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RESEARCH ARTICLE

Conservation in the face of hybridisation: genome-wide study to evaluate taxonomic delimitation and conservation status of a threatened orchid species

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Abstract

This study assessed inter- and intraspecific relationships and genetic structure in an Australian species complex in the helmet orchids (*Corybas*) to clarify the taxonomic and conservation status of the threatened species *Corybas dowlingii*, a narrow endemic from southeast Australia. Taxonomic delimitation between the three closely related species *C. aconitiflorus*, *C. barbarae*, and *C. dowlingii* has been mainly based on floral traits which exhibit varying degrees of overlap, rendering species delimitation in the complex difficult. Genome-wide data for the species complex was generated using double-digest restriction-site associated DNA (ddRAD) sequencing. Phylogenomic, genetic network and genetic structure analysis were carried out as well as co-ancestry analysis and hybridisation detection analysis. The ddRADseq results exhibited fine scale genetic structure within the *C. acotiniflorus* complex and provided evidence for hybridisation and introgression within the complex, resulting in blurred taxonomic boundaries between the three species. Implications of the results for conservation management in the face of hybridisation are discussed.

Keywords Australasia · ddRAD sequencing · Diurideae · Species delimitation · Orchidaceae

Introduction

Hybridisation presents a well-acknowledged conundrum in the conservation of threatened species (Allendorf et al. 2001; Fitzpatrick et al. 2015; Jackiw et al. 2015). On one end of the spectrum, hybridisation can pose a serious threat to species integrity and survival (Rhymer and Simberloff 1996; Todesco et al. 2016) and on the other, it can offer an opportunity for beneficial evolutionary processes and act as an instrument for genetic rescue (Anderson and Stebbins 1954; Mallet 2015; Frankham 2015; Taylor and Larson 2019). Furthermore, hybridisation and introgression can present considerable challenges for effective conservation management through blurring species boundaries and thus obfuscating the target for conservation (Rojas 1992; Isaac et al. 2004; Fitzpatrick et al. 2015). Effective conservation management requires accurate delineation of biologically and ecologically meaningful units, either at species, intraspecific or population level (Moritz 1994; Padial and De La Riva 2006; Funk et al. 2012; Coates et al. 2018), and demands a sound understanding of key threatening factors and processes, including those associated with 'genetic invasions' (Jackiw et al. 2015; Mallet 2015). For species with weak reproductive barriers,

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conservation managers and practitioners are often faced with uncertainties around delimitation of their taxon of conservation concern, and with difficulties in determining whether hybridisation is a friend or foe to their biological system (Allendorf et al. 2001; Fitzpatrick et al. 2015; Jackiw et al. 2015). Recent advances in high throughput DNA sequencing and statistical analysis now facilitate unprecedented resolution of inter-and intraspecific relationships and detection of hybridisation and introgression (Funk et al. 2012; Coates et al. 2018; Taylor and Larson 2019).

Orchids are the second largest flowering plant family and of major conservation concern globally due to their high ratio of threatened genera and high number of threatened species (Swartz and Dixon 2009; Chase et al. 2015; Wraith and Pickering 2018; Plant List 2018). Many orchid species are naturally rare, exhibiting highly specialised ecological interactions with symbionts and pollinators and narrow habitat requirements, which render orchids particularly vulnerable to threats such as habitat degradation and loss, over-exploitation, and climate change (Cribb et al. 2003; Swartz and Dixon 2009). Orchids are a prime example of a taxonomically notoriously difficult plant group in which taxonomic uncertainties often pose a serious obstacle for conservation (Pillon and Chase 2007; Hopper 2009; Ahrens et al. 2017). This is reflected in a high rate of new species descriptions in orchids, with approximately 500 species per year worldwide (Chase et al. 2015), and an increasingly large number of taxonomic synonyms in orchids (Govaerts et al. 2017). Taxon delimitation in orchids still heavily relies on the evaluation of morphological and ecological traits, which can be subject to convergent or parallel evolution and environmental plasticity. Further, taxonomic boundaries can be blurred through hybridisation and introgression, which are frequently observed phenomena in orchids (Dressler 1981; Cozzolino et al. 2006; Pinheiro et al. 2010).

The resulting uncertainties around the taxonomic and conservation status of many orchid species greatly hamper effective conservation management and allocation of scarce resources (Pillon and Chase 2007; Hopper 2009; Ahrens et al. 2017). While progress has been made in developing guidelines for dealing with taxonomic uncertainty and hybridisation in a conservation context (Allendorf et al. 2001; Fitzpatrick et al. 2015; Jackiw et al. 2015; Coates et al. 2018), recent advances in high throughput DNA sequencing and bioinformatics now offer powerful genomic approaches to resolve complex inter- and intraspecific relationships at unprecedented resolution.

Next generation sequencing approaches and statistical analysis facilitate the re-assessment of taxonomic concepts in species complexes and the conservation status of rare and threatened species (Funk et al. 2012; Ahrens et al. 2017; Bateman et al. 2018; Coates et al. 2018; Cozzolino et al. 2020; Taylor and Larson 2019). Restriction-site-associated DNA sequencing (RADseq; Baird et al. 2008) is a next generation sequencing method based on reduced representation library sequencing, which is a cost-effective method to obtain genome-scale data from non-model organisms (Davey et al. 2011; Lemmon and Lemmon 2013). For RADseq, genomic DNA is digested using one or two restriction enzymes (ddRADseq). DNA fragments of a certain size range are selected as subset for library preparation and then subjected to high throughput sequencing (Miller et al. 2007; Baird et al. 2008; Peterson et al. 2012; Lemmon and Lemmon 2013). RADseq approaches have been successfully used to clarify inter- and intraspecific relationships and to assess taxonomic delimitation in species complexes, including in orchids (Wagner et al. 2013; Jones et al. 2013; Eaton and Ree 2013; Escudero et al. 2014; Takahashi et al. 2014; Mort et al. 2015; Herrera and Shank 2016; Beheregaray et al. 2017; Bateman et al. 2018; Hipp et al. 2018; Wagner et al. 2018; Brandrud et al. 2019; Brandrud et al. 2020a, b).

Here we applied a conservation genomic approach to assess taxonomic boundaries in an orchid species complex of conservation concern, the *Corybas aconitiflorus* complex (Acianthinae, Diurideae, Orchidoideae). The complex comprises one threatened species endemic to the east coast of Australia, *C. dowlingii* D.L.Jones, two Australasian species, *C. aconitiflorus* Salisb. and *C. barbarae* D.L.Jones, and one species from Java, *C. imperatorius* (J.J.Sm.) Schltr. Morphologically, *C. dowlingii* is only weakly differentiated from *C. aconitiflorus* and *C. barbarae*, mainly by differences in flower size and colouration, and a later, yet overlapping flowering time (Jones 2004, 2006). In Australia, *C. aconitiflorus* and *C. barbarae* are locally common and widespread, extending over 2000 km along the Australian east coast, broadly in sympatry (Fig. 1; AVH 2018). In contrast, *C. dowlingii* is narrowly endemic in New South Wales, extending ca. 100 km from Bulahdelah north of Newcastle to Freemans Waterhole south of Newcastle (Jones 2004; AVH 2018), and occurs within the distribution range of both *C. aconitiflorus* and *C. barbarae* (Fig. 1; AVH 2018). *Corybas dowlingii* is listed as vulnerable species at federal level (EPBC 1999) and as endangered under the New South Wales Threatened Species Conservation Act (TSCA 1995) due to its highly restricted distribution and anthropogenic pressures on its habitat. So far, no molecular study exists which provides insights into taxonomic delimitation and genomic diversity and differentiation in the species complex. Previous molecular research in *Corybas* was unable to resolve interspecific relationships in the *C. aconitiflorus* complex (Clements et al. 2002; Lyon 2014). A study based on five plastid and three nuclear markers resolved *C. aconitiflorus*, *C. barbarae*, *C. dowlingii*, and *C. imperatorius* as a clade, however relationships among these species remained unclear due to a lack of statistical support (Lyon 2014). While sequence divergence among the three Australian

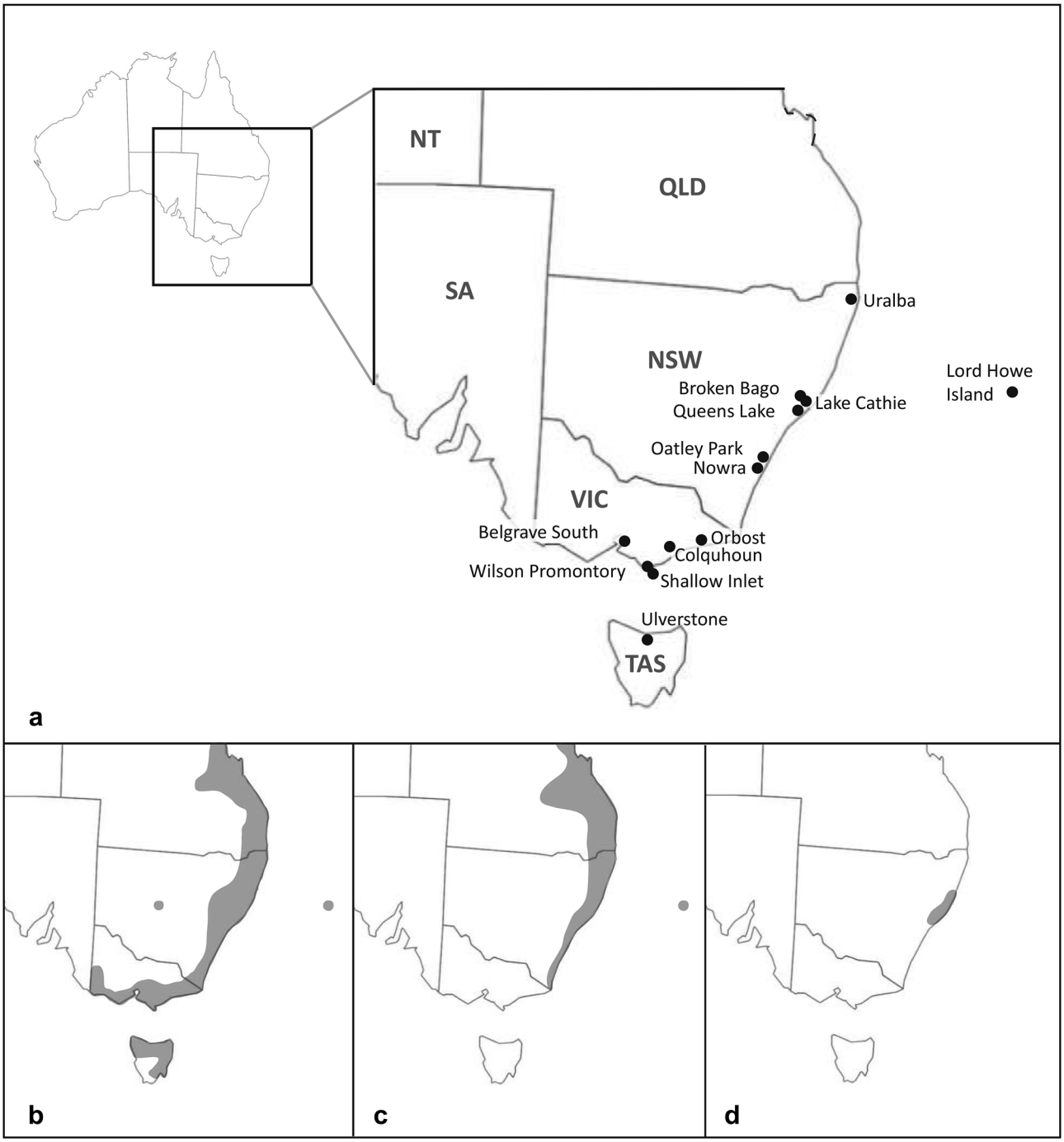


Fig. 1 Study area and collection sites in southeastern Australia (**a**) and distributions of studied *Corybas* species in study area: (**b**) *C. aconitiflorus*; (**c**) *C. barbarae*; (**d**) *C. dowlingii*. Australian states given in bold (*NSW* New South Wales; *NT* Northern Territory; *QLD* Queensland; *SA* South Australia; *TAS* Tasmania; *VIC* Victoria)

species was shallow, *C. imperatorius* was situated on a long branch in the phylogenetic tree reconstruction (Lyon 2014). Due to the low genetic divergence within the *C. aconitiflorus* complex (Clements et al. 2002; Lyon 2014), and the weak morphological differentiation between the three species with partly overlapping character states combined with overlapping distribution ranges, further molecular studies with more informative molecular markers are required to assess species delimitation within the complex to inform conservation management for *C. dowlingii*.

This study aimed to use a conservation genomic approach to assess species delimitation and genetic diversity in the *C.*

aconitiflorus complex to evaluate the taxonomic status of the narrow endemic *C. dowlingii* and to inform its conservation management. The study addresses the following questions:

- Are taxonomic concepts in the *C. aconitiflorus* complex supported by genome-wide evidence, i.e. do currently recognised species within the complex represent separately evolving metapopulations as defined by de Queiroz (2007)?
- Does genome-wide data provide evidence for hybridisation and introgression within the *C. aconitiflorus* complex?
- What are the implications of the genome-wide study on the taxonomic status of the threatened species *C. dowlingii* and its conservation management?

Materials and methods

Material studied

In total, 72 samples were included in the study, of which 70 samples were from the *C. aconitiflorus* complex: *C. aconitiflorus* (24 samples, 9 localities), *C. barbarae* (32 samples, 5 localities), and *C. dowlingii* (14 samples, 2 localities). *Corybas pruinosus* (A.Cunn.) Rchb.f. (2 samples) was included as outgroup based on Clements et al. (2002). Sampling focussed on the south-eastern distribution of the *C. aconitiflorus* complex to which *C. dowlingii* is endemic. It extended from the restricted distribution of *C. dowlingii* (between Port Macquarie and Newcastle, New South Wales) ca. 300 km northwards to the border between New South Wales and Queensland (Uralba), ca. 1200 km southwards to Tasmania (Ulverstone), and ca. 600 km eastwards to Lord Howe Island. We were unable to include the Javan species *C. imperatorius* in this study as we did not have access to fresh tissue material as required for ddRADseq. For each sampled population, one herbarium voucher was taken per species sampled and lodged at CANB. Sampling locations are shown in Fig. 1 and further details on plant material studied are provided in Table 1.

DNA extraction, ddRAD library preparation, and sequencing

Total DNA was extracted from silica-dried leaf material using a modified CTAB protocol (Weising et al. 2005). DNA quality was assessed using a spectrophotometer (NanoDrop, Thermo Scientific) and gel electrophoresis on 2% agarose gels, and DNA quantity was determined using a Qubit fluorometer (Thermo Fisher Scientific).

Double-digest restriction-site associated DNA (ddRAD) sequencing libraries were prepared following Peterson et al. (2012). During the initial establishment phase, double restriction enzyme digests of genomic DNA were carried out for three *Corybas* samples (*C. aconitiflorus* CNS_G04732, *C. barbarae* CNS_G04671, *C. dowlingii* CNS_G04727) testing eight different restriction enzyme combinations comprising a six base cutter (*PstI* or *EcoR1*) and a four base cutter (*MspI*, *HypCH4VI*, *MseI* or *NIaIII*). Digestion was followed by ligation of barcoded adapters compatible with the restriction site overhang, bead purification, and amplification of the non-size selected sequencing library via PCR. Sequencing libraries were evaluated based on gel electrophoresis using TapeStation (Agilent Technologies, Santa Clara, CA, USA) to select the most suitable restriction enzyme combination that indicated the least amount of likely repetitive sequences. After this initial test, the enzyme combination *Pst*I and *Nla*III was selected for the three *Corybas* samples, including ligation of barcoded adapters, purification of the pooled digested-ligated fragments followed by size selection via Blue Pippin (Sage Science, Beverly, Massachusetts, USA) for two ranges, a narrow (280–342 bp) and a wide (280–375 bp) range. The two pooled libraries were amplified via PCR with indexed primers, and sequenced on the Illumina MiSeq platform for single-ended, 150 bp reads at the Australian Genome Research Facility (AGRF; Melbourne, Victoria, Australia). The sequencing data for the narrow and wide size selected libraries was analysed using the Stacks pipeline (Catchen et al. 2011, 2013) to assess the number of ddRAD loci per sample, the average coverage per sample, and the number of unique and shared ddRAD loci across the three samples. The narrow library yielded between 116,991 and 276,091 ddRAD loci per sample and an average coverage of 6.5–9.2 per sample and the wide library resulted in 85,229 to 203,174 ddRAD loci per sample and an average coverage of 6.4–9.4 per sample. The narrow size selection yielded a higher number of shared ddRAD loci across the three samples than the wider size selected sequencing library. Based on the evaluation of two pooled libraries, the narrow size selection was chosen for ddRAD sequencing for the complete sample set. Quality and reproducibility of libraries and DNA sequencing were assessed by running five samples in duplicate (6.9% of all samples). Multiplexed libraries were sequenced on one lane of a NextSeq500 sequencing platform (Illumina Inc., San Diego, CA, USA) as single-ended, 150 bp reads at the Australian Genome Research Facility (AGRF; Melbourne, Victoria, Australia).

Bioinformatics and data filtering

Quality of the sequence reads was examined using FastQC v.0.11.5 (Andrews 2010). Raw sequences were demultiplexed, trimmed and further processed using the ipyrad pipeline v.0.6.15 (Eaton and Overcast 2016). In an initial

Table 1 Material studied

Corybas species	Collection number	DNA number	Provenance	Provenance code
C. aconitiflorus Salisb.	ORG 7362	CNS_G04675	AU: New South Wales, North Coast; near Uralba Nature Reserve	URA
C. aconitiflorus Salisb.	ORG 7364; ORG 7365; ORG 7366; ORG 7366; ORG 7367; ORG 7367; ORG 7368; ORG 7369	CNS_G04732[1]; CNS_G04735; CNS_G04728 CNS_G04728*; CNS_G04730; CNS_G04730*; CNS_G04731; CNS_G04653	AU: New South Wales, Central Coast; near Nowra	NOW
C. aconitiflorus Salisb.	ORG 7361A; ORG 7361B; ORG 7361C; ORG 7361D	CNS_G04668; CNS_G04662; CNS_G04712; CNS_G04708	AU: Tasmania, North West; Stubbs Rd, near Ulverstone	ULV
C. aconitiflorus Salisb.	ORG 7373B	CNS_G04687	AU: Victoria, Gippsland; Wilson Promontory, Five Mile Rd	WIP
C. aconitiflorus Salisb.	Turner 2063A; Turner 2063B; Turner 2063C; Turner 2064A; Turner 2064B; Turner 2064C	CNS_G04680; CNS_G04657; CNS_G04700; CNS_G04672; CNS_G04688; CNS_G04685	AU: Victoria, Gippsland; Oil Bore Road in Colquhoun Forest Regional Park, 9.5 km NW of Lakes Entrance	COL
C. aconitiflorus Salisb.	Turner 2065A; Turner 2065B; Turner 2065C	CNS_G04729; CNS_G04679; CNS_G04674	AU: Victoria, Gippsland; Near Circle Break Track 14.5 km W of Orbost (S of Princes Highway)	ORB
C. aconitiflorus Salisb.	ORG 7374A; ORG 7374B	CNS_G04738; CNS_G04737	AU: New South Wales, Central Coast; Oatley Park, George River	OAT
C. aconitiflorus Salisb.	ORG 7381	CNS_G04747	AU: Victoria, Gippsland; Shallow Inlet	SHI
C. aconitiflorus Salisb.	ORG 7376A	CNS_G04844	AU: Victoria, Gippsland; Belgrave South	BEL
C. barbarae D.L.Jones	ORG 7359A; ORG 7359B; ORG 7359C; ORG 7359D; ORG 7359E; ORG 7359F; ORG 7359G; ORG 7359H; ORG 7359I; ORG 7359J	CNS_G04667; CNS_G04724; CNS_G04714; CNS_G04683; CNS_G04686; CNS_G04665; CNS_G04702; CNS_G04693; CNS_G04663; CNS_G04710	AU: New South Wales, North Coast; Broken Bago State Forest, between Herons Creek & Wauchope	BRB
C. barbarae D.L.Jones	ORG 7363A	CNS_G04709	AU: New South Wales, North Coast; property of C & P Charlie, ajoining Uralba Nature Reserve	URA
C. barbarae D.L.Jones	ORG 7357C	CNS_G04742	AU: New South Wales, North Coast; Edge of Queens Lake, Lakewood	QUL
C. barbarae D.L.Jones	Hutton, I. 946A; Hutton, I. 946B; Hutton, I. 946C; Hutton, I. 946D	CNS_G04743; CNS_G04744; CNS_G04745; CNS_G04746	AU: Lord Howe Island: Ridge between Kim's Lookout and Malabar	LHI

Table 1 (continued)

Corybas species	Collection number	DNA number	Provenance	Provenance code
C. barbarae D.L.Jones	ORG 7357D; ORG 7357D; ORG 7357F; ORG 7357G; ORG 7357G; ORG 7357H; ORG 7357I; ORG 7357J; ORG 7357K; ORG 7357K; ORG 7357L; ORG 7357M; ORG 7357N; ORG 7357o; ORG 7357P; ORG 7357R; ORG 7357T; ORG 7357U; ORG 7357V	CNS_G04655; CNS_G04655*; CNS_G04736; CNS_G04670; CNS_G04694; CNS_G04661; CNS_G04698; CNS_G04722; CNS_G04656 CNS_G04656*; CNS_G04740; CNS_G04671[1] CNS_G04689; CNS_G04673; CNS_G04659; CNS_G04739; CNS_G04741; CNS_G04719; CNS_G04726	AU: New South Wales, North Coast; Queens Lake, Lakewood	QUL
C. dowlingii D.L.Jones	ORG 7358A; ORG 7358A; ORG 7358B; ORG 7358C; ORG 7358D; ORG 7358E; ORG 7358F; ORG 7358G; ORG 7358H;	CNS_G04699; CNS_G04699*; CNS_G04701; CNS_G04707; CNS_G04727[1]; CNS_G04697; CNS_G04733; CNS_G04677; CNS_G04690;	AU: New South Wales, North Coast; Broken Bago State Forest, between Herons Crk & Wauchope	BRB
C. dowlingii D.L.Jones	ORG 7370A; ORG 7370B; ORG 7370C; ORG 7370D; ORG 7370E; ORG 7370F; ORG 7370G;	CNS_G04725; CNS_G04660; CNS_G04654; CNS_G04717; CNS_G04682; CNS_G04695; CNS_G04715;	AU: New South Wales, North Coast; Lake Cathie, Lake Innes Nature Reserve, Corduroy fire trail	LAC
C. pruinosus (R.Cunn.) Rchb.f.	ORG 7374H; ORG 7374M	CNS_G04713; CNS_G04652	AU: New South Wales, Central Coast; Oatley Park, George River	OAT

AU Australia; *CNS* Australian Tropical Herbarium; *ORG* Orchid Research Group, Centre for Australian Plant Biodiversity Research, Canberra

Duplicate samples are marked with an asterisk

[1]Sample used for ddRADseq establishment phase

filtering step, reads with more than five low quality bases (Phred quality score < 20) were excluded from the data set. The phred quality score offset was set to 33. The strict adapter trimming option was selected, and a minimum read length of 35 bp after trimming was chosen to retain a read in the dataset. After these quality-filtering steps, the reads were clustered within and across samples by similarity of 85% using the vclust function in VSEARCH (Edgar 2010). The alignment was carried out using MUSCLE (Edgar 2004) as implemented in ipyrad. Clusters with less than six reads were excluded in order to ensure accurate base calls. The resulting clusters represent putative RAD loci shared across samples. A maximum number of five uncalled bases ('Ns') and a maximum number of eight heterozygote sites ('Hs') was allowed in the consensus sequences. The maximum number of single nucleotide polymorphisms (SNPs) within a locus was set to ten and the maximum number of indels per locus to five. For the sample set including all accessions of the *C. aconitiflorus* complex as well as two accessions of *C. pruinosus* as outgroup ipyrad runs for two different datasets were generated, i.e. based on loci shared by at least 20 individuals (m20) and on loci shared by at least 70 individuals (m70). Additionally, the same settings were used for ipyrad runs excluding the outgroup (*C. pruinosus*, 2 samples). The datasets generated and analysed in the study were deposited at CSIRO's Data Access Portal (https://doi.org/10.25919/5vyn-mh60).

Phylogenomic analysis

Phylogenetic relationships were inferred using maximum likelihood (ML) based on concatenated alignments applying the GTR + Γ model of nucleotide substitution using RAxML v.8.2.4 (Stamatakis 2014) for both datasets (m20, m70) including the outgroup. Statistical support was assessed via a rapid bootstrapping with 100 pseudoreplicates (Stamatakis et al. 2008) under the same ML analysis settings.

Network analysis

Reticulate relationships resulting from hybridization are not well represented by bifurcating tree topologies (Huson and Bryant 2006; McBreen and Lockhart 2006). In a bifurcating tree topology, the hybrid may be placed in an incorrect sister position to one of the parents or even at a basal branch outside parents, depending on its genomic composition (McDade 1992). Combination of conflicting genomic data sets of hybrids likely results in decreased statistical support for clades and loss of resolution (Pirie et al. 2009). To overcome this problem, various network methods have been developed to visualize reticulate relationships (Huson and Bryant 2006; Wen et al. 2016). Split-networks are distance-based and represent incompatibilities in a dataset, which reflects reticulate relationships better than tree-building methods (Huson and Bryant 2006). Among the various algorithms for reconstruction of networks, NeighborNet is widely used as a clustering method for recognition of species-level relationships (Morrison 2014). Thus, we utilised SplitsTree v.4 (Huson and Bryant 2006) in order to reconstruct possible network-like evolutionary relationship among the species. Based on the unlinked SNP data set m20 without outgroups, we generated the split network by implementing NeighbourNet analysis with variance of ordinary least squares. The unlinked SNPs represent one randomly chosen SNP per locus and are therefore considered independent markers. Equal angle split transformation and uncorrelated P distance were selected for the NeighborNet analysis. Bootstrapping was conducted with 1000 replicates as implemented in SplitsTree v.4 on the NeighbourNet to assess statistical support of branches. In all analyses, missing data were treated as unknown.

Genetic structure analysis

Genetic structure was analysed using the Bayesian Markov Chain Monte Carlo (MCMC) clustering method implemented in the program Structure v.2.3.4 (Pritchard et al. 2000) based on the m70 dataset excluding the outgroup. The Structure output format of unlinked SNPs of the ipyrad pipeline was used as input file. Data analysis assumed correlated allele frequencies and admixture and prior population information was not included in the analysis (Hubisz et al. 2009). After preliminary runs with a smaller number of cycles, we conducted three independent runs for each value of K = 2–10 with 100,000 MCMC cycles following a burn-in of 10,000 MCMC cycles for the final analyses. The number of genetic groups best fitting the dataset was determined using the delta K method (Evanno et al. 2005) as implemented in Structure Harvester (Earl and vonHoldt 2012). We refrained from Structure analysis based on the m20 dataset due to decreased performance of Structure analysis based on datasets with higher levels of missing data (Pritchard et al. 2010).

Co-ancestry analysis

To further explore genetic structure, we employed RADpainter included in the package fineRADstructure (Malinsky et al. 2018), which infers population structure from RADseq data. The program creates a co-ancestry similarity matrix based on haplotypes. The analysis compares nearest neighbour haplotypes by finding the closest relative for each allele for a given sample set using SNP data. For more details see Malinsky et al. (2018). We performed the analyses using default settings based on the m70 dataset (alleles.loci file) resulting from ipyrad. First, we prepared the input file using the python script finRADstructure_input.py included in 'finRADstructure-tools' (https://github.com/edgardomortiz/fineRADstructure-tools). With the input file we calculated the co-ancestry matrix employing RADpainter. We then used 'finestructure' for clustering and tree assembly using 100,000 MCMC replicates and a burnin of 100,000 applying the clustering approach '-m T'. The results were visualized with the R script 'fineRADstructurePlot'.

Hybridisation detection

Hybridisation detection with the software HyDe (Blischak et al. 2018) allows testing for hybridisation and introgression at a population or species level based on D-statistics by estimating the amount of admixture (γ). We applied the 'run_hyde_mp.py' script to test for putative parent-hybrid combinations. HyDe uses p-values to test for the significance of results. While a recent 50:50 hybrid is characterized by a γ-value of about 0.5, very low levels of admixture (e.g. 0.1 = close to parent P1; 0.9 = close to parent P2) may be indicators for several processes such as incomplete lineages sorting (ILS). Intermediate values represent older hybridization events and introgression. After initial tests, we used a range of γ = 0.4–0.6 to identify recent hybridization events in the data set, and intermediate ranges of γ = 0.2–0.4 and 0.6–0.8 for older events. We excluded significant values < 0.2 and > 0.8. The SNP data of the m70 data set including two accessions of *C. pruinosus* as outgroup were used

as input data. We tested two approaches: first, we used each individual sample as an entity and, second, we assigned the individuals to species according their determination and provenances resulting in 16 'populations'. The first approach is better able to detect individual admixture. The applied γ-values between 0.4 and 0.6 for recent hybridization are in accordance with recent studies, which showed similar γ-values for modelled and empirical hybrid data (Blischak et al. 2018; Zhang et al. 2019; Wagner et al. 2020b).

Results

An average of 2,98 (±1,01) million filtered Illumina reads per sample were used for the analyses. A total number of 362,412 pre-filtered loci passed the ipyrad pipeline. After subsequent filtering steps, the number of retained loci for the final datasets varied between 3597 (m70) and 12,420 (m20) for the datasets including the outgroup, and between 4293 (m70) and 14,915 (m20) loci for the datasets excluding the outgroup. The latter included 60,489 SNPs (see Table 2). The average read depth per locus was 20.87 (±4.01) reads. Further statistics of the ddRAD datasets are summarized in Table 2.

Table 2 Statistics for the ddRAD dataset for the *C. aconitiflorus* complex (A) including the outgroup (*C. pruinosus*, 2 samples) and (B) excluding the outgroup resulting from the different filtering thresholds in the ipyrad pipeline for loci shared by minimum number of samples. pis: parsimony informative characters, bp: base pairs, m: minimum number of samples

Filtering threshold	m20	m70
(A) *C. aconitiflorus* complex including outgroup (*C. pruinosus*)[a]		
#RAD loci	12,420	3597
#Variable sites	54,177	17,378
#Pis	36,486	12,302
#Aligned bp	1,682,315	487,474
Missing data*	37.66%	4.55%
(B) *C. aconitiflorus* complex (ingroup)[b]		
#RAD loci	14,915	4293
#Variable sites	60,489	18,828
#Pis	39,470	12,252
#Unlinked SNPs	13,708	4066
#Aligned bp	1,967,151	581,128
Missing data*	36.79%	2.30%

*Proportion of gaps and undetermined bases in DNA sequence alignment

[a]Average # filtered reads: 2.98 Mio (+/− 1.01) per sample

[b]Average # filtered reads: 3.01 Mio (+/− 1.01)

Maximum likelihood analysis

The ML analysis based on the m20 dataset including the outgroup retrieved the *C. aconitiflorus* complex as monophyletic clade with maximum bootstrap support (BS 100) (Fig. 2). Genetic divergence between the *C. aconitiflorus* complex and *C. pruinosus* was considerably higher than within the species complex as indicated by branch length (Fig. 2). Within the *C. aconitiflorus* complex, the ML reconstruction did not provide support for the monophyly of the three species *C. aconitiflorus*, *C. barbarae*, and *C. dowlingii* (Fig. 2).

First diverging, a well-supported clade A (BS 96) was depicted which comprised individuals from *C. barbarae* from North Coast (Broken Bago), followed by a well-supported clade B (BS 100) with the remaining *C. barbarae* samples from North Coast (Broken Bago). Next diverging was clade C unifying individuals from *C. barbarae* from North Coast (Queens Lake and Uralba). Within clade C, relationships were not well supported, with the exception of a highly supported clade D, which unified the majority of samples from Queens Lake exhibiting low genetic divergence. Next diverging was a highly supported clade E (BS 97) harbouring individuals from *C. barbarae*, *C. dowlingii* and *C. aconitiflorus*. Within clade E, the first two diverging clades, F and G, were formed by individuals of *C. dowlingii* from North Coast (Broken Bago), both receiving high support (F: BS 92 and G: BS 94). The next diverging clade H was only weakly supported (BS 59) and exhibited a dichotomy with branch I that harboured a moderately supported clade J (BS 85) unifying individuals of *C. aconitiflorus* from North Coast and South Coast (Uralba and Nowra) next to a highly supported clade K (BS 100) comprising individuals of *C. barbarae* and *C. dowlingii*. Clade K split into two highly supported branches (BS 100, each), clade L with all individuals of *C. barbarae* from Lord Howe Island and clade M with individuals of *C. dowlingii* from North Coast (Lake Cathie).

The second main branch within clade H, clade N, was well supported (BS 90) and comprised the remaining samples of *C. aconitiflorus* from Central Coast and South Coast (Sydney and Nowra), all *C. aconitiflorus* samples from south Victoria (Belgrave, Colquhoun, Orbost, Shallow Inlet, Wilson Promontory) and Tasmania (Ulverstone) (Fig. 2). Within clade N, the *C. aconitiflorus* individuals from south Victoria and Tasmania formed a weakly supported clade O (BS 60) (Fig. 2).

The ML analysis of the m70 dataset yielded congruent results for highly supported clades and differed in topology for nodes that remained unsupported or received low statistical support in the ML analyses. Results of the ML analysis of the m70 dataset are presented in Online Resource 1.

NeighborNet analysis

The NeighborNet diagram of the *C. aconitiflorus* complex based on 13,708 unlinked SNPs from the m20 dataset showed two main clusters. Cluster 1 comprised the majority of *C. aconitiflorus* samples corresponding to clade N in the ML analysis while cluster 2 comprised *C. barbarae* from the Australian mainland. Further, several smaller clusters were found, harbouring *C. dowlingii, C. barbarae* from Lorde Howe Island, and *C. acotiniflorus* samples from Uralba and Nowra (Fig. 3).

Within main cluster 1 harbouring *C. aconitiflorus*, samples from south Victoria and Tasmania formed a weakly differentiated subgroup. *C. aconitiflorus* samples from NSW (Nowra, Oatly Park, and Uralba) were found in more proximate position to *C. dowlingii* from Broken Bago, corresponding to relationships found in the ML analysis (Fig. 2).

Main cluster 2 comprised all *C. barbarae* samples from the Australian mainland. Cluster 2 harboured two branches with individuals from Broken Bago (North Coast) corresponding to clade A and B in the ML analysis, as well as three branches with individuals from Queens Lake (North Coast), corresponding to clades C1, C2 and D in the ML analysis. Further, main cluster 2 harboured one branch with *C. barbarae* from North Coast (Uralba), corresponding to lineage C3 in the ML reconstruction.

Corybas barbarae samples from Lord Howe Island and *C. dowlingii* from Lake Cathie formed a cluster, corresponding to clade K found in the ML analysis. Within cluster K, *C. barbarae* samples from Lord Howe Island formed a cluster corresponding to clade L in the ML reconstruction and *C. dowlingii* from Lake Cathie (North Coast) formed a cluster corresponding to clade M in the ML reconstruction. However, genetic distances between the clusters in the NeighborNet diagram were overall low and the NeighborNet diagram exhibited patterns of conflicting signal (Fig. 3). Subclusters within the NeighborNet were largely consistent with well supported clades found in the ML analysis (Fig. 2).

The NeigbourNet analysis based on the m70 dataset yielded highly congruent results to the analysis of the m20 dataset and is provided in Online Resource 2.

Genetic structure analysis

The Bayesian cluster analysis with Structure based on the m70 dataset comprised 4066 loci. The best number of genetic groups (K) for the m70 dataset as determined by the modal Δ K distribution was K = 7 (Online Resource 3).

The majority of *C. barbarae* samples from mainland Australia fell into three genetically distinct groups. Two of these comprised samples from Broken Bago (North Coast), which also formed two well-supported clades in the ML analysis (A and B) and two distinct clusters in the NeighborNet analysis (A and B). The third genetic group of *C. barbarae* in the Structure barplot comprised the majority of samples from Queens Lake (North Coast), which were also found as highly supported clade in the ML analysis (D) and as a cluster in the NeighborNet analysis (D).

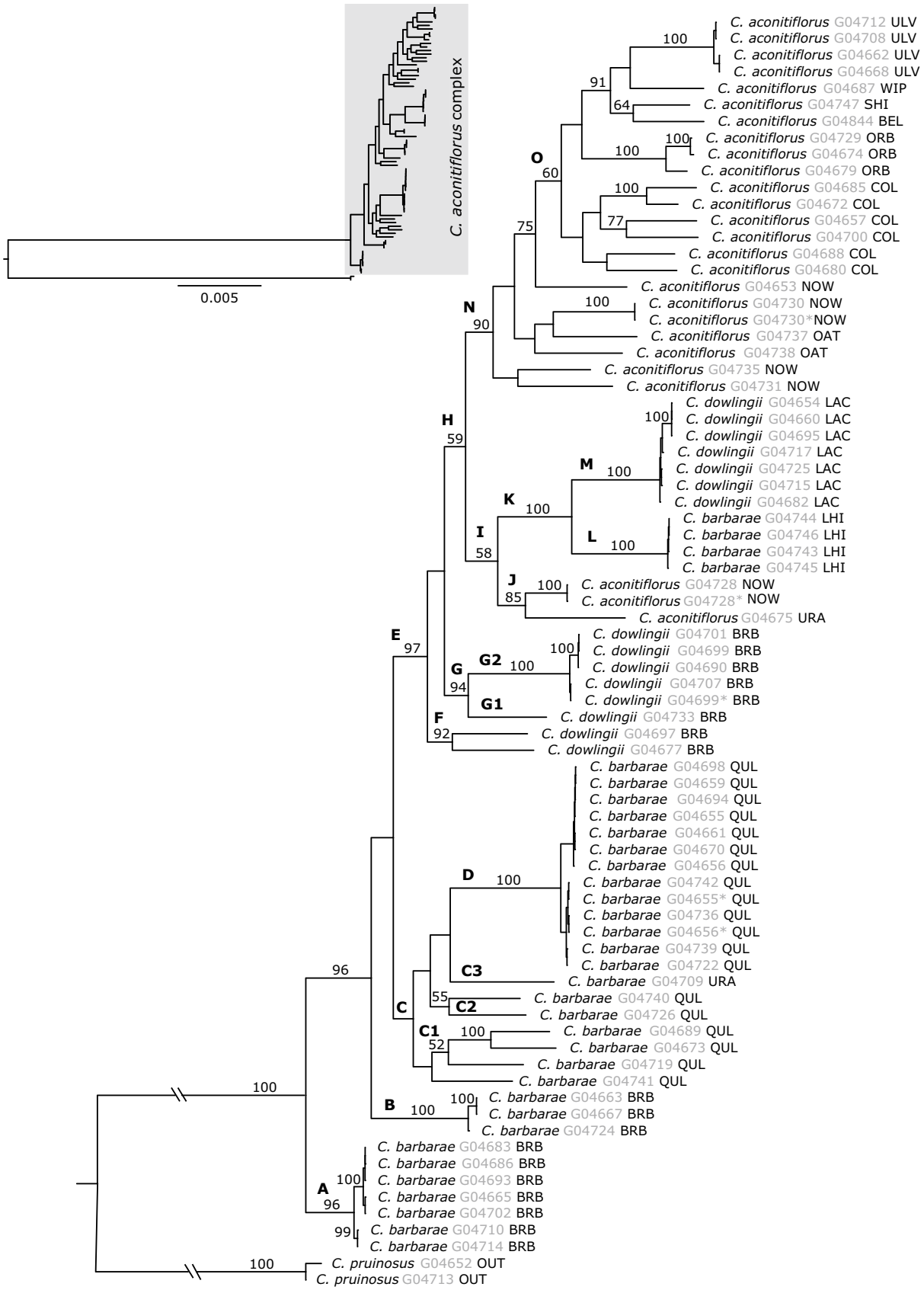
Corybas barbarae samples from Lord Howe Island and *C. dowlingii* samples from Lake Cathie together formed another genetically distinct group in the Structure barplot. These samples were also reconstructed in a highly supported clade K in the ML analysis and as cluster K in the NeighborNet analysis. The remaining *C. barbarae* samples from Queens Lake and from Uralba (North Coast) formed a genetic cluster corresponding to lineages C1, C2, and C3 in the ML phylogenetic reconstruction. This Structure group indicated genetic admixture with other Structure groups including those comprising *C. aconitiflorus*, *C. barbarae*, and *C. dowlingii* samples (Fig. 4).

The largest Structure group comprised all *C. aconitiflorus* samples corresponding to clades N and J in the ML phylogeny and clusters N and J in the NeighborNet analysis. The large Structure group also harboured three *C. dowlingii* samples from Broken Bago (North Coast), corresponding to lineages G1 and J found in the ML analysis and branches G1 and J in the NeighborNet diagram. The Structure group displayed varying levels of genetic admixture with *C. barbarae* samples from Queens Lake (North Coast). Among the *C. aconitiflorus* samples, those from south Victoria and Tasmania displayed the lowest signals of genetic admixture (Fig. 4).

Co-ancestry analysis

Genetic structure within the complex was further inferred with FineRADstructure based on the m70 SNP dataset comprising 4066 unlinked SNP loci. Higher-level relationships based on the co-ancestry similarity matrix yielded three main clusters within the species complex (Fig. 5): a large cluster comprising the majority of *C. acotiniflorus* samples, corresponding to clade N of the ML analysis and cluster N of the NeighborNet; one large cluster comprising the majority of *C. barbarae* samples, and a third main cluster comprising all *C. dowlingii* samples together with the remaining *C. aconitiflorus* samples and *C. barbarae* samples. Thus, higher-level relationships retrieved in the FineRADstructure analysis differed from those inferred by ML analysis.

However, fine-scale population structure shown in the co-ancestry matrix corresponded closely with moderately to highly supported clades retrieved in the ML analysis and observed clusters in the NeighborNet and genetic Structure analyses (Fig. 5, Online Resource 4). Within *C. acontiflorus,* the smaller cluster corresponded to clade/cluster J of the ML and NeighborNet analysis, respectively. Within *C. barbarae*, fine-scale genetic clusters retrieved through co-ancestry


C. aconitiflorus complex
0.005
C. aconitiflorus G04712 ULV
C. aconitiflorus G04708 ULV
C. aconitiflorus G04662 ULV
C. aconitiflorus G04668 ULV
C. aconitiflorus G04687 WIP
C. aconitiflorus G04747 SHI
C. aconitiflorus G04844 BEL
C. aconitiflorus G04729 ORB
C. aconitiflorus G04674 ORB
C. aconitiflorus G04679 ORB
C. aconitiflorus G04685 COL
C. aconitiflorus G04672 COL
C. aconitiflorus G04657 COL
C. aconitiflorus G04700 COL
C. aconitiflorus G04688 COL
C. aconitiflorus G04680 COL
C. aconitiflorus G04653 NOW
C. aconitiflorus G04730 NOW
C. aconitiflorus G04730* NOW
C. aconitiflorus G04737 OAT
C. aconitiflorus G04738 OAT
C. aconitiflorus G04735 NOW
C. aconitiflorus G04731 NOW
C. dowlingii G04654 LAC
C. dowlingii G04660 LAC
C. dowlingii G04695 LAC
C. dowlingii G04717 LAC
C. dowlingii G04725 LAC
C. dowlingii G04715 LAC
C. dowlingii G04682 LAC
C. barbarae G04744 LHI
C. barbarae G04746 LHI
C. barbarae G04743 LHI
C. barbarae G04745 LHI
C. aconitiflorus G04728 NOW
C. aconitiflorus G04728* NOW
C. aconitiflorus G04675 URA
C. dowlingii G04701 BRB
C. dowlingii G04699 BRB
C. dowlingii G04690 BRB
C. dowlingii G04707 BRB
C. dowlingii G04699* BRB
C. dowlingii G04733 BRB
C. dowlingii G04697 BRB
C. dowlingii G04677 BRB
C. barbarae G04698 QUL
C. barbarae G04659 QUL
C. barbarae G04694 QUL
C. barbarae G04655 QUL
C. barbarae G04661 QUL
C. barbarae G04670 QUL
C. barbarae G04656 QUL
C. barbarae G04742 QUL
C. barbarae G04655* QUL
C. barbarae G04736 QUL
C. barbarae G04656* QUL
C. barbarae G04739 QUL
C. barbarae G04722 QUL
C. barbarae G04709 URA
C. barbarae G04740 QUL
C. barbarae G04726 QUL
C. barbarae G04689 QUL
C. barbarae G04673 QUL
C. barbarae G04719 QUL
C. barbarae G04741 QUL
C. barbarae G04663 BRB
C. barbarae G04667 BRB
C. barbarae G04724 BRB
C. barbarae G04683 BRB
C. barbarae G04686 BRB
C. barbarae G04693 BRB
C. barbarae G04665 BRB
C. barbarae G04702 BRB
C. barbarae G04710 BRB
C. barbarae G04714 BRB
C. pruinosus G04652 OUT
C. pruinosus G04713 OUT
O
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G1
F
D
C3
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C2
C1
B
A

◂**Fig. 2** Maximum likelihood phylogenetic reconstruction of the *Corybas aconitiflorus* complex based on 12,420 ddRAD loci (m20 dataset including outgroup). Three letter codes denote sample provenances. *BEL* Belgrave South; *BRB* Broken Bago; *COL* Colquhoun; *LAC* Lake Cathie; *LHI* Lord Howe Island; *NOW* Nowra; *OAT* Oatly Park; *ORB* Orbost; *QUL* Queens Lake; *SHI* Shallow Inlet; *URA* Uralba Nature Reserve; *ULV* Ulverstone; *WIP* Wilson Promontory. Bootstrap support values above 50 are given above branches. Duplicate samples are marked with an asterisk. Clades referred to in the text are labelled A–O

estimation corresponded to clades/clusters A, B, D, L and lineages C1-3 in the ML and NeighborNet analyses. Within *C. dowlingii*, one cluster corresponded to clade/cluster M of the ML and NeighborNet analysis, one cluster to lineage G2, and a third cluster combined lineages F and G1 found in the ML and NeighborNet analysis (Fig. 5).

The clustered co-ancestry matrix revealed varying degrees of co-ancestry within the complex. Highest degrees of shared co-ancestry (displayed in dark red to purple colour; Fig. 5) were found within the same population of a species. However, clusters with high degrees of shared co-ancestry did not always include all samples of a given population. High levels of co-ancestry were found in *C. barbarae* populations from Broken Bago (as two clusters: A and B), Queens Lake (cluster D), and Lorde Howe Island (L). High levels of shared co-ancestry were also found in *C. dowlingii* from Lake Cathy (M), and in one *C. dowlingii* cluster with the majority of samples from Broken Bago (G2). Within *C. aconitiflorus*, only two clusters showed higher levels of co-ancestry and thus stronger isolation. These were the populations from south Victoria (Orbost) and Tasmania (Ulverstone) (Fig. 5).

Further, the clustered co-ancestry matrix displayed moderate levels of shared co-ancestry (depicted in light orange colours; Fig. 5) not only within species but also between species. Most striking was the shared co-ancestry between samples of *C. dowlingii* from Lake Cathy and *C. barbarae* from Lorde Howe Island, which corresponded to clade K/cluster K from the ML and NeighborNet analysis. Further, moderate levels of shared co-ancestry were also evident between samples of *C. aconitiflorus* and *C. dowlingii* and between *C. aconitiflorus* and *C. barbarae* (Fig. 5).

Hybridisation detection analysis

To analyse the dataset for signals of hybridisation we performed a HyDe analysis (Blischak et al. 2018) based on 17,378 SNPs resulting from the m70 dataset including outgroup. In the first analysis with the 'individual approach', 202,575 triplets were tested. Among the tested triplets, HyDe revealed 2,594 significant hybridisation events (Online Resource 5). Of these, 915 (35%) exhibited γ-values between 0.4 and 0.6, which is an indicator of recent hybridisation. Most hybrid samples were detected in *C. barbarae* with a total of 1791 events, of which 612 events yielded γ-values between 0.4 and 0.6. Within *C. aconitiflorus*, a total of 628 hybridisation events were detected, of which 265 events were indicative of recent hybridisation. Only a minor fraction of tested triplets (179 events) assigned *C. dowlingii* samples as hybrids, of which 40 showed γ-values between 0.4 and 0.6. In a second HyDe analysis, in which samples were assigned to species and provenances ('population approach'), 1365 triplets were analysed. Here, HyDe detected only 43 significant results, all of which assigned *C. aconitiflorus* accessions as hybrids.

Discussion

This study provided detailed insights into phylogenetic relationships and genetic structure in the *C. aconitiflorus* complex based on ddRAD data comprising several thousand loci. Previous molecular phylogenetic studies in *Corybas* based on one to few plastid and nuclear loci were unable to resolve interspecific relationships within the complex (Clements et al. 2002; Lyon 2014). Our ddRADseq study provided molecular evidence for interspecific hybridisation and introgression within the *C. aconitiflorus* complex resulting in blurred species boundaries. The phylogenomic analysis found several samples from *C. aconitiflorus* and *C. barbarae* to be more closely related to *C. dowlingii* than to conspecific individuals, thus not supporting the monophyly of the three species. Likewise, our genetic network analysis showed several samples of *C. aconitiflorus* and *C. barbarae* exhibiting close genetic affinities to *C. dowlingii* and the NeighborNet indicated conflicting phylogenetic signal for inferred relationships between these samples. The analysis thus revealed patterns consistent with the presence of reticulation within the complex and did not support the hypothesis that the three species represent separately evolving metapopulations. Other studies also found conflicting signal in NeighborNet analysis among closely related hybridising species, such as in Bromeliaceae and Asteraceae (Schulte et al. 2010; Wagner et al. 2020a). However, a ddRADseq study in hybrid zones of *Melocactus concinnus* (Cacataceae) and four congeneric species found that the five species formed well supported clusters in the NeighborNet analysis not exhibiting conflicting signal despite the occurrence of hybridisation within the complex (Kahn et al. 2020). The study provided evidence that the *Melocactus* species maintained their genetic distance from each other despite frequent hybridisation events due to low bidirectional introgression. Parental genomes were found to be favoured, likely contributing to purging of introgressed alleles among the *Melocactus* species (Kahn et al. 2020).

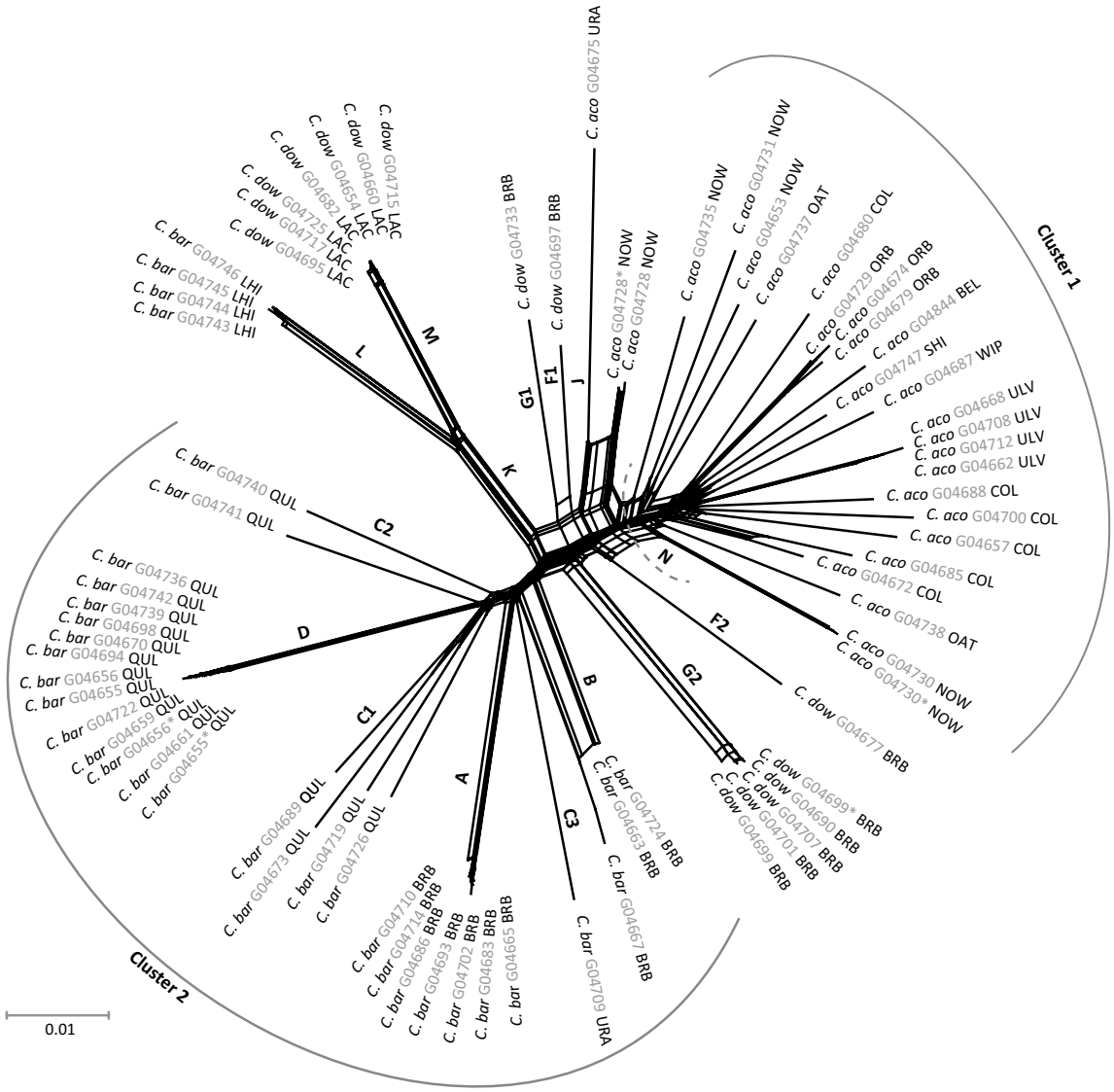

Fig. 3 NeighborNet network for *Corybas aconitiflorus* complex based on 4,293 unlinked SNPs (m20 dataset excluding outgroup). C. aco: *Corybas aconitiflorus*; C. bar: *Corybas barbarae*; C. dow: *Corybas dowlingii*. Letters A-N above branches correspond to clades retrieved in the ML phylogenetic analysis (Fig. 2). Three letter codes denote sample provenances. *BEL* Belgrave South; *BRB* Broken Bago; *COL* Colquhoun; *LAC* Lake Cathie; *LHI* Lord Howe Island; *NOW* Nowra; *OAT* Oatly Park; *ORB* Orbost; *QUL* Queens Lake; *SHI* Shallow Inlet; *URA* Uralba Nature Reserve; *ULV* Ulverstone; *WIP* Wilson Promontory. Duplicate samples are marked with an asterisk

Our genetic structure analysis retrieved genetic groups largely consistent with clades and clusters found in the phylogenomic and network analysis. As in the other analysis, genetic groups found in the Structure analysis did not align with current species delimitation within the complex. Instead, two genetic groups found in the Structure analysis comprised samples from two species (*C. aconitiflorus* and *C. dowlingii*; *C. barbarae* and *C. dowlingii*) and indicated genetic admixture in several *C. aconitiflorus*, *C. barbarae* and *C. dowlingii* samples. Thus, the Structure analysis provided genetic evidence for porous species boundaries within the complex leading to genetic admixture.

The co-ancestry matrix and associated tree reconstruction provided further insights into relationships between genetic clusters. While two main clusters unified the majority of *C. acotiniflorus* and *C. barbarae* samples respectively, the third large cluster retrieved in the co-ancestry analysis comprised *C. dowlingii* and several *C. acotiniflorus* and *C. barbarae* samples, thus providing further evidence for the non-monopyly of the three species. The co-ancestry matrix revealed shared co-ancestry between these outlier samples with

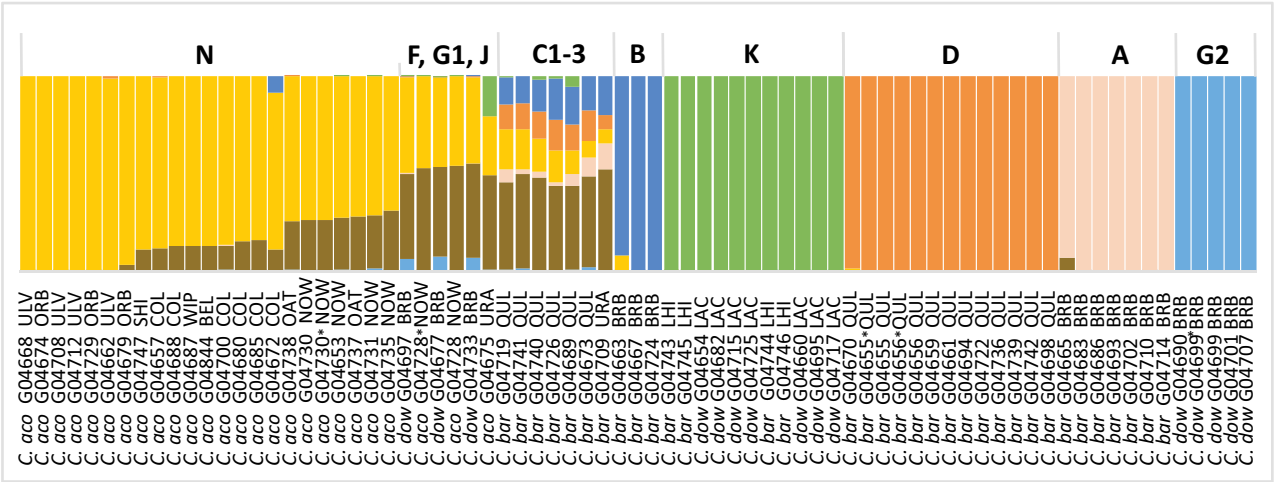

Fig. 4 Genetic structure of *Corybas aconitiflorus* complex based on Structure analysis of 4066 unlinked SNPs (m70 dataset excluding outgroup) for the optimal K value of seven genetic clusters. *C. aco Corybas aconitiflorus*; *C. bar Corybas barbarae*; *C. dow Corybas dowlingii*. Three letter codes denote sample provenances. Three letter codes denote sample provenances. *BEL* Belgrave South; *BRB* Broken Bago; *COL* Colquhoun; *LAC* Lake Cathie; *LHI* Lord Howe Island; *NOW* Nowra; *OAT* Oatly Park; *ORB* Orbost; *QUL* Queens Lake; *SHI* Shallow Inlet; *URA* Uralba Nature Reserve; *ULV* Ulverstone; *WIP* Wilson Promontory. Capital letters above the Structure barplot correspond to clades retrieved in the ML phylogenetic analysis (Fig. 2). Duplicate samples are marked with an asterisk

conspecific samples in the main clusters. The co-ancestry analysis also highlighted signals of shared ancestry between samples of different species, providing evidence for hybridisation and introgression within the complex (Fig. 5). The hybridisation detection analysis provided further evidence for hybridisation within the complex, with signatures of both recent and older hybridisation events. Studies examining hybridisation events within plant genera based on RADseq data in *Myrsine* (Applehans et al. 2020) and *Salix* (Wagner et al. 2020b) also detected recent and older hybridisation events using HyDe, however found a higher prevalence of older hybridisation events. This difference to our study is not surprising as we investigated a recently evolved species complex. Thus, our study shows the applicability of HyDe within species complexes (Blischak et al. 2018a).

Most recent hybrids detected were assigned to *C. barbarae* samples, followed by *C. aconitiflorus* samples, and only 38 of the inferred recent hybridisation events in the triplet analysis were assigned to *C. dowlingii* samples. Overall, this ddRADseq study demonstrated that species boundaries within the complex are porous, resulting in occasional interspecific hybridisation. The molecular evidence for hybridisation and introgression within the species complex found in our study is in line with morphological evidence, which shows intergrading traits between the three species, such as in flower colour, shape, and size (Jones 2004, 2006).

Our genetic results indicate weak pre- and postzygotic barriers within the *C. acotiniflorus* complex allowing for occasional interspecific hybridisation. Orchids are well known to often exhibit weak postzygotic barriers among closely related species, a trait that is exploited widely in the creation of horticultural hybrids (https://www.rhs.org.uk/about-the-rhs/publications/orchid-hybrid-lists). However, highly specific plant-pollinator interactions often act as effective pre-zygotic barriers in orchids. While the pollination strategy of *Corybas* has been regarded as food or brood deceptive based on observations from other *Corybas* species (Pridgeon et al. 2001), recent observations in *C. aconitiflorus* indicate that the species is food-rewarding (Kuiter and Findlater-Smith 2017). Fungus gnats of the genus *Phthinia* Winnertz 1863 (Mycetophilidae) were observed to visit the flowers to forage on the column mound which has been reported to exude nectar. Females where found leaving the flowers with pollinia attached to their thorax (Kuiter and Findlater-Smith 2017). The repeated and directed visiting behaviour of the fungus gnats was regarded as indication that the fungus gnats are attracted through floral scent. Further, the plant-pollinator relationship was found to be specific in the *Corybas* populations in Victoria, where only one *Phthinia* species was observed to visit the flowers (Kuiter and Findlater-Smith 2017). However, the detection of genetically admixed individuals in our molecular study implies that the plant-pollinator relationships within the *C. acotiniflorus* complex is less specific and allows for occasional cross-pollination. Further studies on the pollination biology within the *C. acotiniflorus* complex are required to clarify pollination biology of the species and the extent of pollinator specificity within the complex, especially in sympatric populations.

Our ddRADseq study revealed fine-scale genetic structure within the *C. acotiniflorus* complex. The results of the phylogenetic, genetic network, genetic structure, and co-ancestry

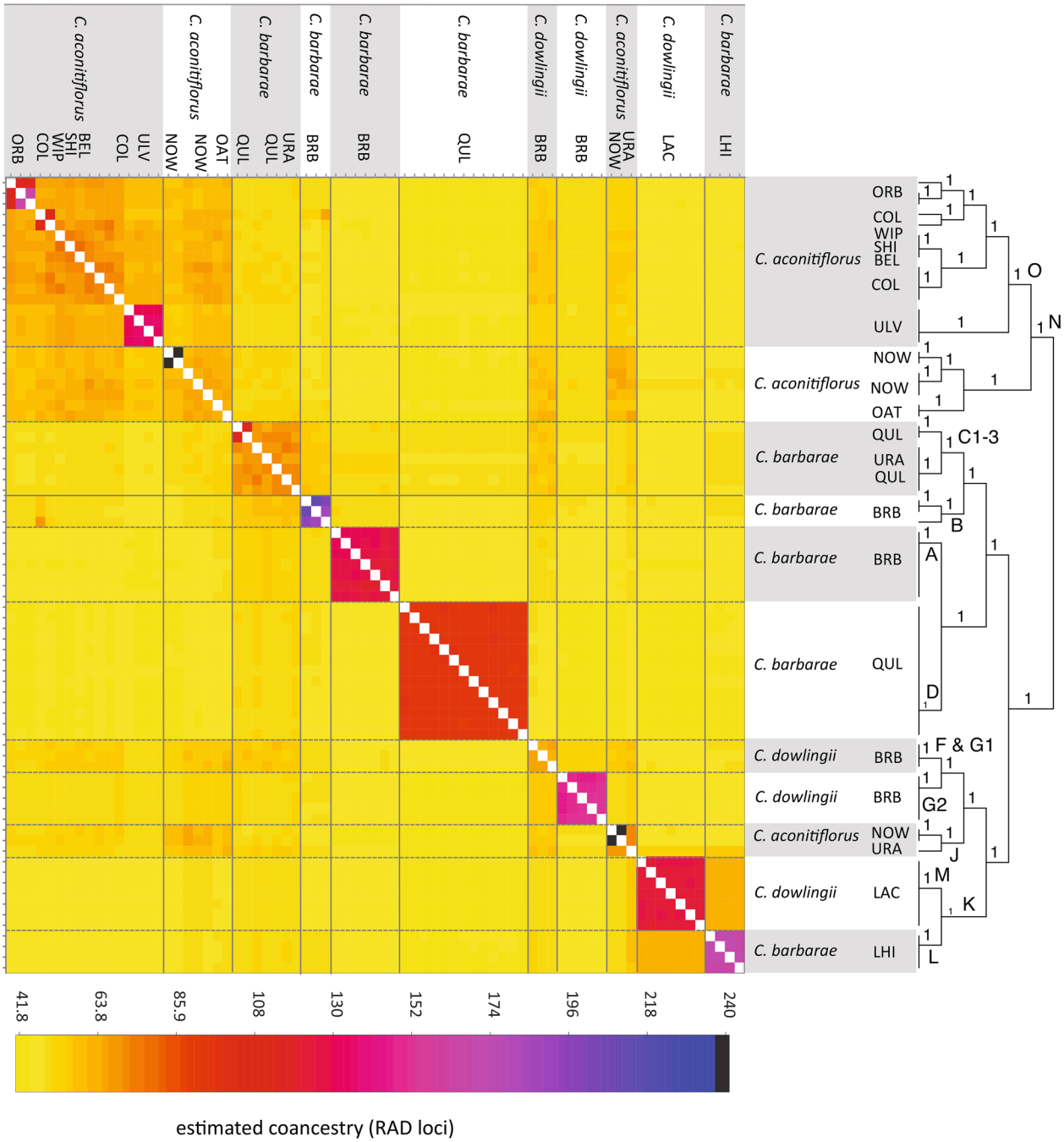

Fig. 5 Clustered co-ancestry matrix derived from a RADpainter analysis based on 4066 unlinked SNP loci of the m70 dataset (excluding outgroup). Three letter codes denote sample provenances. *BEL* Belgrave South; *BRB* Broken Bago; *COL* Colquhoun; *LAC* Lake Cathie; *LHI* Lord Howe Island; *NOW* Nowra; *OAT* Oatly Park; *ORB* Orbost; *QUL* Queens Lake; *SHI* Shallow Inlet; *URA* Uralba Nature Reserve; *ULV* Ulverstone; *WIP* Wilson Promontory. Capital letters above branches in the cladogram refer to clades retrieved in the ML analysis (Fig. 2). Numbers above branches denote posterior population assignment probabilities

analyses largely retrieved the same genetic groups at a fine scale. These genetic groups usually comprised individuals of the same species from the same provenance and were moderately to well supported in our phylogenomic analysis. However, these groups did not always comprise all samples of the species from the same provenance. The co-ancestry matrix clearly showed the highest levels of co-ancestry in samples of species of the same provenance, and these clusters were consistent with clades and clusters found in the phylogenomic and network analyses. Further, the co-ancestry analysis also highlighted instances where conspecific samples from the same provenance exhibited lower co-ancestry and

showed levels of shared co-ancestry with other populations or species within the complex. This indicates the existence of hybrids or introgressed individuals within a population and thus ongoing geneflow (Malinsky et al. 2018).

An unexpected result of our conservation genomic study was the apparent close relationship between *C. barbarae* samples from Lord Howe Island and *C. dowlingii* samples from Australia's east coast (Lake Cathy). Further population genomic studies are warranted to resolve the taxonomic status of *C. barbarae* on Lord Howe Island and to clarify its relationship to *C. dowlingii*.

Based on the results of this study, we conclude that species delimitation in the *C. aconitiflorus* is imperfect due to occasional interspecific hybridisation. Because of the remaining uncertainties regarding the taxonomic status of *C. barbarae* from Lord Howe Island and its relationship to *C. dowlingii*, we refrain from taxonomic changes within the complex and recommend an extended population genomic study to assess species delimitation and levels of hybridisation and introgression within the complex in more detail. For such an extended genomic study, a denser population-level sampling is recommended in *C. dowlingii*, as well as inclusion of samples from the entire geographic range of the complex. Further, the inclusion of other species from the Australasian *Corybas* clade (Clements et al. 2002) should be considered.

A recent review of conservation legislation found that legal definitions of species are quite flexible and can accommodate a range of infra-specific taxa and divergent populations (Coates et al. 2018). While the taxonomic boundaries between *C. dowlingii* and its closest relatives are somewhat fuzzy due to occasional interspecific gene flow within the complex, genomic evidence from this study showed *C. dowlingii* populations as genetically distinct and thus can still be considered to warrant protection due to anthropogenic pressure on their habitat. In the following, we will discuss implications for the protection of a species of a species complex in which hybridisation occurs and will consider whether hybridisation may pose a threat to the survival of a rare species or genetically distinct populations.

While traditionally the protection of pure, genetically distinct species that do not interbreed successfully have been favoured in conservation science and policy (Agapow et al. 2004), it is increasingly recognised that biological diversity generated by hybridisation can also hold conservation value (Allendorf et al. 2001; Agapow et al. 2004). While the evolutionary importance of hybridisation in the diversification of plants has long been recognised, recent genomic studies highlighted the prevalence of hybridisation in the natural world (Taylor and Larson 2019). Further, the detection of ancient hybridisation in genomic studies, which indicate that hybridisation occurred in many taxa at some point in the past, has led to a greater appreciation of the evolutionary importance of hybridisation and introgression and improves our understanding of potential long-term consequences of hybridisation (Taylor and Larson 2019). Hybridisation can lead to greater fitness compared to parental species, i.e. heterosis (hybrid vigour), and is seen as important evolutionary process that promotes adaptation and speciation. Hybrids can exhibit novel adaptive traits that allow for increased ecosystem resilience to environmental stressors and can allow for the successful colonisation of novel habitats (Stebbins 1959). However, hybrids can also have negative impacts on biodiversity, in particular in cases where hybrids pose risks to the survival of their parental species or to other native vegetation (Rhymer and Simberloff 1996). Based on a review of species hybrids and their conservation or management, Jackiw et al. (2015) strongly advocate for a case by case approach in assessing the conservation value of species which may be subject to hybridisation or are the result of hybridisation.

A key ecological consideration is whether hybridisation is likely to pose a risk to the survival of the parental species, for example through decreasing the genetic variability in parental species or by causing extinction through genetic assimilation as long-term consequences of recurrent hybridisation and introgression (Rhymer and Simberloff 1996). This is of particular importance in cases where parental species are already threatened by other factors, as is the case in *C. dowlingii*. The species has only a narrow distribution whereas the other two species of the complex are common and occur over a large distributional range with both sympatric and allopatric distributions. Our genomic study did not clearly indicate whether hybridisation within the complex poses a current threat to *C. dowlingii* through genetic swamping. Therefore, an extended genomic study within the complex is warranted to examine levels of hybridisation and introgression within *C. dowlingii* in more detail.

Another consideration concerns species fitness as hybrids exhibiting a higher fitness than the parental species may have detrimental effects either for the parental species or to other native species, as has been documented for example in invasive weeds (Stebbins 1959; Rhymer and Simberloff 1996). *Corybas dowlingii* is only known from a few populations extending ca. 100 km from Bulahdelah to Freemans Waterhole in New South Wales, overlapping with the geographic distribution of the two other species of the complex and occurring in the same habitat. To date, there is no indication of increased fitness of hybrids within the complex compared to its parental species, however ecological studies are warranted to assess potential risks.

An important consideration from an evolutionary perspective is that hybrids are often regarded as beneficial. They hold the potential to act as catalysts for speciation or more generally as pathway for evolution, for example in cases where hybrids exhibit novel properties such as in floral scent

(Stökl et al. 2008; Vereecken et al. 2010), habitat requirements (Jacquemyn et al. 2012) or genome duplication and rearrangements (de Storme and Mason 2014). These novel traits might lead subsequently to the formation of a new species. Therefore, the maintenance of evolutionary processes becomes a key ethical consideration in the conservation of species which hybridise (Jackiw et al. 2015).

In the following we will examine possible beneficial aspects of hybridisation within the complex such as the potential of the hybrids to act as pathway for evolution. In the New Caledonian hybrid species *Corybas* × *halleanus* E.Faria, a greater tolerance to lower humidity was observed for the species compared to its more moisture dependent parental species (Faria 2016). Consequently, the species exhibits increased resilience to environmental stressors, which enables the hybrid to colonise novel habitat, thus rendering the conservation of this hybrid species beneficial. In contrast, individuals with genetic signatures of recent hybridisation or introgression found in this analysis were in the same habitats as their parental species, and so far, there is no indication that these occupy a novel ecological niche.

In the case of *C. dowlingii*, the conservation of an existing rare genotype needs to be balanced against maintenance of the evolutionary potential, which lies in hybridisation. Given that the two widespread species within the *C. aconitiflorus* complex, *C. aconitiflorus* and *C. barbarae*, occur in sympatry over a large distributional range, maintenance of this evolutionary potential can be safeguarded while at the same time accommodating the protection of *C. dowlingii* from potential genetic swamping.

Conclusions

Our assessment of the phylogenetic relationships and genetic structure within the *C. aconitiflorus* complex indicated occasional gene flow and hybridisation within the species complex, resulting in blurred species boundaries. To further clarify taxonomic delimitation within the complex, an extended population genomic study of the complex across its entire distribution is required using highly resolving markers such as ddRADseq or custom target sequence capture markers. For such an extended genomic study inclusion of other species from the Australasian *Corybas* clade (Clements et al. 2002) should also be considered.

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Author contribution KN and MC conceived and designed the study; LS undertook lab work and contributed to data analysis, NW carried out data analysis; all authors contributed to data interpretation and writing/revision of the manuscript.

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Data availability Sequence data (raw and processed data files) were deposited in the CSIRO Data Access Portal (https://doi.org/10.25919/5vyn-mh60). under the Creative Commons Attribution-Licence 4.0 for non-commercial use.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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