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Spatial patterns of genetic diversity among Australian alpine flora communities revealed by comparative phylogenomics

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ABSTRACT

Aim The alpine region of mainland Australia is one of the world's 187 biodiversity hotspots. Genetic analyses of Australian alpine fauna indicate high levels of endemism on fine spatial scales, unlike Northern Hemisphere alpine systems where shallow genetic differentiation is typically observed among populations. These discrepancies have been attributed to differences in elevation and influence from glacial activity, and point to a unique phylogeographic history affecting Australian alpine biodiversity. To test generality of these findings across Australian alpine biota, we assessed patterns of genetic structure across plant species.

Location The Australian Alps, Victoria, eastern Australia.

Methods We used an economical pooled genotyping-by-sequencing (GBS) approach to examine patterns of genetic diversity among seven widespread species including shrubs and forbs from 16 mountain summits in the Australian Alpine National Park. Patterns of genetic structure among summit populations for each species were inferred from an average of 2,778 independent SNP loci using Bayesian phylogenomic inference and clustering approaches.

Results SNP results were consistent across species in identifying deep evolutionary splits among summit communities from the Northern and Central Victorian Alpine regions. These patterns of genetic structure are also consistent with those previously reported for invertebrate and mammal taxa. However, local genetic structure was less pronounced in the plants, supporting the notion that population connectivity tends to be higher in plant species.

Main conclusion There is deep lineage diversification between the North and Central Victorian Alpine regions, reflecting a high level of endemism. These findings differ from those reported for alpine biodiversity from New South Wales and much of the Northern Hemisphere, and support the notion that genetic diversity is typically greatest in areas least affected by historical ice sheet formation. We discuss the implications of our findings in the context of conservation planning, and highlight the benefits of this rapid and cost-effective genome scan approach for characterizing evolutionary processes at multi-species and landscape scales.

Keywords Alpine flora, Australian Alps, comparative phylogenetics, SNP loci, genotyping by sequencing, Pool-Seq, wildlife conservation

INTRODUCTION

The alpine region of mainland Australia makes up approximately 0.07% of the continental landmass. It is a multi-use landscape of cultural, ecological, geological and hydrological significance, and is recognized as one of the world's 187 biodiversity hotspots (Costin, 1972; Kirkpatrick, 1994; Good *et al.*, 2009). As in most alpine systems, temperature is the primary factor defining Australian alpine environments, where a mean isotherm of ≤10ºC in the warmest months delineates the boundary of treeless alpine habitats (Costin, 1967). Consequently, alpine environments are acutely sensitive to elevated temperatures and the current threats of anthropogenic climate change (Beniston & Price, 1991; Pauli *et al.*, 2001; Grace *et al.*, 2002; Beniston, 2003; McDougall *et al.*, 2005). Despite the historical climatic oscillations associated with glacial cycling they have endured, it is uncertain how well Australia's alpine biota can adapt to climate change threats. In addition, this biota has faced other anthropogenic pressures over the last century including exotic plant and animal incursions, cattle grazing, and infrastructure development associated with hydroelectricity, water extraction and ski resorts (McDougall *et al.*, 2005; Williams *et al.*, 2014).

In contrast to the Northern Hemisphere where alpine systems are typically characterized by steep elevation gradients and have been historically influenced by heavy glaciation, the Australian Alps form a complex of flat-topped and rounded mountain ranges with mostly shallow elevational gradients (Ollier, 1987; O'Sullivan *et al.*, 1995). They are part of an ancient (40–90 Ma) and geologically stable system, which originated by the process of uplift through continental splitting, rather than continental collision (Ollier, 1987; Holdgate *et al.*, 2008). Furthermore, glacier formation was minimal during the Pleistocene epoch due to the system's low elevation and was restricted to areas above 2000 m in the vicinity of Australia's highest peak, Mt Kosciuszko (2228 m) in New South Wales (Colhoun *et al.*, 1996; Barrows *et al.*, 2002). Because of the region's unique geomorphology and climatic history, it has been suggested that the evolutionary processes responsible for shaping Australian alpine biodiversity are likely to differ from those in Northern Hemisphere systems (Endo *et al.*, 2015).

Genetic analyses of Australian alpine fauna indicate high levels of genetic structuring at fine spatial scales, with evidence of deep lineage splits among summit communities, including those connected by high-elevation plateaus (Osborne *et al.*, 2000; Chapple *et al.*, 2005; Mitrovski *et al.*, 2007; Koumoundouros *et al.*, 2009; Endo *et al.*, 2015; Hatley & Murphy, 2016). These patterns differ markedly from Northern Hemisphere alpine systems, where shallow genetic differentiation is typically observed among populations owing to a history of post-glacial expansion (e.g., Buckley *et al.*, 2001; Ikeda *et al.*, 2008; Kubow *et al.*, 2010; Todisco *et al.*, 2012). It is suspected that shallow elevation gradients and minimal fragmentation by historical glacial activity have helped maintain high levels of genetic diversity and local genetic structure in Australian alpine communities (Endo *et al.*, 2015). These findings point to a unique phylogeographic history affecting Australian alpine faunal biodiversity, and the importance of conserving biota from multiple mountain summits to preserve patterns of endemism and evolutionary potential in a changing climate.

These patterns still need to be investigated across multiple plant taxa. The Australian Alps have a rich mosaic of plant species and communities (Carr & Turner, 1959; McDougall &Walsh, 2007; Williams et al., 2014) and is recognised as one of the continent's 11 centres of plant diversity. The plant communities provide critical habitat as well as ecosystem services that underpin soil quality and stability, and overall catchment health (Costin, 1958; Carr & Turner, 1959; Williams *et al.*, 2014).

While alpine grasses show minimal genetic differentiation between summit populations, possibly because of their capacity for long-distance pollen and seed flow (Griffin & Hoffmann, 2014), general patterns of population connectivity among Australian alpine plant communities have not yet been investigated.

Previous attempts to characterize evolutionary processes at landscape scales based on multispecies genetic analyses have been limited by the coarse resolution of genetic markers such as mitochondrial/chloroplast DNA sequence data and restriction fragment length polymorphism (Vandergast *et al.*, 2008; Lorenzen *et al.*, 2012; Manel *et al.* 2012; Reitzel *et al.*, 2013; Wood *et al.*, 2013). Next generation sequencing (NGS) technologies now facilitate the genotyping of individuals across populations for hundreds to millions of genetic markers. In particular, reduced representation sequencing protocols, such as genotyping by sequencing (GBS) and restriction site-associated DNA sequencing (RADseq), are effective options for characterizing patterns of genetic variation within and among populations at a genome-wide scale (Elshire *et al.*, 2011; Peterson *et al.*, 2012). Yet the costs associated with such analyses can still be substantial for ecological studies involving large sample sizes, such as multispecies, multi-population studies. In such cases costs can be reduced by genotyping pooled samples (e.g. Pool-Seq: Futschik & Schlötterer, 2010; Schlötterer *et al.*, 2014). Pool-Seq has been used widely for gaining accurate single nucleotide polymorphism (SNP) allele frequency estimates and population genomic parameters (Futschik & Schlötterer, 2010; Rellstab *et al.*, 2013; Schlötterer *et al.*, 2014). As far as we are aware, this method has not yet been applied at the multispecies level.

In this study we examine patterns of genetic diversity among seven widespread shrub and forb species from 15 mountain summits in the Australian Alpine National Park. We hypothesize that genetic structuring across mountain summits will be shallower in plant species compared to that in ground dwelling terrestrial fauna groups, given that many plant species have a greater dispersal capacity facilitated by aerial pollination and/or seed dispersal (Endo *et al.* 2015; Griffin & Hoffmann, 2014; M'Baya *et al*. 2013; Nicotra *et al.* 2015). We use an economical Pool-Seq GBS approach and characterize levels of genetic structure among summit populations for each species using a combination of phylogenomic and population genomic methods. Findings from this study highlight the variability in connectivity across species but also identify some broad patterns of differentiation that build on the previous work of Endo *et al*. (2015) on invertebrates. We discuss the

management implications of our findings, and the benefits of the Pool-Seq approach for characterizing population genetic processes and biogeographic patterns across multiple species and landscape scales.

MATERIALS AND METHODS

Study area

Patterns of genetic structure and diversity were investigated across seven alpine species spanning 16 summit locations within the Victorian Alpine National Park (Table 1, Supporting Information S1, Fig. 1a). The majority of the sampling occurred within the Bogong High Plains (BHP) at sites undergoing regular monitoring as part of the Long Term Ecological Research Network (LTERN) scheme. Additional sites adjacent to the BHP comprising Mt. Fainter, Mt. Hotham and Mt. Loch as well as representative peaks in the Central Alps including Mt. Buller, Mt. Stirling and Mt. Howitt, were also included to provide coverage of all distinct peaks in the Victorian alpine region. This site combination provides a basis for investigating genetic connectivity at both fine and broad scales across multiple discrete sky-island populations.

Study species

Seven endemic alpine plant taxa that have broad distributions and are common to high elevation heathland communities in the Victorian Alpine National Park (Costin, 1957; Williams & Ashton, 1987) were selected for genomic analysis. These included four shrubs (*Hovea montana* (Hook.f.), *Asterolasia trymalioides* (F. Muell), *Grevillea australis* (R. Br) and *Pimelea alpine* (F. Muell)), and three forbs (*Scleranthus biflorus* (Hook.f.), *Oreomyrrhis eriopoda* (Hook.f.), and *Stylidium armeria* (Labill). All seven species are distributed across the Northern and Central alpine regions of Victoria, except for *Oreomyrrhis eriopoda* whose distribution is limited to the Northern region. Additionally, six of the species were expected to be diploid based on inferences from karyotypes in related species, while *Stylidium armeria* is a confirmed tetraploid (Raulings & Ladiges, 2001). These species represent a variety of life-forms, dispersal and pollination syndrome traits, that capture a broad representation of common Australian alpine flora (Table 2).

Field collection

Leaf tissue was collected over the summer period between December 2012 and February 2013, with additional sampling at Mt. Stirling and Mt. Howitt in October 2013. For each species, a minimum of 30 ~ one gram samples of fresh growth was collected within an area of approximately 100 m^2, avoiding adjacent individuals to reduce possible sampling of close relatives. This is particularly important for prostrate spreading species such as *Pimelea alpina*, where individuals can be difficult to distinguish. GPS coordinates were logged at each site to facilitate spatial analysis (Table 1 and Supporting Information S1). Individual samples with unique identifiers were preserved in paper coffee filters and rapidly desiccated with silica gel ensuring minimal degradation of genetic material.

DNA extraction and genotyping

Genomic analyses were conducted on samples of 10 individuals per site for each species. Genomic DNA was extracted from 30 mg sample of tissue for individual specimens using the NucleoSpin® 96 Plant II protocol (Machery-Nagel Inc., Düren, KO, GER) and DNA quantitation was performed as per the QuantiFluor® dsDNA System (Promega Inc, Madison, NY, USA). For each species the genomic DNA from the ten individuals representing each collection site were pooled in equal proportions to a total of 500 ng. Reduced representation genome libraries for each pooled sample were prepared using the modified genotyping by sequencing (GBS) protocol of Elshire et al. (2011) (Supporting Information S2). Five hundred nanograms of genomic DNA from each individual was digested in a 20 μL reaction containing four units of PstI-HF® (New England BioLabs, Ipswich, MA, USA) for 2 h at 37 °C, without a heat kill step. Digestion products were then ligated to modified P1 and P2 adapters with 80 unique barcode combinations to allow for subsequent multiplexing of all individuals. Fifty microlitre ligations were performed containing the PstI-HF digested DNA, 1.125 ng of P1 and P2 adapters, 400 units of T4 ligase and 19 T4 buffer (New England Biolabs, Beverly MA, USA). Ligations were incubated at 16 °C for 90 min followed by 30 min of denaturation at 80 °C. Adapter-ligated DNA fragments were purified using a Qiagen MinElute® PCR purification kit (Redwood City, CA, USA), eluted in 20 μL of ddH20 and subsequently used for PCR amplification. Fifty microlitre PCRs were performed using 29 MyTaqTM HS Mix (Bioline, Taunton, MA, USA), and containing 0.2 μM each of Illumina Dual Index

Sequencing Primers 1 & 2 (Illumina Inc., San Diego, CA, USA) and 10 μL of above purified DNA. PCR conditions were as follows: 95 °C for 1 min, 24 cycles of 95 °C for 30 s, 65 °C for 30 s, 72 °C for 30 s and a final extension step of 72 °C for 5 min. DNA quantitation and qualitative analysis of individual PCR products were performed on an MCE®-202 MultiNA with a DNA-1000 kit (Shimadzu, Kyoto). Samples were then pooled in equimolar amounts, and library amplicons between 300 and 600 bp were extracted from an agarose gel prior to sequencing on a single HiSeq™ 2500 (Illumina, San Diego) lane at the Australian National University Biomolecular Resource Facility (Australian Capital Territory, Australia).

Data processing

Sequence alignment, assembly, and SNP genotyping were conducted using the UNEAK pipeline implemented in the TASSEL 3.0 command line interface (Lu *et al.*, 2013) (see Supporting Information S3 for the command-line arguments used). The UNEAK pipeline overcomes some of the challenges of *de novo* sequence alignment: Illumina reads are trimmed to 64 bp in order to eliminate the majority of sequencing errors, which accumulate at the end of reads. In addition, the truncated length of 64 bp increases computational efficiency—large raw sequences can be quickly parsed to SNP genotypes on a desktop computer (Lu *et al.*, 2013). Then, consensus reads are merged as reference tags. After pairwise alignment, candidate SNPs are identified in tag pairs that contain a single base pair discrepancy; markers are restricted to biallelic polymorphisms. The UNEAK pipeline is not explicitly designed for pooled samples and the default settings do not discriminate against SNPs with low coverage. Therefore, for this study, reference tags containing less than ten reads were excluded in order to remove low-coverage SNPs which would have had low-accuracy genotype calls.

The UNEAK pipeline enforces a strictly biallelic criterion for the identification of putative SNP loci, in line with evidence that most real SNPs are biallelic (Krawczak, 1999; Morin *et al.*, 2004). The most frequently observed allele is denoted as the ‘major’ allele and the less frequent one as the ‘minor’. In light of this, we re-assessed ambiguous genotype calls using a custom Python script (Supporting Information S3). We applied a binomial test ($P < 0.05$) to assess whether the ratio of minor allele coverage to major allele coverage was equal to or greater than 1:10. This

step reduced spurious or singleton SNPs that could otherwise be misinterpreted as biologically informative loci (as well as removing true low-frequency alleles).

SNP data was converted from the TASSEL HapMap to PHYLIP format using the TASSEL 3.0 graphical user interface, and subsequently converted to Nexus format using PGDSPIDER 2.0.5.2 (Lischer & Excoffier, 2012) to facilitate compatibility with phylogenetic software. Removal of within-population fixed loci and loci with missing data was conducted in SeaView version 4.0 (Gouy *et al.*, 2010) for assessments of genetic diversity and genetic distance.

Phylogenomic analysis

Phylogenomic reconstructions were conducted for individual species SNP datasets by Bayesian inference (BI) methods using MRBAYES version 3.2.2 (Ronquist *et al.*, 2012). All SNP loci showing intra-population polymorphisms were converted to missing data prior to this analysis, as ambiguous nucleotide codes are not recognized as valid character states (Lischer *et al.*, 2014). The generalised time reversible model with a gamma-distribution of rate heterogeneity (GTR+G) was used as the nucleotide substitution model for all species. Being the most generalised and neutral model, GTR+G is broadly applicable and robust for phylogenetic reconstruction, and is more suited to the independent nature of SNP loci than models that have been designed for single locus alignments (Tavaré, 1986; Liò & Goldman, 1998). This choice was confirmed with JMODELTEST version 2.1.5 (Guindon & Gascuel, 2003; Darriba *et al.*, 2012).

BI analyses consisted of two parallel Metropolis coupled Markov chain Monte Carlo (MCMCMC) runs with four chains each at a temperature setting of 0.02, chosen to improve chain mixing and the efficiency of coupling. All analyses started with a random starting tree with no specified root. A total of 10 million generations were run with convergence of parallel runs indicated by average standard deviation of split frequency values being < 0.01. Burn-in and convergence for each run was determined via assessment of likelihood score stabilization in Tracer version 1.6 (Drummond & Rambaut, 2007). Post-burn-in trees discarding the first 25% of cold-chain samples were summarized as a 50% majority-rule consensus tree with posterior probabilities providing nodal support.

Genetic diversity and distance estimates, and clustering analyses

Pairwise genetic distance matrices between each population pair were calculated in POFAD version 1.06 (www.plantevolution.org/en/pofad.html). Given the pooled nature of our genotyping method, intra-population polymorphisms were common across loci, and we used the genpofad algorithm to include these loci in our distance calculations (Joly & Bruneau, 2006; Joly *et al.*, 2014). Allelic diversity was calculated in the TASSEL 5.0 GUI, scored as the proportion of polymorphic loci for each sample site for each species. Estimates were derived from datasets excluding within-population fixed loci and loci with missing data (Bradbury *et al.*, 2007).

For a complementary analysis to the phylogenomic reconstructions, we performed a spatial principal component analysis (sPCA) available in the R package 'adegenet' 1.4-2 (Jombart, 2008) in R 3.1.0 (Team, 2013). This approach performs a PCA to decompose the function $C(x)$, which measures both genetic variability (x is the allele matrix) and spatial structure (by incorporating the matrix of geographic distance weights). An sPCA was performed for each species separately, retaining as many positive and negative eigenvalues as possible to capture >80% of the genetic and spatial variance. Because the first positive eigenvalue in an sPCA result reflects the dominant global geographic structure, we took the first positive eigenvalue scores for each species and transformed them to a common (-1, 1) scale for graphical display, except for *Oreomyrrhis* which was transformed to a (0, 1) scale to match, because the Central populations were not represented in this species (see Fig. 3A).

RESULTS

Genotyping and bioinformatics

SNP data was successfully generated for 92 pooled samples of 10 individuals representing seven plant species across nine to 16 summit populations per species. The raw count of SNP markers averaged across all species was 16,065 (Table 3). After filtering by coverage an average of 2,778 polymorphic markers were retained for the phylogenetic analysis (minimum: 2215 for *S. biflorus*; maximum: 4135 for *G. australis*). The genetic distance and diversity measures employed an average 956 markers (Table 3).

Phylogenomic analyses

Bayesian phylogenomic reconstructions across six species were consistent in supporting a deep evolutionary split between sites from the Northern (Bogong High

Plains, Mt. Fainter, Mt. Hotham and Mt. Loch) and the Central (Mt. Howitt, Mt. Stirling and Wellington Plains) alpine regions of Victoria. High statistical support (posterior probability (PP) ≥ 0.9) for the reciprocal monophyly of the Northern and Central ancestral clades was observed for *H. montana, A. trymalioides, P. alpina*, *S. biflorus* and *S. armeria* (Fig. 2A-B, D-E). This topology was also observed for *G. australis*, however statistical support for the clade monophyly was markedly lower (Fig 2C). Interrelationships among sites within these ancestral clades were largely unresolved, potentially indicating historical and/or ongoing gene flow, which is expected given these are intraspecific trees. *Oreomyrrhis eriopoda* was an exception where strong statistical support (PP ≥ 0.9) for the monophyly of the sites at the western extreme of the sampling range of the Northern alpine region was observed (Fai, Loc, Hot; Figures 2G). *Scleranthus biflorus* was also an exception where the interrelationships among the majority of internal nodes were well resolved (eight of nine nodes with PP ≥ 0.9; Figures 2E), but in this case the topology did reflect clear geographic patterning.

Genetic diversity and distance estimates, and clustering analyses

Overall allelic diversity, calculated as the proportion of polymorphic loci, ranged from 0.65-0.69 for the four shrub species. In contrast, allelic diversity estimates for the herbaceous species *O. eriopoda, S. biflorus* and *S. armeria* were 0.35, 0.25, and 0.69 respectively. The average pairwise genetic distance among sample locations ranged from 21-22% for the shrubs, except *H. montana*, which exhibited an average distance of 10%. Pairwise genetic distances were higher in the herbaceous species, with estimates for *O. eriopoda*, *S. biflorus*, and *S. armeria* being 39%, 40%, and 25%, respectively.

A spatial principal coordinates analysis (sPCA) was performed to investigate global geographic patterns of genetic structure. For most taxa, a clear pattern of separation was visible on the first positive eigenvalue axis between Northern and Central region populations (Figure 3A). For *G. australis, H. montana* and *P. alpina,* the split was located north of the Wellington Plains (WP) site (with the (WP) populations grouping with the other Central region populations); however for *O. eriopoda* the WP population did not diverge particularly strongly from the Central populations. WP was not sampled for *S. armeria* or *A. trymalioides* but these taxa did display a Northern-Central division, with Mt Loch clustering closer to the Central

populations in *A. trimalioides*. For *S. biflorus*, Mt Bogong clustered among the central populations despite this area being firmly in the northern cluster for most other taxa.

DISCUSSION

Genomic analyses performed in this study have revealed deep lineage divergence in a number of unrelated Australian alpine plant species from seven families. Despite differing habits and life histories, the species showed patterns of genetic structure that were largely consistent and suggest a lack of historical gene flow between the Northern and Central alpine regions of Victoria. Specifically, summit populations from the Bogong High Plains appear to be genetically distinct from those occurring on Central highland summits including Mt Buller, Mt Stirling, Mt Howitt and Wellington Plains. This pattern of genetic structure is also common to a variety of insect and mammal species (Osborne *et al.*, 2000; Mitrovski *et al.*, 2007; Endo *et al.*, 2015), suggesting that Victorian alpine species share a common phylogeographic history at the broad scale regardless of taxonomy and life history.

Victoria's Northern and Central alpine regions are currently separated by discontinuous alpine habitat; steep valleys and land cleared for agriculture dissect the alpine region and present strong contemporary barriers to connectivity between summit populations. However, during the most recent glacial advances the snowline was approximately 800 m lower than in the present day, connecting much of the alpine habitat across the region (Galloway, 1965; Fig. 1B). These intermittent periods of habitat connectivity are expected to have provided opportunities for gene flow between summit populations for some species, while shorter spring and summer seasons during glacial periods may have compromised opportunities for others. Regardless alpine communities appear to have remained isolated, diverging genetically over time. Although the phylogenies presented in this study are not time calibrated due to the anonymity of loci, Endo *et al.* (2015) used mitochondrial markers to estimate that the isolation of invertebrate communities from summits likely dates back to the early Pleistocene. Patterns of genetic structure described here, and at an even finer scale in animals (Mitrovski *et al.*, 2007; Endo *et al.*, 2015; Hatley & Murphy, 2016), differ from Northern Hemisphere alpine systems where shallow genetic differentiation is typically observed among populations, owing to a history of

post-glacial expansion (Tribsch & Schönswetter, 2003; Rovito, 2010; Schoville *et al.*, 2011; Shafer *et al.*, 2011; Schoville *et al.*, 2012).

For most plant species included in our study, we found little evidence for differentiation among summit populations within the Northern and Central Victorian alpine regions. These findings are consistent with those reported for Australian alpine *Poa* grasses, also noting limited genetic differentiation within regions (Griffin & Hoffmann, 2014). Only *O. eriopoda* and *S. biflorus* were exceptions in displaying patterns of intra-regional genetic structuring. Unlike other plant species examined in our study, pollination in *O. eriopoda* and *S. biflorus* is not known to be assisted by winged pollinators. This suggests that rates of gene flow might be lower in these species compared with other plants. Similarly, genetic structuring among summits within regions has been reported for ground dwelling and flightless terrestrial invertebrate taxa (Endo *et al.*, 2015), and obligate freshwater invertebrates (Hatley & Murphy, 2016). Rates of gene flow are also expected to be lower in such taxa compared with most plant species included in this study.

The east coast of Australia is known to consist of eight major biogeographic barriers that delineate distinct habitat types, aligning with inter-population geographic structure and/or species boundaries (reviewed in Bryant & Krosch 2016). In many cases, support for barrier locations comes from multiple species and a broad taxonomic base (Bryant & Krosch, 2016). We suggest that multiple lines of evidence have now accumulated to support a new biogeographic breakpoint separating Northern and Central alpine communities in Victoria occurring less than 50 km apart (Osborne *et al.*, 2000; Mitrovski *et al.*, 2007; Endo *et al.*, 2015). Previous genetic studies of alpine grasshoppers and skinks indicate that such barriers are unlikely to exist in the New South Wales alpine region, supported by a lack of genetic structuring (Chapple *et al.*, 2005; Haines *et al.*, 2017; Tatarnic *et al.*, 2013; Slatyer *et al.*, 2014). In the Kosciusko alpine region of New South Wales, habitats are largely contiguous, and it is the only part of mainland Australia where glacial formations are known to have occurred during the Pleistocene (Galloway, 1965; Good, 1992). Endo *et al.* (2015) proposed that the low levels of genetic diversity observed in the region may reflect recolonization of glaciated areas from glacial refuge populations, as seen in Northern Hemisphere systems (Schoville *et al.*, 2012).

Genetic studies from the Northern Hemisphere indicate that the highest levels of intraspecific genetic diversity are generally found in areas least affected by

Pleistocene ice sheets, such as Beringia and large parts of northern Siberia (Brochmann & Brysting, 2008). In heavily glaciated areas, numerous species show evidence of repeated bottlenecks, resulting in extremely depauperate and virtually identical populations over vast geographic areas following post-glacial range expansions (Abbott *et al.* 2000; Ehrich et al. 2007; Schönswetter et al. 2003). In contrast, high levels of genetic diversity have been maintained in areas less affected by glacial ice sheets for these same species (Abbott *et al.* 2000; Ehrich et al. 2007; Schönswetter et al. 2003). Our observations from the Australian Alps are highly consistent, and highlight the need for further characterization of intraspecific genetic divergence across both the Victorian and New South Wales Alpine regions. Such studies will help quantify the true extent of glacial activity on genetic diversity in the Australian Alps, and provide further insights on the regions phylogeographic history.

The current study highlights the value and feasibility of multi-species analyses in understanding the evolutionary history of an area. Such studies are being increasingly undertaken to inform management and conservation planning. For instance, in North America genetic data across a range of vertebrate and invertebrate species have been used to assess the adequacy of wildlife reserves (Vandergast *et al.*, 2008; Wood *et al.*, 2013). To date such studies have been limited to a small number of genetic markers, often comprising of single mitochondrial and nuclear genes (Vandergast *et al.*, 2008; Lorenzen *et al.*, 2012; Reitzel *et al.*, 2013; Wood *et al.*, 2013). As shown here it is now also possible to undertake comparative phylogeographic studies with rapid and cost effective NGS methods to ensure that patterns are established based on large numbers of independent loci.

The GBS Pool-Seq method (Futschik & Schlötterer, 2010; Elshire *et al.*, 2011; Schlötterer *et al.*, 2014) utilized in this study yielded an average of 2,778 independent SNP loci across seven species, based on small pools of up to 10 individuals from 16 locations multiplexed on a single illumina HiSeq 2500 sequencing lane. Costs for this analysis equated to ~US$3.50 per sample (in June 2016), which is approximately one third of the cost associated with traditional Sanger sequencing used in previous multi-species genetic investigations targeting only single DNA regions. The Pool-Seq method has been applied in population genetic and phylogenetic research (Gould *et al.*, 2016; Guo *et al.*, 2016; Rellstab *et al.*, 2016), and we suggest that it also has wide applicability in multi-species analyses to understand processes affecting natural

communities and to identify areas that are particularly unique from an evolutionary perspective. We acknowledge that there are some limitations to the methodological approach taken in our study. Larger pool sizes and corresponding deeper genome sequencing would minimize variance in allele frequency estimates (Schlötterer *et al.*, 2014), enabling more accurate reconstruction of genetic diversity patterns within and between populations. Potential PCR bias could also have been minimized by performing multiple PCR reactions with fewer cycles on each population pool template. However, cost was a significant factor in our study and we opted for the most cost-effective approach along with the following precautions to minimize sources of error. First, sample bias due to unequal starting DNA amounts was minimized by equimolar pooling of individual samples informed by careful assessments of DNA quality and quantity. Second, the bioinformatic data processing approach was very conservative, effectively re-classifying the allele counts (which gave inaccurate estimates of allele frequency due to small pool size) into artificial homozygote/heterozygote "population genotype" calls. This meant that only common minor alleles were retained and effectively only large differences in allele frequency between populations were considered. The fact that seven independent species datasets were consistent in resolving common patterns of deep genetic structure in the Australian Alps, suggests that a strong genetic signal can still be detected despite the limitations of small pool sizes and potential PCR bias in such experiments.

The limited historical gene flow we have identified among seven common and broadly distributed plant species from the Victorian mountain regions has implications for management. Genetically distinct populations need to be managed separately particularly when considering risks associated with climate change and land-use practices. Summit populations can represent isolated, self-recruiting gene pools, and the deep lineage diversification between the North and Central Victorian alpine regions highlights the need to treat these regions separately to maintain evolutionary diversity across plant communities.

CONCLUSION

We used an economical pooled genotyping-by-sequencing (GBS) approach to examine patterns of genetic diversity among seven widespread species including shrubs and forbs from 16 mountain summits in the Australian Alpine National Park.

Patterns of genetic structure were consistent across species in identifying deep evolutionary splits among summit communities from the Northern and Central Victorian Alpine regions. Similar genetic patterns were previously reported for invertebrate, reptile and mammal taxa, indicating shared phylogeographic histories across broad taxonomic groups. These findings differ from those reported for alpine biodiversity from New South Wales and much of the Northern Hemisphere, supporting the notion that genetic diversity is typically greatest in areas least affected by historical ice sheet formation. We advocate the need for independent management of Victorian summit populations, and the adoption of pooled genotyping-by-sequencing (GBS) methods for conducting rapid, and cost-effective multi-species biogeographic research.

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Table 1 Summary of collection sites from the Victorian Alpine National Park from which plant species were collected for genomic analysis

Table 2 Summary of life history traits for species used in the study

Table 3 Summary pool compositions, and numbers of tags and SNPs retained following filtering steps.

Figure 1 Topographic map of the Victorian Alpine region of south-eastern Australia, with collection sites labelled by site code (refer to Table 1). The red contours in panel (b) represent the Last Glacial Maximum expected snowline at 700 m, highlighting the extent of historical connectivity across the region. Grey contours indicate the current-day snowline.

Figure 2 Unrooted Bayesian Inference (BI) trees generated from SNP dataset for each species, a) *Hovea montana*, b) *Asterolasia trymalioides*, c) *Grevillea australia* d) *Pimelea alpine*, e) *Scleranthus biflorus*, f) *Stylidium armeria*, g) *Oreomyrrhis eriopoda*. Terminal nodes are labeled with site codes corresponding with those provided in Table 1. Nodal support ≥ 0.9 is indicated by the presence of black circles with white centers. Blue and Red branches indicate lineages belonging to the Northern and Central regions of the Victorian Alpine National Park respectively.

Figure 3 Broad-scale landscape genetic patterns, site location and population diversity across the study species. A: Transformed sPCA axis 1 score for each sample site for each species. sPCA scores were centred and scaled to a common range for clarity of display here. Coloured lines link values for the same sample site across species, matching colours in panel B. The north--central division is visible in the majority of species, with the Wellington Plains (WP) site sometimes clustering with the northern and sometimes with the central sites. B: Map of the Victorian Alps showing the sample sites with grey shading indicating the Australian Alpine Bioregion (IBRA 7). C: Allelic diversity (proportion of polymorphic loci) plotted against longitude for each species.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 A summary of collection sites for each species included in the study. A tick indicates a collection was made and included in downstream analyses.

Appendix S2 Work flow diagram study includes methodological approaches adopted in the wetlab and bioinformatics

Appendix S3 UNEAK pipeline command-line arguments used to filter and identify SNP loci using the program TASSLE 3.0

Appendix S4 Python code for depth filtering, along with TASSEL 3.0, MrBayes and POFAD command line arguments.

BIOSKETCH

The Climate Change and Adaptation Group at The University of Melbourne (http://pearg.com/ccagroup) is led by Australian Research Council Laureate Fellow Professor Ary Hoffmann. The primary research focus of the group is on the adaptation of organisms (particularly invertebrates) to environmental stress, with an emphasis on examining new ways to predict species distribution shifts and adaptive capacity under climate change.

Author contributions: This project was conceived by A.D.M. and A.A.H.; N.B. and A.D.M conducted the data collection; N.B. performed the bioinformatic data processing; P.G., N.B. and A.D.M performed the data analyses and all authors contributed to the writing of the manuscript.

Table 1 Summary of collection sites from the Victorian Alpine National Park from which plant species were collected for genomic analysis. *Note GPS coordinates have been converted from WGS84 datum to decimal degrees.

Site Name	Altitude (m)	Latitude ºN	Longitude ºE	Code
Northern region				
Bogong High Plains				
Mount Bogong	1987	-36.7325	147.30641	Bog
Buckety Plain	1540	-36.9402	147.33783	BP
Mount Buller	1789	-37.1444	146.42712	Bul
Mount Cope	1844	-36.9271	147.28127	Cop
Marums Point	1799	-36.8688	147.33885	Mar
Mount McKay	1824	-36.8744	147.2433	McK
Mount Nelse	1892	-36.8303	147.32423	Nel
Pretty Valley	1672	-36.8998	147.24317	PV
Rocky Knobs	1746	-36.89	147.28694	RK
Rocky Valley	1674	-36.8919	147.30167	RV
Bogong High Plains Adjacent				
Mount Fainter	1872	-36.8538	147.18987	Fai
Mount Hotham	1866	-36.9756	147.12841	Hot

Mount Loch	1885	-36.9566	147.15649	Loc
Central Region				
Mount Howitt	1742	-37.1757	146.65099	How
Mount Stirling	1723	-37.127	146.49925	Sti
Wellington Plains	1459	-37.4903	146.84167	WP

838 **Table 2** Summary of life history traits for species used in the study. *Note that all species included in the table are perennials.

Species	Family	Life Form	Pollen vector	Selfing Propensity	Seed dispersal
Asterolasia trymalioides	Rutaceae	Low shrub	Bee and fly	Self-incompatible**	Ballistic
Grevillea australis	Proteaceae	Shrub	Bee and fly	Protandrous*	Gravity
Hovea montana	Fabaceae	Low shrub	Bee and fly	Undetermined	Ballistic
Oreomyrrhis eriopoda	Apiaceae	Semelparous herb	Undetermined	Autogamous	Gravity
Pimelea alpina	Thymelaeaceae	Spreading prostrate shrub	Moth**	Gynodioecious*	Gravity
Scleranthus biflorus	Caryophyllaceae	Perennial cushion or mat forming herb	Ant	Facultative autogamous*	Gravity
Stylidium armeria	Stylidiaceae	Perennial herb	Bee	Protandrous, Gynostemium	Gravity

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840 *Denotes traits inferred literature on closely related species with the genus. **Traits inferred from floral morphology

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850 **Table 3** Summary pool compositions, and numbers of tags and SNPs retained following filtering steps.

Species	Sample locations in pool	Individuals in pool	Raw reads in pool	Total number of error free 64bp reads with barcode and cut-site	Raw UNEAK reference tags	UNEAK reference tags with ≥10 reciprocal reads	Number of tags with exactly one SNP	Number of SNPs after filtering loci with ≥10x coverage depth	Proportion of ambiguously -genotyped SNPs	Proportion of unambiguously -genotyped SNPs	Number of SNPs reained for Mr Bayes analysis[1]	Number of SNPs retained for Diversity/SCPCA/analysis[2]
Asterolasia trymalioides	11	110	29839345	27170114	830627	225663	17790	7190	0.61	0.39	2240	1459
Grevillea australis	16	160	44771747	40444193	442712	249485	22343	9219	0.659	0.341	4135	2950
Hovea montana	13	130	33153265	30120404	593879	255592	20574	7262	0.69667	0.30333	2929	1640
Oreomyrrhis eriopoda	13	130	28724054	25860869	423873	88979	10514	6983	0.63892	0.36108	2895	3223
Pimelea alpina	16	160	32580381	29458695	378257	117365	14743	7386	0.65	0.35	2494	1112
Scleranthus biflorus	14	140	34685597	31528654	1505689	210926	15229	4955	0.28693	0.71307	2215	707
Stylidium armeria	9	90	18259009	16820259	1269652	138246	11263	6017	0.52	0.48	2540	932

851 [1] (>⅓ of samples represented per locus, no ambigous calls, no invariable sites)

852 [2] (>⅓ of samples represented per locus, no invariable sites, no missing data)