, and several samples were

taken within large clumps that appeared to comprise multiple individuals or in weed-infested areas that made visual plant separation challenging (Fig. 1). For this purpose, we defined a visually distinct plant as any individual that was not physically connected above ground to neighboring plants. Additionally, GPS coordinates of each sample were recorded with the Garman Handheld GPSMap 67i GPS (Kansas, USA) (accuracy ±5 m). For improved fine-scale accuracy, the maximum distance between ramets within each genet (i.e., largest distance between identifiable stems within a genotype) and the maximum width of each group of ramets (i.e., multiple clumps all with the same genotype) were measured manually using a tape measure. In total, all known 19 sites were visited, but only 16 sites were sampled because three previously recorded sites had no living plants. This resulted in 12 sites sampled across the northern region and four sites in the southern region (Fig. 2). Sample sizes varied among populations (Table 1), yielding a total of 244 samples. Of these, 211 were from suspected ramets, while 33 were collected from apparent single plants to determine whether they represented a single individual or multiple intermixed genets.

For the augmented site H, 60 samples were collected in total, 51 of these were from known translocated plants (see section *Study species* for further details), five were from suspected wild individuals within the fenced translocation areas, and four were sampled from outside the fenced translocation areas and were assumed to be wild as they over 35 m from the nearest translocated plant, (see section *Genomic variation - Translocated populations* for further details). Monitoring the translocation in 2024, after this study was completed, indicated that five individuals were not sampled during our study.

DNA extraction, library preparation and sequencing

Genomic DNA was extracted from freeze-dried tissue using a 2% CTAB protocol (Doyle and Doyle, 1987) modified by the addition of 2% w/v polyvinylpyrrolidone, 2% 2-mercaptoethanol and 0.1 M sodium sulfite to the extraction buffer (Byrne et al. 2001). DNA samples were sent to Diversity Arrays Technology Pty Ltd (DArT, Canberra, Australia) for DArTseq analysis. DArT uses a complexity reduction DNA hybridisation-based method, whereby thousands of anonymous single nucleotide polymorphisms (SNPs) are preferentially assayed in low-copy genomic regions, primarily from the nuclear genome (Jaccoud et al. 2001; Sansaloni et al. 2010). For our samples, genome complexity was reduced using DNA digestion with two methylation-sensitive restriction enzymes, *Pst*I and *Mse*I. The resulting "sticky-ends" of the digested fragments were ligated with uniquely barcoded adaptors. Following PCR and quantification, these samples were standardised and pooled for sequencing on the Illumina Novaseq X+ platform. Read assembly, initial quality control and SNP calling were undertaken by DArT using their proprietary DArTsoft14 software. Within this pipeline, technical replicates (ca. 25% of samples) were used to assess genotyping reproducibility, while the modelled Mendelian behaviour of DArTseq markers was used to filter sequencing errors and paralogous regions, which do not fit the expected results for a diploid reference sequence and may otherwise increase the number of false negative and false positive variants (Alkan et al. 2011). The DArTsoft14 pipeline produced a dataset that consisted of 72,362 SNPs with 19.8% missing data and 37.60x mean read depth for 241 samples (three samples, two from site B and one from the translocation site H, were removed for low quality).

Clone identification

To identify genetically identical samples in the dataset, we used 2 × the average genetic distance between the paired technical replicates to represent a threshold (≤ 0.02) for the level of sequencing error expected between known identical samples. This threshold was applied to all pairwise combinations of samples across the full dataset using the 'Poppr' package v2.9.6 (Kamvar et al. 2014) in R v4.4.1 (R Core Development Team, 2024). This resulted in the identification of 147 distinct genetic individuals overall, including 58 distinct genetic individuals (wild and translocated) within the augmented site H. All 33 samples collected from apparent single plants were assigned to a

single genet, indicating that each represented one genetic individual. Clonal diversity was assessed by calculating the genotypic richness of each subgroup as the proportion of plants with unique genotypes as per Arnaud-Haond et al. (2007). Lastly, we calculated Pielou's evenness, how evenly represented each of the genotypes are within each site, from Shannon diversity index and the number of multilocus genotypes for each site as per Arnaud-Haond et al. (2007).

Genomic differentiation in the wild and translocated sites

To remove any bias from the over-representation of clonal genotypes, all genotypic replicates were removed from the dataset for downstream population genetic analyses of wild *A. volubilis*. This resulted in the removal of 96 samples. Additional quality control filtering was applied to the dataset using the 'dartR' package v1.0.8 (Gruber et al. 2018) in R to obtain high quality datasets suitable for downstream analyses. This filtering removed secondary SNP loci using method 'best', retained samples with a read depth between 5 - 100, reproducibility ≥ 0.99, locus call rate ≥ 0.90, minor allele frequency ≥ 0.02, removed any resulting monomorphic loci and subsequently, retained samples with an individual call rate ≥ 0.80 and used the function 'gl.recalc.metrics' in 'dartR' to recalculate the locus-level metrics for the wild and translocated samples. This produced dataset A, consisting of 7,556 SNP loci with 2% missing data and 42.66x mean read depth for 145 genetically distinct samples. The filtering was then repeated, excluding the 52 distinct samples from site H that were known to have been from translocated plants. The function 'gl.recalc.metrics' in 'dartR' was subsequently to used recalculate the locus-level metrics for the wild samples only. This produced dataset B with 7,420 SNP loci with 2% missing data and 42.61x mean read depth for the 94 genetically distinct wild plant samples across all sites. Of the five suspected wild individuals within the fenced translocation area at site H, two were revealed to be clones of the same individual. Therefore, dataset B retained three samples within the translocation area that were suspected of being wild (see section *Sampling* for further details), with the aim of determining their origin.

To visually represent the genetic variation across the range of *A. volubilis*, we employed a principal component analysis (PCA) on all wild individuals (dataset B) using the function 'gl.pcoa' in 'dartR'. This analysis provides a useful way to initially assess the variation among samples without the assumptions of evolutionary models (see Jombart et al. 2009). To identify the number of genetic clusters amongst the wild samples, we conducted an admixture analysis using sparse non-negative matrix factorisation (sNMF) implemented in the R package 'LEA' v3.18.0 (Frichot and Francois, 2015), which is similar to the Bayesian clustering STRUCTURE (Pritchard et al. 2000), to detect *K* genetic clusters in the data. Admixture coefficients were generated with 100 iterations for each *K*-value. We applied 10 as the upper limit for *K* and selected the optimal *K*-value based on the point of plateau in the cross-entropy scores against *K*-max (Frichot et al. 2014).

We applied population-level analyses to the three genetic clusters identified in the PCA and sNMF analyses (see Results), rather than the 16 sampled sites, due to low census numbers and clonality reducing many sites to very low sample sizes (see Table 1). A hierarchical analysis of molecular variance (AMOVA) using 'Poppr' was used on dataset B to assess how genetic variation was partitioned within and among the three genetic clusters of wild samples. The significance of the components of variation were assessed using the 'randtest' function with 1000 repeats in the R package 'ade4' v1.7.22 (Dray and Dufour, 2007). We then estimated pairwise F_{ST} values across the three wild genetic clusters and the translocation (dataset A).

Genetic diversity in the wild and the translocation

Observed and expected heterozygosity estimates from genetic data are typically based on variable loci only and can be biased by sample size and filtering parameters (Schmidt et al. 2021; Sopniewski and Catullo, 2024). To reduce these biases, we filtered each of the three genetic clusters and the translocation (original dataset with 96 clones removed) individually using procedures recommended by Sopniewski and Catullo (2024). This approach allowed us to generate both SNP-based heterozygosity, which is the more widely recognised metric in the literature, and autosomal heterozygosity, which assesses both variant and invariant sites, a more accurate measure of genetic diversity. The locus call rate was set to 1 to remove all missing data, and the number of individuals was kept consistent (N = 12). Any cluster with more than 12 genetically distinct individuals (the maximum number of individuals in cluster 3), were normalised to 12 through random sub-setting. Following filtering for each cluster and the translocation, SNP-based and autosomal-based observed heterozygosity (Ho), expected heterozygosity (He) and inbreeding coefficients (F_{IS}) were estimated using 'dartR'. We assessed the significance of SNP and autosomal Ho and He between the wild populations and the translocation (dataset A) using non-parametric Kruskal-Wallis tests in the R package 'FSA' v0.10.0 (Ogle et al. 2023). Lastly, we calculated the percentage of polymorphic loci (% Poly. Loci) and allelic richness for the three wild genetic clusters and the translocation (dataset A) using the R package 'hierfstat' v0.5.11 (Goudet 2005).

RESULTS

Genetic Distance and Clonality

Clonality was detected within all wild sites except sites A, K and M, with more extensive clonality in the northern sites compared to the southern sites. On average, genotypic richness per wild site was 0.39 (± 0.11 SE), with six (38%) of these sites found to be monoclonal (Table 1). After taking clonality into account, the number of unique genets per wild site ranged from 1 – 34, with 81% of the sites found to have less than 10 genetically distinct individuals. Genotypic evenness values averaged 0.49 (± 0.11 SE) and were generally higher in the southern sites (sites M-P; Table 1). On average, for wild sites, genet size was 2.19 m (± 0.31 m SE) and the average maximum distance between two ramets in the same genet was 0.79 m (± 0.12 m SE).

Genomic variation - Wild populations

The PCA indicated clear population structuring across the wild range of *A. volubilis* (Fig. 3A). The first two axes explained 28% of the total variation and together, indicated three genetic clusters. Individuals from all northern sites (A - L and the wild individuals in H) grouped together in a broad cluster (hereafter, cluster 1), with some minor spread across both axes that reflected a geographic cline. The remaining *A. volubilis* individuals separated into two clusters: cluster 2 consisted of southern sites M and N, which were separated from cluster 1 on PC1 (20.3%), while cluster 3 separated along PC2 (7.3%) and consisted of southern sites O and P. The two unknown samples in site H clustered with wild samples in the translocation and were treated as wild samples for the remainder of the analyses (Fig. S1).

The three clusters identified in the PCA were also detected by the sNMF analysis (K = 3), with no meaningful structure detected at higher K values (Fig. 3B). The majority of individuals from the northern sites showed complete assignment to cluster 1. However, 14 individuals, largely from the southernmost sites within cluster 1 (sites E, F, G, I, J, K, L, and the wild individuals from H), showed some minor affinity with clusters 2 and 3 (Fig. 3B). All individuals from the southern sites (O, P, M and N) showed complete assignment to either cluster 2 or 3, as per the PCA (Figs. 3A and 3B). The AMOVA analysis partitioned 61.71% of the variation within sites with a further 33.57% of the total genetic variation partitioned among the three clusters, and just 4.72% among sites within clusters. All components of variation were statistically significant ($P \leq 0.01$) at the 95% confidence level. The high among-cluster component

was supported by the moderate to strong differentiation detected among clusters (F_{ST} range: 0.197 – 0.352) (Table 3).

Genetic diversity varied among the three genetic clusters. Northern cluster 1 showed observed heterozygosity levels that were lower than expected heterozygosity, generating the highest F_{IS} estimates of all the wild clusters (Table 2). For the southern clusters 2 and 3, a heterozygote deficit was also evident, although to a much lesser extent. Observed and expected heterozygosity estimates from both SNP- and autosomal-based measures did not differ significantly between the southern and northern clusters ($P > 0.05$ in all instances). The percentage of polymorphic loci was higher in cluster 1 compared to the southern subpopulations (Table 2). Allelic richness was also found to be slightly higher in cluster 1 (Table 2).

Genomic variation - Translocated populations

Consistent with the known source of most plants (site C) for the translocation, site H showed weak differentiation from wild cluster 1 in the north ($F_{ST} = 0.029$) and strong differentiation from the wild clusters in the south ($F_{ST} =$ 0.263 and 0.216; Table 3). Additionally, both SNP- and autosomal-based estimates of observed and expected heterozygosity for the translocated plants at site H did not differ significantly from the northern or southern wild genetic clusters ($P > 0.05$). Translocated plants from site H showed no evidence of clonality and exhibited high levels of polymorphic loci compared with the northern and southern wild clusters (Tables 1 and 2). Allelic richness in the translocated plants of site H was similar to the primary source site (site C) (Table 2).

DISCUSSION

Our research has documented the genetic variation remaining across the highly fragmented distribution of *A. volubilis*. Range-wide sampling and genome-wide SNP genotyping fill a large knowledge gap surrounding the extent of clonality, the number of genetically distinct wild individuals and the genetic diversity available for conservation management. Despite evidence of clonality and low genetic diversity within some sites, there remains significant genetic variation across the species' range that can be utilised for conservation management of the species. In addition, evaluation of the translocation has confirmed that genetic diversity has been adequately captured from the primary source population with no signal of clonality. We discuss this genetic assessment in terms of improved understanding of the species' conservation status and informing ongoing management, including SPA design, which remains a necessary step to increase seed production and ultimately support future translocations for *A. volubilis* and other highly threatened exceptional species.

Clonality reduces census estimates and clarifies genotypic diversity

Our data confirmed that clonality is common in *A. volubilis*; although the patterns of clonality (i.e., frequency and distance) varied considerably within and between sites. The presence of clonality reduced the total abundance estimates of *A. volubilis* in the wild from 184 individuals counted in the 2024 census to fewer than 100 individuals. Though, we note that in addition to clonality, the 2024 census estimate may have been overinflated because weeds made it difficult to distinguish some individuals. With permanent tags now installed to identify plants with unique genotypes during ongoing monitoring, future seed collections will benefit from knowing how much seed is available from each genotype and be able to plan accordingly. This added knowledge will help avoid situations like that described by Krauss et al. (2002), where over half of the *Grevillea scapigera* individuals introduced into the species' primary translocation were genetically identical. Consequently, the F1 generation in that translocation showed a

substantial decline in genetic variation compared to the parental generation, threatening the long-term viability of the species (Krauss et al. 2002). While genotypic diversity within most *A. volubilis* sites is low, there remains sufficient diversity across sites and genetic clusters that can be harnessed in management actions to facilitate recovery. Our fine scale clonality assessment will support the future collection of diverse propagation material by helping to maximise the unique maternal lines available for inclusion in both the SPA and translocations.

Increased extinction risk for small, disturbance-prone road verge populations

Accurate population size estimates for *A. volubilis* now provide a more comprehensive understanding of the species' conservation status and highlight the sites at the greatest risk of extinction. Our genomic assessment indicates that sites B, E, I, and J, which were estimated by visual assessment to contain 50 plants collectively, contain just eight genetically unique individuals when accounting for clonality. In addition, it was previously thought that there was only one monoclonal site (site L) and our assessment has shown that there are in fact six monoclonal sites (sites D, E, F, J, L and P). Though the reduction in abundance at most sites is indicative of a greater risk of extinction than previously thought, these monoclonal sites face the highest risk of local extinction by a single, stochastic event. Low genetic diversity is also pervasive among many of the remaining sites. All three wild genetic clusters are heterozygote deficient and exhibit moderate levels of inbreeding. Typically, small numbers of isolated individuals have limited opportunities for widespread outcrossing and a greater likelihood of near-neighbor matings (Ellstrand and Elam 1993; Manoel et al. 2012). Over time, frequent near-neighbour matings (often between related plants) can increase the levels of inbreeding and result in a greater number of homozygotes for mildly deleterious alleles with lower evolutionary fitness (Wang et al. 1999; Arcos-Burgos and Muenke, 2002; though see Madsen et al. 2023). Most sites are therefore at a high risk of local extinction, with the exception of site M, which has the largest number of wild genets, shows no signal of clonality, produces seed, and is the only site in relatively undisturbed habitat within a conservation reserve. These qualities indicate that *A. volubilis* can thrive under better conditions and that the extensive clonality observed is likely a response to high disturbance and poor habitat conditions, especially in the northern clusters. Thus, site M provides encouragement that recovery actions to increase the number of populations in suitable sites could be a viable means of conserving this species.

Genetic variation supports a regional admixture approach for A. volubilis

Threatened plant populations are often small and isolated and thus, may have a limited genetic base for conservation management actions (Coates et al. 2018; Monks et al. 2019). Between the ordination and admixture analyses, we identified three distinct genetic clusters across the geographic range of *A. volubilis*. This population structuring is unlikely to be a consequence of recent land clearing limiting gene flow. Prior to European settlement, much of this region would have been dominated by eucalypt woodlands, interspersed with low kwongan shrublands that included *A. volubilis* (Brown 1989; Harris and Brown 2003). This naturally patchy distribution, in conjunction with the major paleo-drainage system that separates the two southern clusters, would likely have acted as barriers to gene flow over evolutionary time, similar to other species in the region (Binks et al. 2026). Neutral genetic differentiation is generally used as a predictor for adaptive genetic differentiation and guidelines often caution against a regional admixture approach that mixes highly differentiated populations due to the risk of outbreeding depression (Frankham et al. 2011; Weeks et al. 2011; Ottewell et al. 2016; St. Clair et al. 2020). In the present study, the strong genetic differentiation among the three *A. volubilis* genetic clusters might caution against their mixing in the planned SPA and future translocations. Frankham et al. (2011) presented a risk framework for outbreeding depression that suggests a high risk for populations separated from gene flow for more than 500 years, which would

be the case for *A. volubilis*. However, more recent evidence suggests that concerns of outbreeding depression have been overstated and that mixing divergent populations may be necessary, and highly successful, when all other options lead to a higher extinction risk from inbreeding (Weeks et al. 2016; Kronenberger et al. 2017; Ralls et al. 2018). Indeed, the current geographic extent of *A. volubilis* is narrow and lacks strong climatic or environmental gradients, meaning that the likelihood of these clusters harboring critical local adaptations is low, despite their historical separation. Moreover, our results indicate that the available pool of genetic variation within any given cluster is highly limited and that the differentiation among clusters presents an opportunity for novel genetic compositions that could be harnessed using a regional admixture approach to increase both genetic variation and evolutionary potential for the species (St. Clair et al. 2020). Similar mixing strategies have already proven successful for *Acacia montana* and *Dianthus deltoides*, where mixing several source populations increased outcrossing (*A. montana*), improved genetic diversity (*D. deltoides*), and reduced correlated-paternity inbreeding (*A. montana*) in the resulting F1 generations (Broadhurst et al. 2017; Van Rossum and Le Pajolec 2021). Additionally, the benefits of genetic rescue, through outcrossing, can persist beyond the F1 generation. Frankham et al. (2016) showed a median benefit of outcrossing on fecundity and survival of 86% in F3 generation plants. In the case of *A. volubilis*, we suggest that the long-term benefits of increasing genetic diversity, and possibly seed production, by mixing the three genetic clusters outweighs the low risk of outbreeding depression. Nevertheless, it would be wise to monitor plants produced from seed from such a mixed-source translocation or SPA for any fitness effects downstream.

Diversity successfully captured within translocation with further augmentation possible

Translocations are generally designed with the goal of maintaining or potentially enhancing genetic diversity, and effective genetic monitoring remains an important tool for evaluating outcomes and helping to ensure successful conservation results (Milinkovitch et al. 2007; Bell et al. 2019; Ralls et al. 2020; Van Rossum and Pajolec 2021). Genetic diversity in the translocation of *A. volubilis* was comparable to the northern genetic cluster, which was the dominant source of germplasm, and no clonality was detected. Thus, the approach taken to establish this augmentation was successful in capturing the genetic diversity of the species that was available at the time. Whilst relatively few plants were sourced from sites G (cluster 1) and sites O and P (cluster 3), it is possible that those plants survived to reproduce and may still have contributed to the next generation of seed that is currently sitting in the soil seed bank. Ideally, the current translocation would benefit from further augmentation using the mixed approach already mentioned. Though, with limited seed availability, the first priority should be establishing the planned SPA. Subsequently, seed derived from the SPA could then be used to augment the translocation and establish additional in situ translocations to help reduce the high risk of local extinction for many of the remaining small verge sites (Harris and Brown 2003).

Informing SPA design

Our genetic assessment of the remaining *A. volubilis* sites shows that the genetic diversity within any given site is low, with the exception of site M, but collectively there is sufficient diversity across the species' range to establish a diverse SPA. The central challenge facing the planned SPA, however, will be similar to that which constrained the 2004 translocation, namely the difficulty in obtaining sufficient germplasm from all sites when most plants do not set seed (Godfrey et al. 2002; Harris and Brown 2003). Techniques for cryopreservation of cuttings and tissue culture material remain in development, although success to date has been limited. Thus, the current priority is to collect both seed and cuttings from as many clusters/sites as possible, especially the southern sites that were not known about during the initial establishment of the translocation. Once collected, plants from different clusters should be arranged adjacent to one another in the planned SPA to encourage outcrossing and generate admixed

seed. Though, this approach may still be limited by uneven representation of different clusters in the SPA (based on germplasm availability), unpredictable pollination patterns, and the possibility that plants from different sites may exhibit slightly different flowering times. Thus, to ensure that admixture proceeds as intended and that SPA plant arrangements do not inadvertently generate inbreeding or outbreeding depression in F1 individuals, ongoing genetic monitoring remains critical.

Conclusions

Our study highlights the value of obtaining genetic data to inform the conservation management of a threatened species. For *A. volubilis*, our approach allowed for an accurate population assessment by reliably differentiating between ramets and unique genotypes. The genetic data obtained in this study forms a critical foundation for the ongoing conservation management of *A. volubilis*, particularly a planned SPA that will be an important interim step to support future translocations of this exceptional species that is currently constrained by low seed production. A regional admixture approach is recommended for the SPA with the aim of increasing both seed production and the genetic diversity in the resulting progeny. Importantly, these data provide a baseline against which future studies can detect potential inbreeding and genetic bottlenecks in the SPA, thereby allowing for the selection of only the most genetically diverse progeny for future translocation plantings. Nevertheless, genetic monitoring of SPAs of threatened species remains an emerging area of research, with more studies needed to empirically test plant fitness and signals of outbreeding or inbreeding depression in order to support the effective and appropriate decision-making surrounding utilisation of germplasm produced in such areas.

Declarations

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contribution

J.H.K. - Conceptualization, methodology, visualization, writing—original draft. A.C. - Conceptualization, methodology, writing—review & editing. L.M. - Writing—original draft, writing—review & editing. K.M.T. - Methodology, writing—review & editing. B.P. - Writing—review & editing. R.M.B. - Conceptualization, writing—original draft, methodology, writing—review & editing.

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Data Availability

The genetic data underlying this article will be made available on the SRA database upon acceptance.

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Tables

Table 1 Summary of sampling and genotypic richness across all sites. Sites were defined by a separation of individuals by distances exceeding 500 m. The management code is a unique code given by the Department of Biodiversity, Conservation and Attractions to help manage threatened species. All sites are included except where indicated by letters. We include it here to allow easy crossmatching of our sampled sites to their internal codes for management purposes. Genotypic richness of each site was calculated as the proportion of plants with unique genotypes as per Arnaud-Haond et al. (2007), and Pielou's evenness was derived from Shannon diversity index and the number of multilocus genotypes as per Arnaud-Haond et al. (2007).

Site	Management code	Sampled ramets	Unique genets	Genotypic evenness	Genotypic	richness
A	1A	2	2	1	1	
B	1B & C	25	4	0.29	0.13	
C	2	32	27	0.93	0.84	
D	3	4	1	0	0	
E	5	10	1	0	0	
F	6	7	1	0	0	
G	7	3	2	0.58	0.5	
H_Wild*	8	9	7	0.80	0.75	
H_Translocation*	8	51	51	1	1	
I	9	7	2	0.26	0.17	
J	11	8	1	0	0	
K	12	1	1	-	0	
L	14	2	1	0	0	
M	15	34	34	1	1	
N	16	2	2	1	1	
O	13 (northern)	12	11	0.99	0.91	
P	13 (southern)	2	1	0	0	

*H_Wild and H_Translocation are co-occurring and are presented here as two groups for convenience of interpretation.

Table 2 SNP and Autosomal-based genetic diversity statistic calculated for each genetic cluster of *Acacia volubilis*. Genetic clusters were subset to 12 individuals and analysed in four subsets as per Sopniewski and Catullo (2024). SNP and autosomal measures of F_{IS} were similar, thus for simplicity, we report only autosomal F_{IS}.

Cluster	Sampled ramets	Unique genets	% Poly. Loci	Allelic Richness	SNP Ho ± SE	SNP He ± SE	AUTO Ho. ± SE	AUTO He. ± SE	AUTO F_{IS} ± SE
Northern Cluster 1	101	41	84.7	1.13	0.150 ± 0.002	0.186 ± 0.002	0.000442 ± 0.000109	0.000541 ± 0.000123	0.16 ± 0.01
Southern Cluster 2	36	36	44.8	1.07	0.272 ± 0.003	0.292 ± 0.003	0.000214 ± 0.000132	0.000230 ± 0.000135	0.09 ± 0.01
Southern Cluster 3	15	12	33.0	1.07	0.268 ± 0.003	0.284 ± 0.003	0.000229 ± 0.000137	0.000244 ± 0.001370	0.08 ± 0.01
Translocation	52	52	78.3	1.11	0.214 ± 0.002	0.234 ± 0.002	0.000461 ± 0.000143	0.000513 ± 0.000148	0.11 ± 0.01

Table 3 Pairwise F_{ST} values for *Acacia volubilis* populations assigned on the basis of clustering.

Cluster	Northern Cluster 1	Southern Cluster 2	Southern Cluster 3	Translocation
Northern Cluster 1	-	-	-	-
Southern Cluster 2	0.246	-	-	-
Southern Cluster 3	0.197	0.352	-	-
Translocation	0.029	0.263	0.216	-

Figures

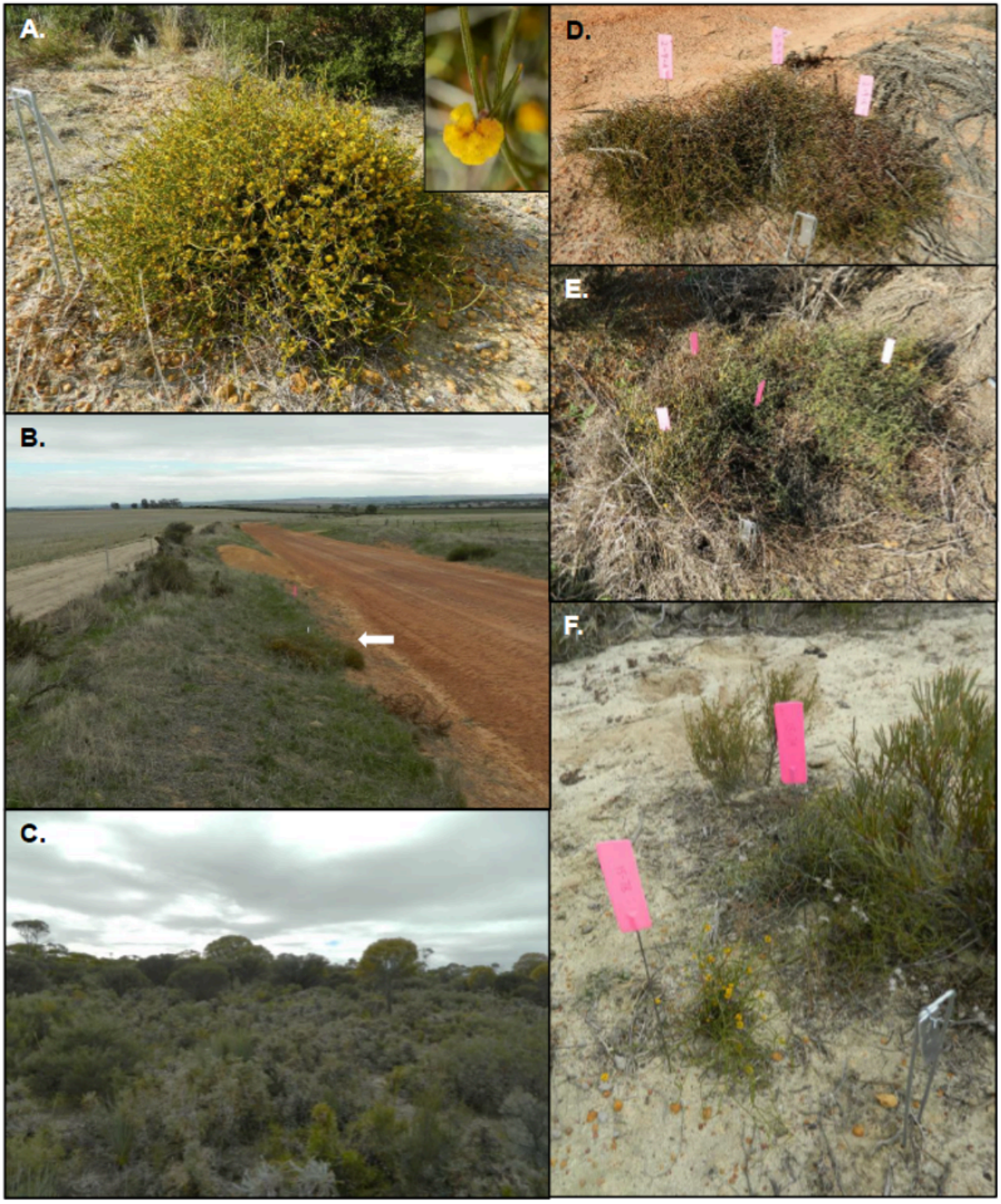


Figure 1

A) *Acacia volubilis* is a small dome-shaped shrub 0.4 m high and growing up to 1 m wide with golden globular inflorescences (inset). B) Due to widespread clearing in the region, all but one of the *A. volubilis* populations are restricted to highly degraded, and often weed infested, road verges. The white arrow indicates the location of the *A. volubilis* plant. C) Native vegetation where *A. volubilis* naturally occurs. D) *A. volubilis* exhibits a wide range of growth patterns including multiple connected domed mounds, E) interconnected plants without distinct shapes, and F) physically separate plants that are clonal. Photo credit DBCA/Andrew Crawford/Joshua Kestel.

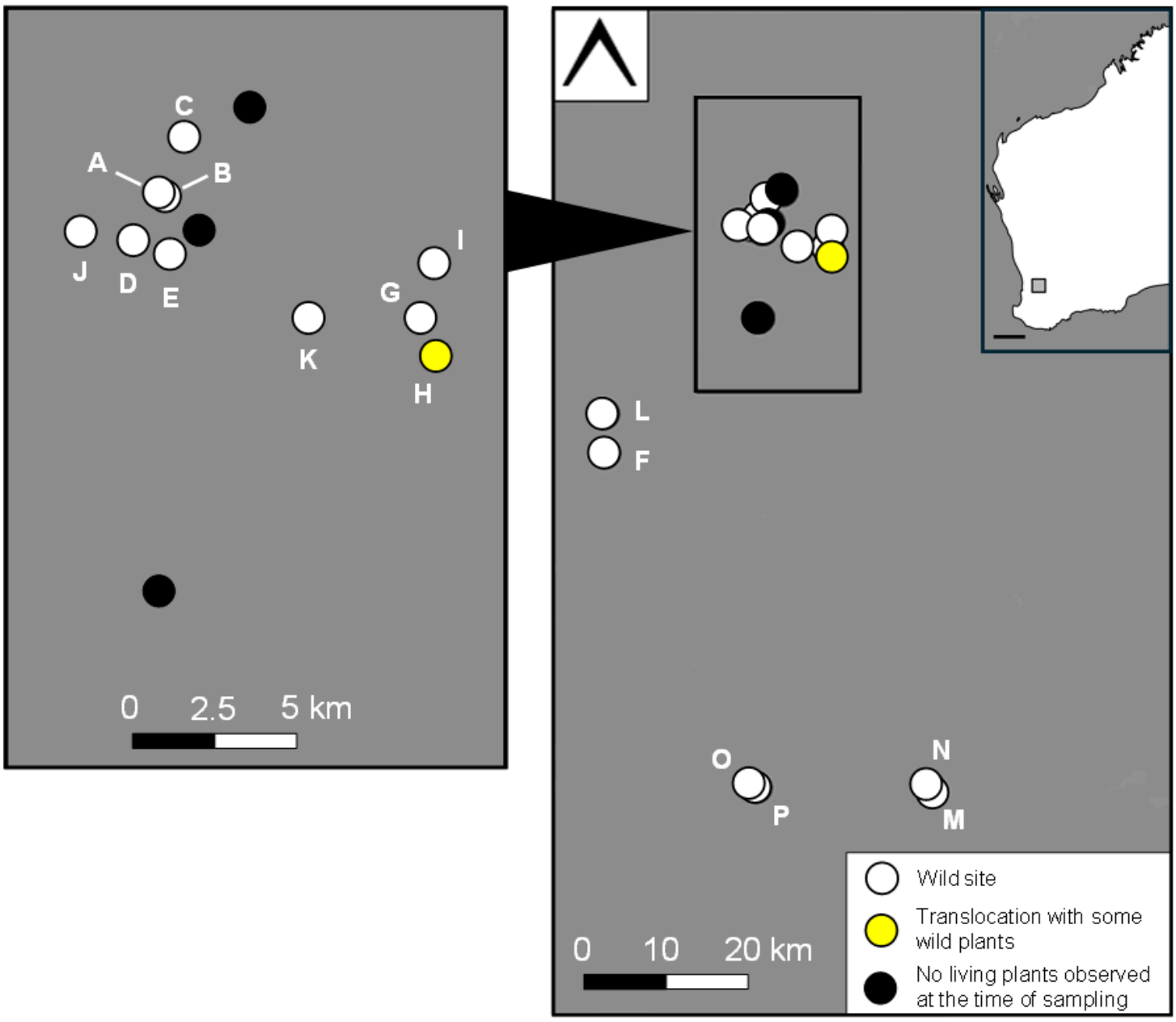


Figure 2

Remaining 16 *Acacia volubilis* sites that were sampled in the Wheatbelt region of Western Australia, denoted in top right panel (scale bar is 500km). White circles denote extant sites, black circles denote sites at the time of sampling with no living plants, and the yellow- circle denotes the translocation (an augmentation of a wild site).

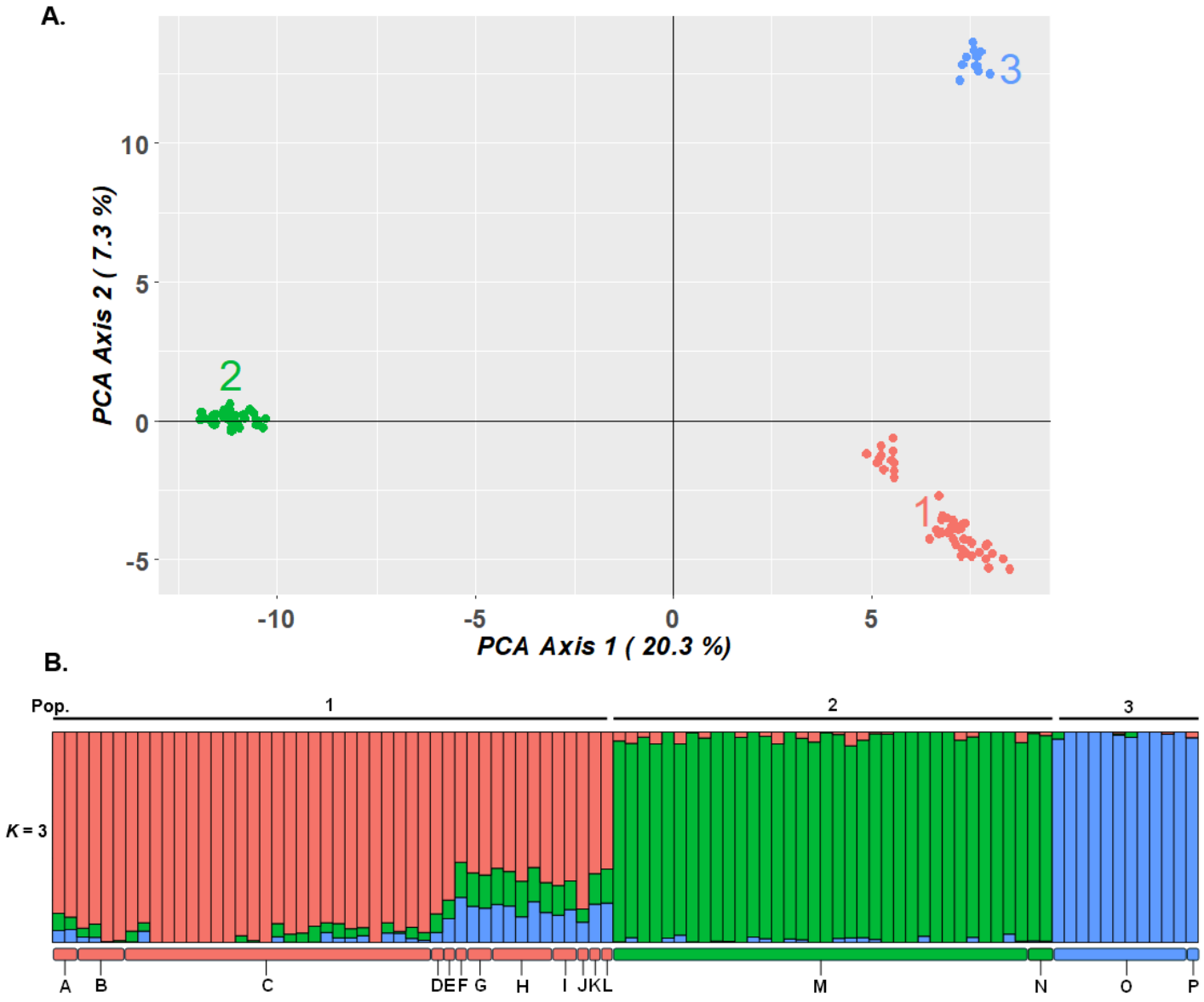


Figure 3

A) Ordination for 7,420 SNPs for 94 wild genotypes (2 % missing data). B) Results of sNMF runs for *A. volubilis*. Sample ancestry assignments for the three major clusters detected (*K* = 3), shown as vertical bars for each sample. Sampling codes (A - P) correspond to the 16 sites sampled (see section *Sampling*).

Supplementary Files

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- 002Supplementary.docx