Understanding the distribution of genetic diversity in South Australian populations of quandong (*Santalum acuminatum*), to inform genetic resource management.

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Abstract

The quandong (*Santalum acuminatum*) has a natural distribution across southern Australia. This geographical distribution encompasses a variety of habitats ranging from coastal, high altitude ranges to desert landscapes. Many dispersal vectors contribute to the mobility of quandong throughout the landscape, such as small mammals, birds, and humans. As an important plant for humans, the quandong has been subject to constant trading and movement across its natural range for centuries, facilitating the species' mobility across very large distance. The morphology of quadong is variable. Some specimens from the Nullarbor plain, a geologically distinctive region shared between South Australia and Western Australia, exhibit intermediate traits between *S. acuminatun* and its sister species, *S. spicatum*. This so-called Nullarbor form is thought to be a hybrid between these species and has been noted as a good rootstock in some plantations in South Australia. Because of its long history among the people of Australia and the efforts for its continued cultivation, we considered it crucial to conduct an assessment of what genetic diversity exists in wild populations of quandong in South Australia and to find out if the Nullarbor form is indeed a hybrid. This is part of a larger project exploring genetic diversity in the quondong and the wider genus. Wild populations in the South Australia Nullarbor region were collected and genotyped using High Throughput Sequencing (HTS) techniques with the Ion Torrent platform (PGM and Ion Proton) to determine potential hybrids in the Nullarbor populations and to estimate genetic diversity. Single nucleotide polymorphisms (SNPs) were detected and used as markers to begin to develop an understanding of the genetic diversity of quandong. The *Santalum acuminatum* Nullarbor form, does not appear to be a hybrid and we determined the baseline genetic diversity to further assess extant populations as they are quite scarce, potentially making specimens to rely on other reproductive factors such as vegetative propagation, which can have an impact on genetic diversity. As a semi-cultivate, quandong can face challenges to its long term successful cultivation due to climate change, introduced fauna and other human-induced changes to the landscape. It is important to conserve extant wild populations to ensure the future survival of the species and related species in the genus. The results of this component of our study suggests that continued research of quandong and its relatives, particularly in the dry lands of South Australia, are necessary to preserve its seeds banks and with this assist on continued improvement of quandong in cultivation.

Introduction

The quandong (*Santalum acuminatum*) has a predominant place as so-called bush tucker, food of native Australia, which has been known and utilised by Aboriginal Australians and later on was adopted by European settlers. The fruit of *S. acuminatum* has an important place in Aboriginal Australian plant traditional knowledge (Latz, 2004; Clarke, 2007). This species is highly regarded as, among other attributes, the fruit can be dried and stored for long periods of time, still preserving much of its nutritional value. In contemporary Australia, jams, chutneys and liquors are among some of the products produced from the fruits of the species. Other parts of the tree have been useful, the seeds contain a nut high in nutritious oils, seeds grinded, leaves and bark were used for medicine by Aboriginal Australians (Latz, 2004; Clarke, 2007), demonstrating both its economic value and cultural significance.

Long recognised as one of the most economically exploited genera across its natural range (Harbaugh and Baldwin, 2007), members of the genus *Santalum* are facing many challenges to their survival. Extensive wild harvesting, human-fragmented landscapes with consequent introduction of non-native species, are some of the most important factors that have contribute to the decline of natural populations. Because of the economic relevance of quandong, as a plant for food and medicine, serious domestication efforts have been conducted since the 1980's (Sedgley, 1982; Ahmed, 2000). *Santalum* species are hemiparasitic hampering domestication efforts and making it difficult to bring the species into a large-scale cultivation. Seed germination requires work and once root shoots emerge, the young plant will require a host for it to establish during its first year. It can then take several more years for the plant to reach maturity, making it long term tending for cultivation.

As the quandong is a rootstock, capable of reproducing clonally, horticulturalists have experimented with grafting, which has been a preferred strategy because it ensures solid establishment of the trees, and is more efficient than cultivation from seedlings (Graham, S. *pers. comm*). However, once the trees establish they are long-lived, and like other hemiparasitic plants, they can play important roles in the ecosystem (Watson et al., 2011). As with many plants exploited by humans, it is crucial to study them and their related species in the wild, in order to determine the distribution of genetic diversity that can ensure their continued survival and adaptation to changing conditions.

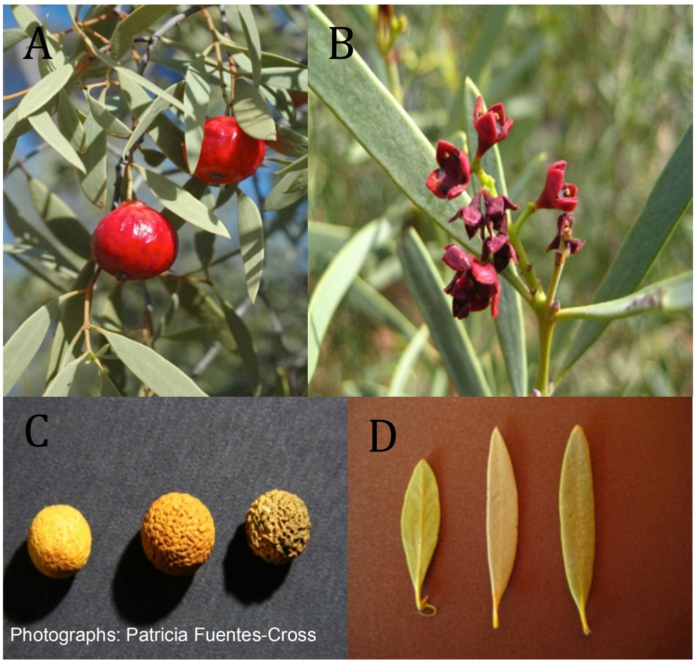

Fig 1. *Santalum acuminatum* Nullarbor form traits. A) Mature fruit exhibits smooth and thin skin as opposed to thick and wrinkled as in common quandong. B) Red flower as opposed to the more common white flower in quandong. C) Seed texture of *S. acuminatum* Nullarbor form (center) compared to *S. spicatum* (far left) and *S. acuminatum* (far right). D) Leaf of *S. acuminatum* Nullarbor form (center) compared with *S. spicatum* (far left) and *S. acuminatum* (far right).

This is the first study, to our knowledge, that utilises high throughput sequencing (HTS) technologies to assess the genetic diversity in wild populations of quandong in South Australia. As the *S. acuminuatum* Nullarbor form (Fig 1) is well known and utilised among horticulturalists, the focus of this component of a larger study falls on the populations of the Nullarbor plain with the aim to assist on future management of wild populations and cultivars in South Australia.

Materials and Methods

Plant tissue sampling and DNA extraction

Fresh leave samples from quandong were collected and preserved in silica gel from extant populations, along the dry lands of the Nullarbor plain of South Australia (Fig 2). The aim was to include as many samples as we encountered in the routes traced along both, highways as well as undisturbed habitats in the Nullarbor plain, where the species exhibits unique morphological traits. In remote areas, the quandong is uncommon to rare and we collected leaves from what individuals that could be found. Leaf samples were sent to the South Australia node of the Australian Genome Research Facility (AGRF), Adelaide, for DNA extraction.

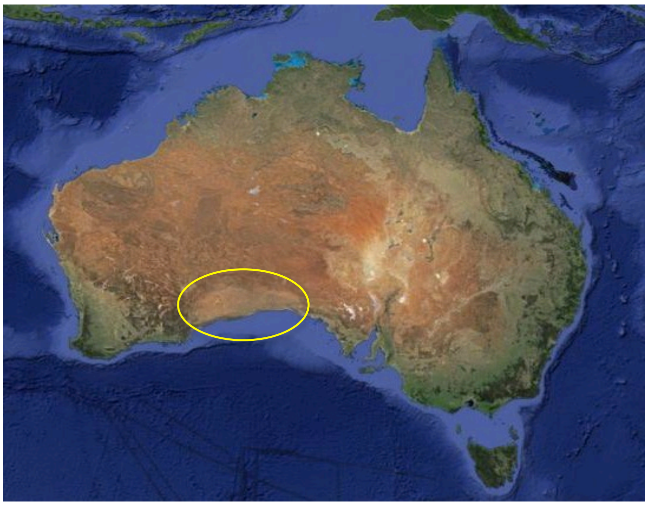

Fig 2. Nullarbor plain region (from *nullus* 'no' and *arbor* 'tree': a region with no trees), a large karst formation between Western Australia and South Australia. Scarce quandong populations live in this region.

Molecular techniques and data analysis

In order to maximise data for this component of our larger project, a novel approach in molecular genetics was utilised to enable simultaneous discovery and screening of variable molecular markers in higher quantities than with previous techniques.

The DNA of most individuals of each population were genotyped using a method that involved enzymatic digestion followed by selective PCR amplification (Amplified Fragment Length Polymorphism high-throughput sequencing, or AFLPseq) as described by Cross (Cross et al, 2016 *in press*) and then sequenced on Ion Torrent HTS machines. The resulting sequences obtained from the HTS platforms were then demultiplexed, trimmed, and aligned to obtain consensus sequences, following bioinformatics pipelines described by Cross (Cross et al, 2016 *in press*). The sequences were then compared to identify variability among individuals and populations, and potential genetic mutations or single nucleotide polymorphisms (SNP).

The SNPs identified were then utilised as markers to determine genetic variation. Relatedness between populations was estimated with an analysis of genetic population structuring, an assessment of regional clustering within populations, performed on the software STRUCTURE. This analysis identifies relatedness of individuals and gene pools by allelic distribution. Quantification of how likely it would be for any particular specimen to belong to each group, the optimal value of K (or cluster) was estimated with the ΔK method by Evanno et al. (2005) with variation levelled off at K=6. Markers were also utilised to build an evolutionary tree (using a phylogenetic analysis) to describe relationships among populations and identify any potential hybrids.

Results

Genetic Structure and relatedness

The data analyses revealed less genetic admixture in the quandong Nullarbor plain populations when compared with other populations trialled for the larger study. The Nullarbor plain populations showed genetic contributions from two geographically distant populations: from Western Australia and from eastern South Australia (Fig 3).

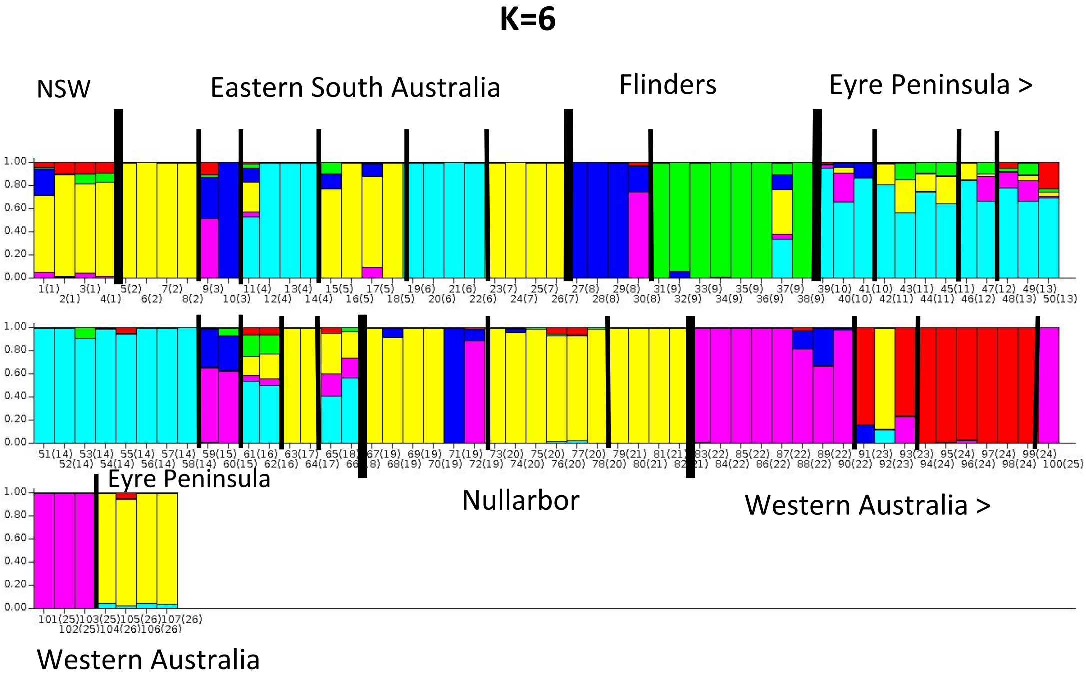

Fig 3. Quandong populations diversity results from STRUCTURE analysis, K=6. Populations demarked by a thin line, regions demarked by thick black line. Nullarbor populations show admixture from South Australia and Western Australia.

Interspecific hybridisation

The phylogenetic analysis showed that the quandong individuals from the Nullarbor plain trialled under our HTS experiments were not hybrids between *S. spicatum* and *S. acuminatum* (Fig 4), however variation within species was detected.

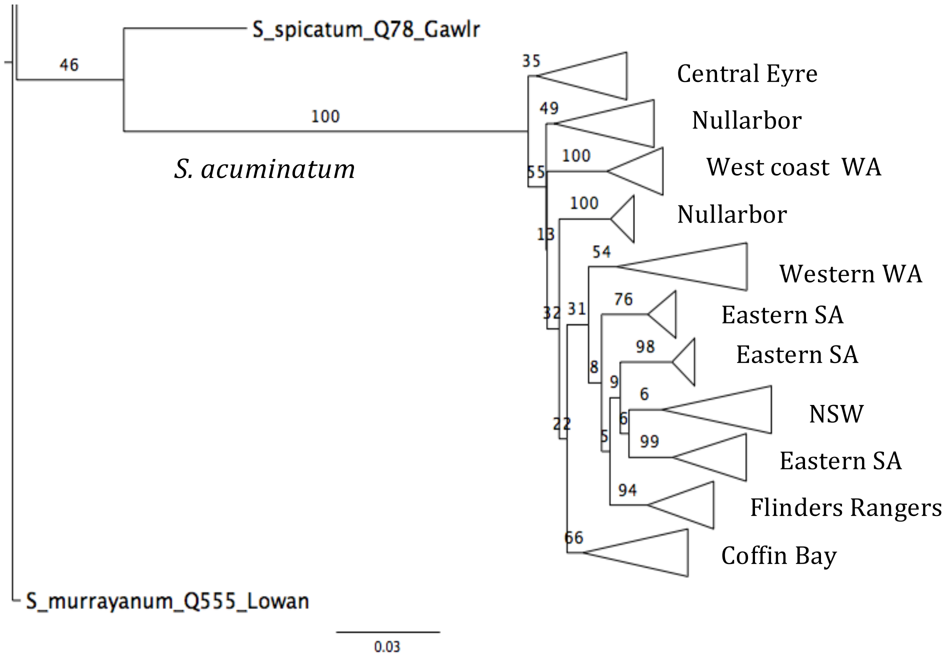

Fig 4. Detailed snapshot of phylogeny constructed from HTS data. Phylogenetic tree rooted on *S. murrayanum.* Putative hybrids removed before analysis. Locations of *S. acuminatum* populations and other species are indicated. Individuals from the Nullarbor plain fall within the *S. acuminatun* clade.

Discussion

Our study is the first of its kind, providing an analysis of the genetic diversity and structure of the quandong across its southern distribution using high-throughput sequencing techniques. With the data obtained from our efforts (within this component and of the larger study) this specific component concentrated on the Nullarbor plain to determine possible interspecific hybridisation, and examine the diversity existing in the wild populations of this region in relation to other populations. The main objective was to assist in our understanding of the historical and future sustainable management of *Santalum acuminatum* using novel technologies that are cost effective and that provide, at the same time, important information on the genetic reservoir in the wild.

Photograph: Patricia Fuentes-Cross

Fig 5. *S. acuminatum* trees across fragmented landscapes towards the Nullarbor plain.

Exploring the utility of SNPs to help unravel the genetic diversity and structure of natural populations of quandong was an important aspect of our research. Because of their slow rate of mutation, SNPs can be good markers for analysis of demographic histories of natural populations (Helyar et al., 2011). It is less likely that multiple mutations occur at a single site and SNPs, being bi-allelic, offer the advantage of efficient high-throughput genotyping, allowing for less recurrent substitutions at a single locus that can confound population history (Brumfield et al., 2003). SNPs have a different information content compared to other markers (i.e. microsatellites), which can be of great assistance for understanding the genetic structure of populations and reconstructing their relationships (Haasl and Payseur, 2011; Heylar et al., 2011).

Genetic diversity in Santalum acuminatum

Population substructure and inferring distinct populations, are important tools for predicting the opportunities for further diversification of the species (Beebee and Rowe, 2009). The populations trialled in this specific component were included in the larger project's analysis to obtain an overall picture of the state of the quandong's genetic diversity. The population structure in *S. acuminatum* was revealed to be clustered in six groups (as determined by the STRUCTURE and the AMOVA test on K clustering). Genetic distance correlation with the geographical distribution appears in some genotypes, but some populations such as the Nullarbor appear to have admixtures related to populations geographically distant and we found this surprising considering the very large geographical distance between some populations (e.g. over 1000 km between the Nullarbor populations and collection sites in eastern South Australia).

A widespread species found in arid, semiarid, coastal, and high altitude locations, the *S. acuminatum* (quandong), is distributed over a large part of the Australian continent, with seemingly few natural barriers. The genetic structure detected in the Nullarbor could be related to their geographical remoteness as it appears more homogeneous, but the genetic contributions from WA and eastern SA is an interesting aspect we discovered in these populations when compared to populations in close proximity to human settlements. This could be related to dispersal vectors capable of facilitating long distance mobility in the species.

Seed production and recruitment are vital, and natural populations living in proximity to human habitats can face stressors such as bushfires and field clearance for pastoral or agricultural activities, which could lead to a loss of some important populations or individuals from the landscape. This can potentially impact on any important traits for 'local adaptation' (Lowe et al., 2005). But pollination is also important and considering that *Santalum* are generally entomophilous (Sedgely, 1982) (needing insects for pollen distribution), changes in habitat, such as land fragmentation and introduction of exotics, can have an important impact on the pollinator's ability and availability, posing a potential stressor to quandong's mating system, apart from seed recruitment.

Natural barriers, such as the Nullarbor plain between SA and WA, appear not to have acted as an isolation factor between quandong populations, and gene flow could be facilitated by dispersal vectors such as animals capable of moving long distances, like the emu (known to favour the quondong fruit). Humans also have travelled this region since prehistoric times, and could also have acted as dispersal vectors. The quandongs are long-lived trees and individuals remaining in remote populations can themselves be important contributors to the species' genetic stock. Another interesting result from our work is that although the individuals still present in the Nullarbor plain show morphological traits that suggest interspecific hybridisation, our results showed that they are not hybrids. Further, we discovered that there is a slight variation within the species, potentially due to local adaptations, in ecotypes of particular regions. This could suggest strong adaptability of this species.

Implications for conservation

The quandong, or *Santalum acuminatum*, being a hemiparasitic plant capable of clonality and with a widespread distribution, is a very interesting species to study. But one of the most compelling traits in *S. acuminatum* is that is has had a long relationship with humans, being one of the few species in the genus producing edible, palatable fruit, big and resilient enough to be carried long distances. Other species of *Santalum*, such as *S. spicatum* and *S. murrayanum,* also have edible fruits and kernels (the nut inside the fruit also has many uses) and though they are less palatable, all of them have a long history of significance for Australia. The quandong is an iconic bush tucker (Fig 5), the 'poster child' of Australian native food plants and this makes it very appealing for wild harvest, posing a problem for conservation of the wild populations' genetic diversity.

Fig 5. Fruits of *Santalum acuminatum* are used in production of various bush tucker foods.

As hemiparasitic plants, the quandong and its relatives can provide important services to the ecosystem by redirecting nutrients, important for the survival of other organisms in most cases producing big, attractive and highly nutritious fruit (Watson et al., 2013; Ahmed and Johnson, 2000). *Acacia* are the most visible hosts available to quandongs, but also casuarinas and eucalypts, and other various shrubs of low stature. Hence, the vegetation mosaic where the quandong and its relatives are found in Australia is of high conservation value and requires continuing monitoring and management.

Preserving diversity in natural populations is crucial for the continuing improvement of potential cultivars. Policies need to be supported to avoid wild-harvesting of the quandong fruits, to continue monitoring of its conservation status, to control invasive species that can inhibit seed recruitment and other factors constraining pollination. Populations in remote areas such as the Nullarbor need to be conserved and constantly monitored. It is also critical to provide support for seed banks and local plantations so they can contribute to a maintenance of genetic diversity. Indeed, local plantations and home gardens could be reservoirs of an important genetic diversity and any efforts towards a domestication of the plant needs to be mindful of the importance of preventing wild harvesting.

Conclusion

Considering the importance that quandong has as a semi-domesticate, conservation efforts are urgently needed to prevent the further decline of wild populations. The remaining genetic diversity held among wild and remote populations should be further investigated and maintained for an economically sustainable use of quandong, since it is possible that it will remain difficult to fully domesticate the species. Our research shows the need of further work, including extensive taxonomic treatment of the genus, as it gave path to many more questions of scientific interest, which we are at present pursuing.

The hope is that this study contributes to *in situ* conservation of the quandong in remote areas and wherever they occur naturally. Is important to support collaborative efforts between horticulturalists, molecular botanists, and other people interested in Australian native plants and their conservation, along with Aboriginal Australian traditional knowledge and to be mindful of the cultural relevance behind native flora. With the affordability that HTS platforms provide, genomics work to support endangered native vegetation is now even more achievable.

Acknowledgements

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