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The *Crinum flaccidum* (Amaryllidaceae) species complex in Australia

Jamie Simpson[A,B,*], John G. Conran[A], Ed Biffin[C], Kor-jent van Dijk[A] and Michelle Waycott[A,C]

For full list of author affiliations and declarations see end of paper

***Correspondence to:**
Jamie Simpson
School of Biological Sciences, The University of Adelaide, Adelaide, SA 5005, Australia
Email: simpsjc015@gmail.com

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ABSTRACT

Crinum flaccidum Herb. is widespread across southern and eastern Australia, but suffers from taxonomic uncertainty. This ambiguity is in part due to widespread hybridisation in the genus, but also morphological variability within and among populations. Morphological and molecular analyses of the complex using 24 morphological characters and 59 chloroplast coding and non-coding regions (~50 000 bp) provided support for the separation of *C. flaccidum* from *C. luteolum* Traub & L.S.Hannibal ex Traub, with the latter representing populations from the Flinders Ranges and Lake Eyre Basin. Within *Crinum flaccidum*, there was greater inferred genetic structure at the population level for New South Wales flood plain accessions, compared with South Australian populations from along the Murray River. The greater structure of the South Australian populations is theorised to be attributed to lower seed-dispersal rates leading to lower gene flow.

Keywords: Amaryllidaceae, *Crinum*, molecular phylogenetics, morphology, New South Wales, South Australia, species complex, taxonomy.

Introduction

Amaryllidaceae is a family of mostly bulbous geophytes with ~70 genera. Species complexes are prevalent in the family, owing to blurred species boundaries and frequent hybridisation, with *Lycoris* Herb., *Allium* L. and *Crinum* L. being just some of the genera with ongoing species-resolution issues (e.g. Shi *et al.* 2006; Marques *et al.* 2007; Lykos 2011; Smirnov *et al.* 2017; Khorasani *et al.* 2018).

The characteristic waterproof, corky-layered seeds with chlorophyllous embryos of *Crinum* have allowed for pantropical dispersal out of Africa (Meerow and Snijman 2001; Meerow *et al.* 2003), with 14 species being recognised in Australia, 13 of which are native (Lykos 2011; Lehmiller *et al.* 2012*a*, 2012*b*; Australasian Virtual Herbarium, AVH, Council of Heads of Australasian Herbaria, CHAH, see https://biodiversity.org.au/nsl/services/APC). The native *C. flaccidum* Herb. has had an ambiguous relationship with *C. luteolum* Traub & L.S.Hannibal ex Traub., the taxonomic status of the latter ranging from species to a minor variant of the former (Hewson 1987, 2020; Lykos 2011; Plants of the World Online, see http://www.plantsoftheworldonline.org; CHAH, see https://biodiversity.org.au/nsl/services/APC).

Hannibal (1963) described *C. flaccidum* as having four 'variants' that possess '…thin-segmented flowers' and being 'a highly polymorphic species' (p. 46). Although Hannibal's (1963) morphological descriptions were vague, the localities given for each morph were more explicit, as follows: Gilgandra and Quirindi, New South Wales; Murray River, South Australia; Andamooka, South Australia; and Spencer Gulf, South Australia, to West-Central Queensland. *Crinum flaccidum* was listed subsequently by Hewson (1987, 2020), as one of the following five species present in Australia: *C. pedunculatum* R.Br., *C. flaccidum*, *C. angustifolium* (now *C. arenarium* Herb.), *C. venosum* R.Br. and *C. uniflorum* F.Muell. Hewson (1987, p. 373) reported the distribution of *C. flaccidum* as 'north-western Western Australia, eastern Northern Territory, Queensland, New South Wales, north-eastern Victoria and eastern South Australia' but also suggested that *C. flaccidum* was 'genetically unstable, hybridising easily, forming many eco-variants',

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as well as noting several morphologically unusual specimens across its wide range that needed further study. Hewson (1987) covered current records (minus eastern Victoria) in AVH (CHAH, see https://avh.ala.org.au/) and expanded the distribution given by Hannibal (1963) (Supplementary Fig. S1). The north-western Western Australian population noted by Hewson (1987, 2020) is an outlier and closer geographically to populations of *C. arenarium* than to the majority of *C. flaccidum* records (CHAH, see https://avh.ala.org.au/). *Crinum arenarium* is a northern Australian species with which *C. flaccidum* has been synonymised previously (CHAH, see https://biodiversity.org.au/nsl/services/APC).

Crinum luteolum Traub & L.S.Hannibal ex Traub was first described by Traub (1965) from Pichi Richi (South Australia), 29 km from the tip of the Spencer Gulf (South Australia), which was one of the locations for the 'variants' of *C. flaccidum* described by Hannibal (1963). Hewson (1987) regarded *C. luteolum* as a desert eco-variant of *C. flaccidum*, but it was recognised by Jones (pers. comm. 2006, cited in CHAH, see https://biodiversity.org.au/nsl/services/APC). Hamilton (2010) and Lykos (2011) further recognised two variants within *C. luteolum*: a northern desert morph with broad yellow tepals, and a southern morph with thinner, paler yellow tepals. Currently, *C. luteolum* is listed as a synonym of *C. flaccidum* in the Australian Plant Census (APC; CHAH, see https://biodiversity.org.au/nsl/services/APC) and the Census of South Australian Plants (State Herbarium of South Australia, see http://flora.sa.gov.au/).

Morphology-based taxonomic issues are common within Amaryllidaceae (Hirschegger *et al.* 2010; Khorasani *et al.* 2018), but molecular studies by Meerow *et al.* (2003) and Kwembeya *et al.* (2007) suggested a South African ancestry for *C. flaccidum*, distancing the species from other Australian natives that have relationships with South-East Asian taxa (e.g. *C. asiaticum* L., *C. pedunculatum* and *C. venosum*). *Crinum luteolum* has yet to be sequenced. This study aims to resolve the relationships between and within *C. flaccidum* and *C. luteolum* through molecular chloroplast sequencing and morphological analyses.

Materials and methods

Plant material

Crinum collections of up to 10 individuals per location were made in South Australia (14 populations) and New South Wales (five populations; Fig. S1). The South Australian samples consisted of 10 *C. luteolum* populations, representing five of the northern and five southern morphotypes of Hamilton (2010) and Lykos (2011), plus four *C. flaccidum* populations from along the Murray River. At each of these locations, leaves of 10 separate individuals were sampled and a population voucher specimens were also collected. Where possible, individuals were collected >3 m apart to reduce the chance of sampling clones. In New South Wales, leaf material was collected for *C. flaccidum* from 3 to 7 individuals per population.

Outgroup taxa included species of *Crinum*, *Calostemma* R.Br., *Amaryllis* L. and *Allium* (Supplementary Table S1), on the basis of relationships inferred by previous studies (Meerow *et al.* 2003; Kwembeya *et al.* 2007), and sourced from the Adelaide Botanic Gardens and State Herbarium of South Australia (Table S1). The morphological study used voucher specimens from the 14 sites studied in South Australia and three sites from New South Wales, together with herbarium material from the State Herbarium of South Australia (Table S1).

Molecular analyses

All material, both fresh and fragments of herbarium specimens, was dried fully prior to DNA extraction. A pilot test was conducted to find the optimum weight of plant material for DNA quantification. High levels of secondary metabolites can interfere with polymerases and restriction enzymes, creating difficulties in isolating high-quality DNA (Varma *et al.* 2007; Moyo *et al.* 2008). Equipment was cleaned with sodium hypochlorite, reverse osmosis (RO) water and 70% ethanol. Samples were limited to 4 mg of tissue per extraction becaue of the large reduction in dsDNA isolated for samples containing more than 6 mg of leaf tissue.

DNA extraction, hybridisation capture and preparation of libraries for sequencing generated data for up to 59 chloroplast regions, as per Waycott *et al.* (2021). DNA sequences were generated using Illumina paired-end sequencing (2 × 150) on a single lane of a HiSeqX Ten at the Garvan Institute for Medical Research in Sydney.

The raw sequence data were processed and mapped using CLC Genomics Workbench (ver. 7.5.1, Qiagen, see https://www.qiagenbioinformatics.com). Following demultiplexing and quality trimming (Phred-score threshold of 20), the paired reads from a single sample (*Calostemma purpureum* R.Br. AD214055) were mapped to the chloroplast genome sequence of *Lycoris aurea* (L'Hér.) Herb. (GenBank number MN158985) by using the following settings: length fraction = 0.5, similarity fraction = 0.8, match score = 1, mismatch cost = 2, insertion–deletion cost = 3. Consensus sequences were then extracted using conflict resolution = vote, a low coverage threshold of 50 and splitting the consensus into separate sequences around regions of low coverage. Consensus sequences of <300 bp were removed, resulting in a set of 37 consensus sequences ranging in length from 323 to 4884 bp, which were used as a mapping reference for all samples. Sequence reads from all samples were mapped to the *Calostemma purpureum* references using parameters as above and consensus sequences were extracted using a low coverage cut-off of eight and inserting ambiguity codes (Ns) for low coverage regions. All consensus sequences were imported into Geneious (ver. 2021.1.1, Dotmatics, see

https://www.geneious.com), aligned using the Geneious MUSCLE (ver. 3.8.425, Dotmatics, see https://www.geneious.com/prime/; Edgar 2004) plugin and each aligned region was concatenated for downstream analyses.

The concatenated alignment was analysed under maximum likelihood (ML) with IQ-TREE (ver. 2.1.3, B. Q. Minh, J. Trifinopoulos, D. Schrempf and H. A. Schmidt, IQ-Tree, see http://www.iqtree.org/; Nguyen *et al.* 2015) using a custom model selected by ModelFinder (ver. 2.1.3, IQ-Tree, see iqtree.org/ModelFinder/; Kalyaanamoorthy *et al.* 2017). Ultrafast non-parametric bootstrap replicates (1000×; Hoang *et al.* 2018) were implemented to assess branch support on the ML tree. A MrBayes Markov-chain Monte Carlo (MCMC) output based on a GTR model with gamma-distributed rate variation and an estimated proportion of invariant sites (GTR + I + G; Ronquist *et al.* 2012) was also run. Analyses were performed with uninformative priors on model parameters, and two independent runs (each with four chains, one cold and three heated) of 2×10^6 generations, sampling every 500 steps. Convergence between independent runs and the appropriate burn-in was performed using the post-run 'sump' command in MrBayes. The resulting trees from these analyses were then exported to FigTree (ver. 1.4.4, A. Rambaut, Molecular evolution, phylogenetics and epidemiology, see http://tree.bio.ed.ac.uk/software/figtree) for viewing and interpretation.

Morphological analyses

Leaf and floral characters were scored with continuous variables transformed through $\log(n + 1)$ to help linearise allometric measurements (Tables S2, S3). The data (Table S3) were imported into the software package PAST (ver. 4.08, Ø Hammer, University of Oslo, see https://www.nhm.uio.no/english/research/resources/past/) and a dendrogram was created using the unweighted pairwise group method arithmetic (UPGMA) algorithm with Gower similarity. Non-metric multidimensional scaling (NMDS) was also run in three dimensions incorporating a biplot analysis derived for the 16 variable characters (Hammer 2021).

Results

The final DNA sequence data set was generated for 163 samples, including 59 chloroplast coding and non-coding regions, representing an aligned length of 52 981 bp (Table S4). Both ML and Bayesian analyses of the chloroplast gene regions resolved a *C. flaccidum* + *C. luteolum* clade (Fig. 1), sister to a *C. arenarium* and *C. pedunculatum* + *C. mauritianum* G.Lodd. clade. Within the *C. luteolum* subclade, 'northern' and 'southern' morphotypes were not distinguished from each other (Fig. S2). The *C. flaccidum* subclade recovered four well-supported groups, one comprising samples from South Australia and three groups of individuals sampled from New South Wales. Relationships among and within these groups were not resolved (Fig. S2).

The morphological cluster analyses separated the *C. luteolum* samples from *C. flaccidum*, with further separation of the New South Wales and South Australia *C. flaccidum* collections (Fig. 2). The 10 populations of *C. luteolum*

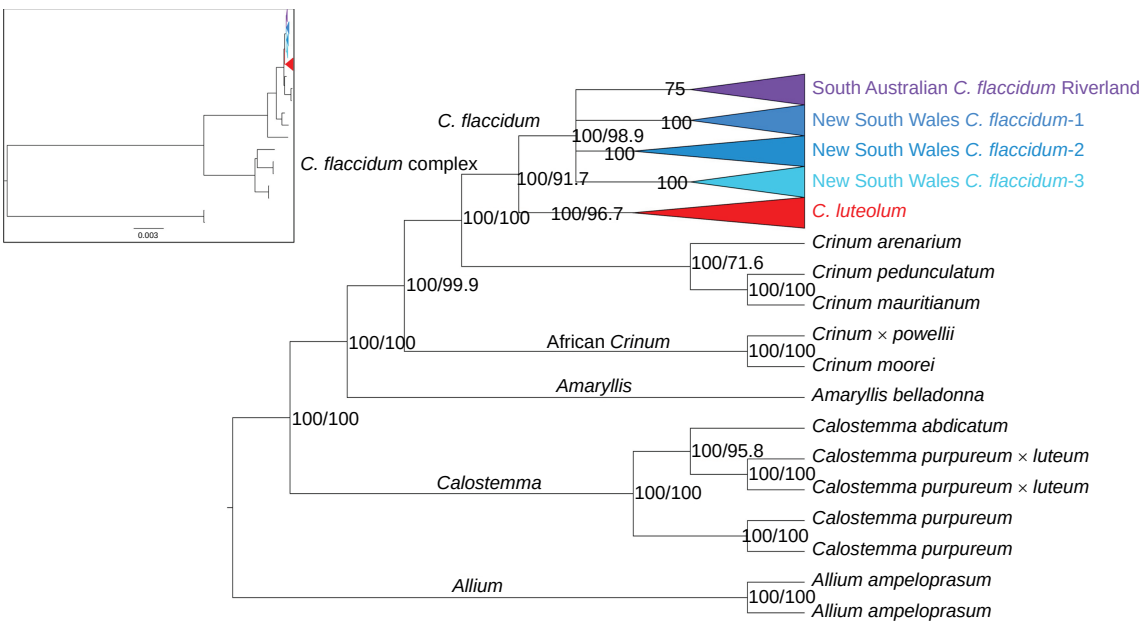

Fig. 1. Cladogram of MrBayes and maximum-Likelihood analysis of *Crinum flaccidum* species complex with respective branch support values; inferred using K3Pu + F + I + G4 best-fit model. MrBayes/UFBoot2. For the *C. flaccidum–luteolum* complex, nodes have been collapsed to indicate the main well supported groupings. There is an inset phylogram to show inter-species differences.

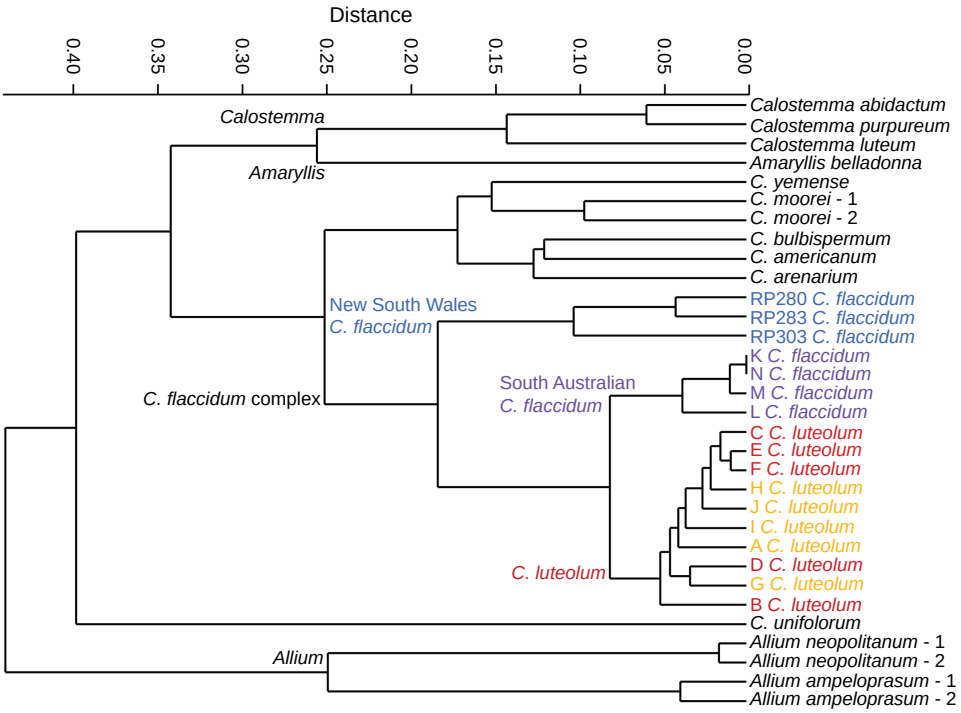

Fig. 2. Dendrogram of the morphological data using unweighted pair group method with arithmetic mean and a Gower similarity based on 24 morphological characters. All characters were independent and weighted equally. The *Crinum flaccidum* species complex has been separated into three clusters, namely, New South Wales, South Australia and *C. luteolum*. Within *C. luteolum*, the northern morphotype is red and the southern morphotype orange. The letter(s) and numbers at the start of *C. flaccidum* and *C. luteolum* samples indicate the population (Table S1).

displayed no internal coherence between 'northern' and 'southern' morphotypes. The NMDS with all sampled *Crinum* species and outgroups resulted in three *C. flaccidum* species-complex clusters (Fig. S3). A similar dendrogram (not presented) and clearer ordination plot resulted from the exclusion of outgroup *Crinum* species (Fig. 3). The New South Wales *C. flaccidum* samples (Group 1; Fig. 3) were correlated with style, filament and flower colour, flower symmetry and leaf length. Group 2 (South Australian *C. flaccidum*) was correlated with perianth, style and filament-length vectors. Group 3 consisted of all *C. luteolum* samples and was placed centrally in the ordination plots for Coordinates 2 and 3, with only the perianth lobe vector showing correlation when viewed through Coordinates 1 and 2. The OTUs representing *C. luteolum* all had pale-yellow styles and filaments, whereas South Australian *C. flaccidum* populations all possessed white filaments and styles, the morphological differences between *C. flaccidum* (South Australia and New South Wales) and *C. luteolum* are summarised in Table 1.

Discussion

The analyses all produced a well supported split between *C. flaccidum* and *C. luteolum*, but there was low chloroplast divergence within the *C. luteolum* clade and no support for the recognition of separate 'northern' and 'southern' lineages (Fig. S2). By contrast, there is a clear split between the South Australian and New South Wales samples within the clade formed by *Crinum flaccidum* samples (Fig. S2), with the New South Wales samples showing structure and organisation at the population level (Fig. S2). South Australian *C. flaccidum* populations showed very low levels of sequence divergence (Fig. 1 inset and Fig. S2), suggesting a high level of connectivity (gene flow) along the lower Murray River. Snijman and Linder (1996) concluded that *Crinum* primarily disperses through water-mediated means. Many *Crinum* species have buoyant seeds capable of long-distance hydrochory (Bjorå *et al.* 2009; Huang *et al.* 2021) and it is plausible that *C. flaccidum* has dispersed along the lower Murray, on the basis of the low genetic variation within the South Australian populations (Fig. 1, Fig. S2). All the sampled New South Wales *C. flaccidum* populations were from flood-plain environments with minor water systems nearby and these populations are likely to rely on major flood events for dispersal, which are known to occur in the region, albeit infrequently (Speer and Leslie 2000; O'Gorman 2012). The differential gene flow for the riparian South Australian *v.* New South Wales floodplains populations was also observed in *Acacia stenophylla* A.Cunn. ex Benth and *Duma florulenta* (Meisn.) T.M.Schust by Higgisson *et al.* (2020). Higher gene flow was seen for riparian *A. stenophylla* than in *D. florulenta*, which grows on the Murray–Darling floodplains. However, hydrochorous riparian species are also effective dispersers on floodplains when floods do occur (Schwab *et al.* 2018) and *C. flaccidum* may have dispersed downstream to South Australia from flood events in the upper Murray–Darling Basin. Most of the *C. luteolum* populations also grew on arid-zone flood plains or close to ephemeral watercourses.

The morphological cluster analysis indicated that South Australian *C. flaccidum* was closer to *C. luteolum* than to the

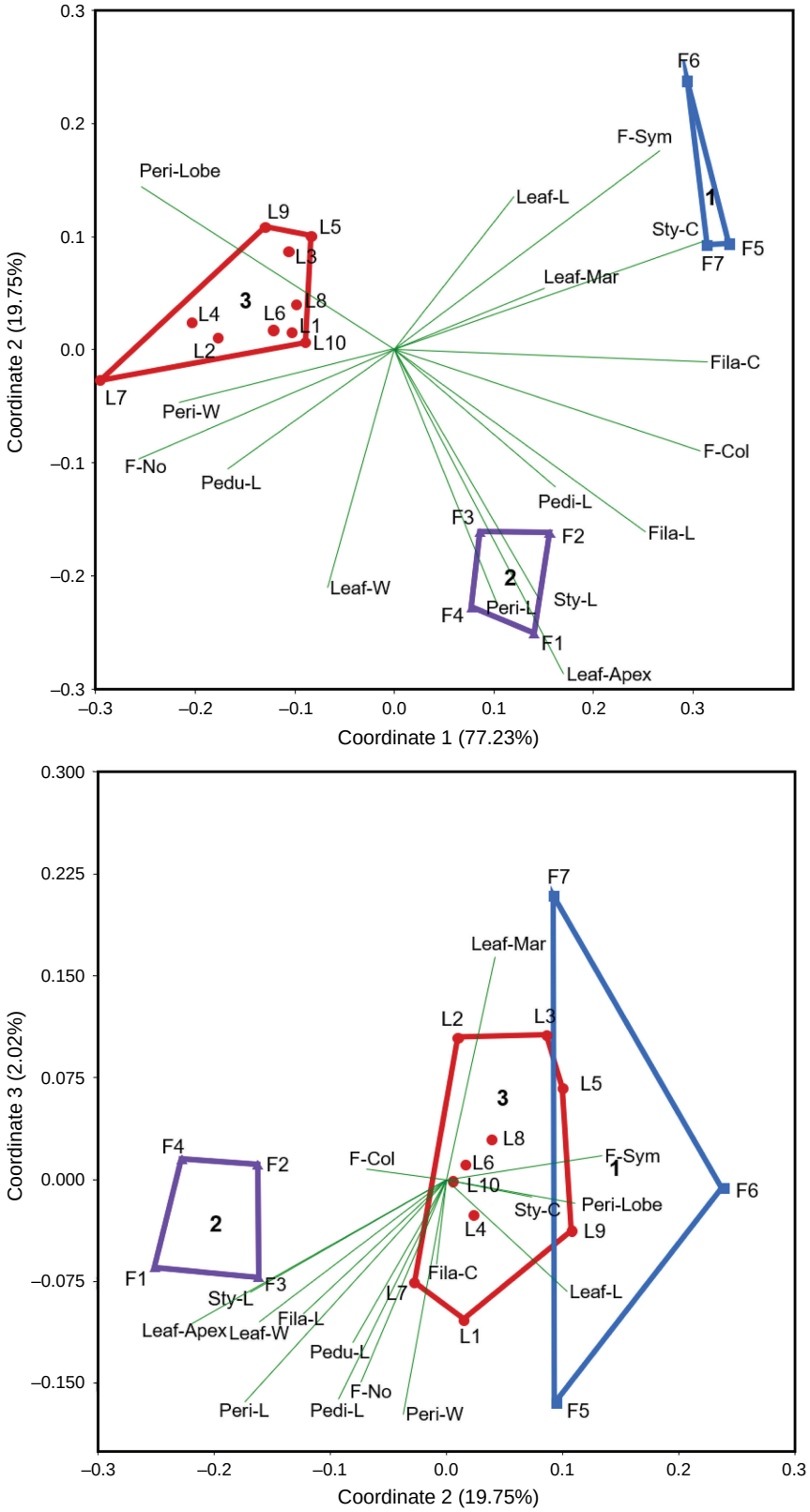

Fig. 3. Three-dimensional NMDS of *Crinum flaccidum* and *C. luteolum* with biplot analysis of the morphological data using Gower similarity. The vectors are the biplot analysis of the 16 variable morphological characters (Table S3), where direction and length are the extent to which the characters are affecting the species complex in the dendrogram (Fig. 2). Group 1 (light blue) consists of all New South Wales *Crinum flaccidum* samples; Group 2 (dark blue) comprises all South Australian *C. flaccdium*; and Group 3 was formed using all *C. luteolum*. The ordination plot has a STRESS score of 11.84%. The polygons represent the area covered by each group. Sample and character codes can be found in Tables S1 and S3 respectively.

New South Wales *C. flaccidum* samples, reflecting the taxonomic confusion within this species complex. The ordination plots (Fig. 3) further help explain the separation of the complex into their respective groups. New South Wales *C. flaccidum* (Group 1) flowers were all clearly zygomorphic and white, as opposed to the more actinomorphic, yellow to

Table 1. Distinguishing features between *Crinum flaccidum* and *C. luteolum*.

Character	*C. flaccidum*	*C. luteolum*
Tepal colour	Light pink–white	Yellow–pale yellow
Flower symmetry	Zygomorphic	Actinomorphic
Filament colour	White–pink	Pale yellow
Style colour	Dark pink–purple (New South Wales); white (South Australia)	Pale yellow
Umbel	6–13 (mean 10)	9–17 (mean 13)
Floral tube length (mm)	70–120	50–100
Filament length (mm)	35–50	25–35
Mean style length (mm)	100	75

A collection of the key features distinguishing *Crinum flaccidum* and *C. luteolum*. The features are a collection of characters from this study and that of Lykos (2011).

pale yellow *C. luteolum* samples (Group 3). The leaves of the New South Wales populations of *C. flaccidum* were also longer than those of the South Australian populations of *C. flaccidum* and *C. luteolum* and the flowers had white–pink filaments and dark pink–pale purple styles. However, style, perianth and peduncle length, perianth and leaf width and the number of flowers per umbel were too variable to show strong correlations with the NMDS ordination space. The *C. flaccidum* South Australia samples were correlated most strongly with vectors for perianth, style and filament length. Perianth lobes were consistently oblanceolate, but the correlation was weak, because some specimens in Groups 1 and 3 also had oblanceolate perianths.

These results are consistent with the broader conclusions of Lykos (2011) in recognising two taxa, but the study did not identify the South Australian *C. flaccidum* and New South Wales split seen here. Lykos (2011) also noted that the number of flowers per umbel for *C. luteolum* was higher on average (~12), being congruent with the data observed in this study (~13). Additionally, this study did not find any evidence for separating *C. luteolum* into 'northern' and 'southern' morphotypes.

Sphingophily is a widely recognised pollination phenomenon in *Crinum* (Manning and Snijman 2002; Huang *et al.* 2021). Three species of hawkmoth and honeybees were observed visiting *Crinum flaccidum* in Kootingal, New South Wales (Howell and Prakash 1990), with pollen found on the proboscis of *Hippotion scrofa* (Boisduval, 1832). Similarly, multiple individuals of *Hyles livornicoides* (T.P. Lucas, 1892) were seen visiting flowers at several populations of *C. luteolum* in the current study (J. C. Simpson and J. G. Conran, unpubl. obs.). All of the hawkmoth species seen visiting members of the *C. flaccidum* complex are widespread within Australia and capable of long-distance migration, in some cases at least as far as Norfolk Island and New Zealand (Fox 1978; Moulds *et al.* 2020). The inferred genetic structure and organisation seen between New South Wales populations (Fig. S2) suggests that pollen exchange between populations is not regular, and although pollen transfer between populations has not been confirmed in the current study, the distances involved are plausible.

Species complex

The *C. flaccidum* species complex can be split confidently into the following two taxa: *C. luteolum*, including all the 'northern' and 'southern' morphotypes originally described by Hamilton (2010) and Lykos (2011); and *C. flaccidum* for the New South Wales and South Australian Riverland forms. This split is clear in both the molecular and morphological data sets and morphological features clearly distinguish these groups (Table 1).

The low genetic difference between *C. luteolum* and *C. flaccidum* (Fig. 1) implies that they may be associated with very recent speciation, but genetic variation among nuclear markers might be able to detect finer-scale diversity (Nge *et al.* 2021). *Crinum luteolum* is genetically distinct from *C. flaccidum* populations, reflecting both the large distances between populations of the two taxa and their isolation in different drainage systems (Lake Eyre *v.* the Murray–Darling Basin). Expansion of the study to examine the relationships of this complex with *C. arenarium* might provide insight into the origins of and gene flow within *C. flaccidum* and *C. luteolum*.

Conclusions

Crinum flaccidum (Darling lily) and *Crinum luteolum* (Andamooka lily) are supported as separate species, on the basis of analyses of 59 coding and non-coding regions of the chloroplast genome (~50 000 bp) and 24 morphological characters. Previous proposed divisions within *C. luteolum* into 'northern' and 'southern' morphotypes were not supported. *Crinum flaccidum* showed greater structure among New South Wales floodplain populations than South Australian riparian populations, possibly as a result of hydrochorous dispersal along the River Murray. The two taxa (*C. flaccidum* and *C. luteolum*) are postulated to have arisen from a common ancestor with *C. arenarium* through allopatry following flood-mediated long-distance dispersal and isolation.

Supplementary material

Supplementary material is available online.

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Data availability. Upon publication the data will be available for public access on the European Nucleotide Archive.

Conflicts of interest. Michelle Waycott is an Associate Editor of *Australian Systematic Botany* but did not at any stage have editor-level access to this manuscript while in peer review, as is the standard practice when handling manuscripts submitted by an editor to this journal. *Australian Systematic Botany* encourages its editors to publish in the journal and they are kept totally separate from the decision-making processes for their manuscripts. The authors have no further conflicts of interest to declare.

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Author affiliations

[A]School of Biological Sciences, The University of Adelaide, Adelaide, SA 5005, Australia.

[B]Clinical and Health Sciences, The University of South Australia, Adelaide, SA 5001, Australia.

[C]Department for Environment and Water, Botanic Gardens and State Herbarium, State Herbarium of South Australia, Adelaide, SA 5001, Australia.