

# Transfer of Cotula alpina to the genus Leptinella (Asteraceae: Anthemideae)

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#### ABSTRACT

Tribe Anthemideae (Asteraceae) is represented in Australia by only nine indigenous species of *Cotula* and *Leptinella*. The generic placement of *Cotula alpina* (Hook.f.) Hook.f. is considered problematic, because it shares the stoloniferous and scapose habit of *Leptinella*, but lacks corollas in female florets, a trait traditionally considered defining of *Cotula*. A previous phylogenetic analysis of *Leptinella* using *ITS* and chloroplast data showed that the species nested in that genus, but some uncertainty remained because of incomplete sequence data and missing cytological information, and no taxonomic change was made. Here, we use target-capture data from three different sequencing initiatives to reconstruct a phylogeny of Australian native and introduced Anthemideae to resolve this outstanding question. We confirm previous results with a high degree of support and formally transfer *Cotula alpina* to the genus *Leptinella*. A lectotype is selected for the basionym, *Ctenosperma alpinum* Hook.f.

**Keywords:** Asteraceae, Australian flora, Compositae, *Cotula*, Cotuleae, *Leptinella*, phylogenetics, taxonomy, typification.

## Introduction

Tribe Anthemideae of the Asteraceae family comprises an estimated 1800 species (Oberprieler *et al.* 2007) with a predominantly Old World extra-tropical distribution (Oberprieler *et al.* 2009). They are herbs or shrubs with frequently dentate to deeply divided leaves, lack pappus bristles, and are often aromatic. Because of the latter trait, members of the tribe have variously been used as medicinal or tea plants (e.g. chamomile), flavour or spices (e.g. absinth, tarragon–estragon), and for scent (cotton lavender) (Simpson 2009). Other species are popular ornamentals (e.g. chrysanthemums, marguerites), and some are significant weeds such as the ox-eye daisy *Leucanthemum vulgare* Lam. (Stutz *et al.* 2021).

Despite their diversity at the global level, Anthemideae is poorly represented in the Australian flora. Only the following nine species from two genera are indigenous: *Cotula alpina* (Hook.f.) Hook.f., an alpine to montane species of south-eastern mainland Australia and Tasmania (Fig. 1*a*); *C. australis* (Sieber ex Spreng.) Hook.f., which is widespread (Fig. 1*e*); *C. cotuloides* (Steetz) Druce, occurring in saline areas and swamps of south-western Western Australia (Fig. 1*f*); *C. vulgaris* Levyns, growing in wet saline areas of South Australia, Victoria, and Tasmania; *Leptinella drummondii* (Benth.) D.G.Lloyd & C.J.Webb of south-western Australia and Tasmania (Fig. 1*b*); *L. longipes* Hook.f., of wet saline areas ranging from South Australia to Queensland and Tasmania (Fig. 1*d*); *L. plumosa* Hook.f. of Macquarie Island; and *L. reptans* (Benth.) D.G.Lloyd & C.J.Webb of South Australia, New South Wales, Victoria, and Tasmania (Fig. 1*c*). Both these genera belong to the Cotulinae, a subtribe of 10 genera and 137 species with a southern hemisphere distribution (Oberprieler *et al.* 2009).

Over the past two decades, the generic placement of *Cotula alpina* has come under scrutiny. The species is unusual in *Cotula* L., whose members are generally erect to



**Fig. 1.** Six of the nine Australian indigenous representatives of Asteraceae subtribe Cotulinae and state or territory where the photograph was taken. (a) Cotula alpina (Hook.f.) Hook.f., New South Wales. (b) Leptinella filicula (Hook.f.) Hook.f., New South Wales. (c) Leptinella reptans (Benth.) D.G.Lloyd & C.J.Webb, Tasmania. (d) Leptinella longipes Hook.f., New South Wales. (e) Cotula australis (Sieber ex Spreng.) Hook.f., Australian Capital Territory. (f) Cotula cotuloides (Steetz) Druce, Western Australia. Note procumbent, stoloniferous, and rosette-and-scape 'Leptinella' habit in a-d and upright to ascending 'Cotula' habit with branched, leafy aerial stems in e, f. All photos were taken by the first author.

ascending herbs with branching, leafy aerial stems (Fig. 1*e*, *f*). Instead, *C. alpina* shares with members of *Leptinella* Cass. a prostrate, stoloniferous (or sometimes long-rhizomatous), rosette-and-scape growth form (Fig. 1*a*–*d*). In overall habit,

*C. alpina* is very similar to *L. filicula* (Fig. 1*a*, *b*), generally distinguished most easily by plants of the former being entirely glabrous, and the two species are also geographically close. However, like other *Cotula* and unlike

*Leptinella*, *C. alpina* does not produce corollas in female florets, a character that has traditionally been considered defining of *Cotula* (Lloyd and Webb 1987; Thompson 2007).

A comprehensive molecular phylogenetic study of *Leptinella* using the nuclear ribosomal internal transcribed spacer region (ITS), chloroplast *psbA–trnH*, and *trnC–petN* regions found *Cotula alpina* nested within the former genus (Himmelreich *et al.* 2012). However, the authors provided the caveat that its base chromosome number of x = 9 would match those known for *Cotula* better than the x = 13 of *Leptinella* (Oberprieler *et al.* 2009). Another potential caveat is that the ITS sequence of the species in the study (Genbank accession HE860701), which evolves faster than the chloroplast regions and thus provides the largest number of informative characters, comprises only the first half of the entire region (ITS1), in contrast to other ITS sequences from the same study. The taxonomic affiliation of *C. alpina* therefore remains unresolved.

In the present study, we used target enrichment to recover genetic data for hundreds of nuclear genes to provide confidence in the phylogenetic placement of *Cotula alpina* relative to other species of Australian Anthemideae and resolve this outstanding taxonomic question.

#### Materials and methods

#### Sampling

To comprehensively cover Australian indigenous and introduced Anthemideae species and add a phylogenetic context of non-Australian species, we combined homologous data from three different sources. Gene sequences published by the Plant and Fungal Tree of Life consortium (PAFTOL) were from https://treeoflife.kew.org/ obtained and their sequence names were reformatted to make them compatible with other datasets. These sequences were added to the dataset before the paragone-nf analysis (see below). For samples sequenced by the Genomics for Australian Plants consortium (GAP), raw reads were obtained from the Bioplatform data portal (see https://data.bioplatforms. com/) and added to the dataset before the quality-filtering step (see below). Introduced Australian species and some additional indigenous species including Cotula alpina were sampled from herbarium specimens (Appendix A1) lodged at the Australian National Herbarium (CANB, including CBG) and processed in the laboratory as described below.

#### Laboratory procedures

Genomic DNA was extracted from 5 to 15 mg of silica-dried leaf tissue or herbarium material by using Invisorb Spin Plant Mini Kit (Stratec, Berlin, Germany), following the manufacturer's instructions. Libraries were built from <1 to 5 ng of DNA by using the QIAseq FX DNA Library UDI-A Kit 96 (Qiagen, Melbourne, Vic., Australia) that included a DNA digestion step to a fragment size of ~200 base pairs. Sequence capture was conducted on pools of 16 libraries by using the Angiosperms353 (Johnson *et al.* 2019) MYbaits kit (Daicel Arbor Biosciences, Ann Arbor, MI, USA), following the manufacturer's instructions. Enriched libraries were sequenced on Illumina NovaSeq 6000 SP with ver. 1.5 paired-end  $2 \times 150$  cycle chemistry.

#### **Bioinformatics**

Reads were quality filtered and paired with TRIMMOMATIC (ver. 0.39, see https://github.com/usadellab/Trimmomatic; Bolger *et al.* 2014) with illuminaclip:adapters, fa:4:20:10, minimum length of 30, and average quality of 25, and then further filtered with bbduk (ver. 38.90, see https://github.com/BioInfoTools/BBMap/blob/master/sh/bbduk.sh) with entropy of 0.8, entropy window of 20, and entropy mask t. Reads were assembled against target sequences by using hybpiper-nf (see https://github.com/chrisjackson-pellicle/ hybpiper-nf; Jackson *et al.* 2021, 2023), a Nextflow pipeline adapted from HybPiper (ver. 1, see https://github.com/mossmatters/HybPiper; Johnson *et al.* 2016) against a target file designed for broad representation of Asteraceae by mining transcriptome data for Angiosperms353 targets (McLay *et al.* 2021).

The results of HybPiper's paralog finder were analysed with the monophyletic outgroups (MO) algorithm as implemented in paragone-nf (see https://github.com/chrisjackson-pellicle/paragone-nf; Jackson *et al.* 2023), a Nextflow pipeline for the four gene tree-based paralogy-resolution algorithms collated by Yang and Smith (2014). We chose this algorithm because it returns at most one ortholog group for each locus, producing a more complete sample  $\times$  gene matrix than do alternative algorithms that return more ortholog groups with, on average, fewer sequences.

For both paralogy resolution and phylogenetic analysis, three representatives of tribes closely related to Anthemideae were used, namely *Bellis perennis* L. (Astereae, Sequence Read Archive, SRA, accession ERR7621192), *Calendula arvensis* L. (Calenduleae, ERR5033757) and *Tussilago farfara* L. (Senecioneae, SRR9113366).

Custom-written Python scripts (see https://bitbucket. csiro.au/projects/NRCA/repos/bioinformatics-and-phylogenetics/browse) were used to ensure that gene alignments were in frame and to concatenate them into a supermatrix. The concatenated dataset comprised 40 terminals and 169 548 characters, of which 33 850 were parsimony informative, 32 835 variable but uninformative, and 102 863 constant.

#### **Phylogenetic analysis**

A phylogeny of the concatenated supermatrix was inferred with IQTREE (ver. 2.2.0.5, see http://www.iqtree.org/;

Minh *et al.* 2020), partitioning the alignment by codon positions and under automatic partition and model testing (Lanfear *et al.* 2017). Testing resulted in the three codon position partitions being maintained, with the first two under the GTR + F + I + G4 model, and the third under GTR + F + G4. In total, 1000 UltraFast Bootstrap (UFB) replicates were used to estimate branch support (Minh *et al.* 2013).

### **Results**

The phylogeny (Fig. 2) showed the ingroup divided into the following two large, strongly supported clades: one comprised the genera Achillea L., Anthemis L. (including Tripleurospermum maritimum (L.) W.D.J.Koch), Argyranthemum Webb ex Sch.Bip., Artemisia L., Matricaria L., and Tanacetum L.; the other comprised Cotula, Hippia L., Leptinella, Schistostephium Less., and Soliva Ruiz & Pav. Cotula and Leptinella were reciprocally monophyletic except for the placement of C. alpina in the latter genus. The monophyly of Leptinella including C. alpina was strongly supported (UFB = 100), but the Cotula clade without C. alpina received a UltraFast Bootstrap value of only 93. Conspecific samples of Anthemis arvensis L., C. alpina, L. filicula, L. reptans and Soliva sessilis Ruiz & Pav. were not placed as sister terminals.

## Discussion

Although neither *Cotula* nor *Leptinella* was comprehensively sampled across their global diversity, our results provided strong support to confirm the results of Himmelreich *et al.* (2012), namely that *C. alpina* is a sister lineage to or potentially nested within the genus *Leptinella*, as suggested by its growth habit. Within *Leptinella*, *Cotula alpina* was part of a small clade sister to the remainder of the genus, the 'filicula-group' of Himmelreich *et al.* (2012). In addition to the Australian *C. alpina* and *L. filicula*, the clade also included *L. altilitoralis* (P.Royen & D.G.Lloyd) D.G.Lloyd & C.J.Webb and *Leptinella wilhelminensis* (P.Royen) D.G.Lloyd & C.J.Webb, both of New Guinea, which we did not sequence.

This relationship is not replicated in our phylogeny because our two samples of *Cotula alpina* and our two samples of *Leptinella filicula* formed a grade under the remainder of *Leptinella*. A caveat of our dataset is that it includes only a single sample of most species, and that duplicate samples from the same species were rarely placed as sister terminals. Possible reasons are the use of protein-coding gene regions, which can be expected to evolve slowly, and the patchy nature of the sample  $\times$  gene matrix, because the median sample had data for 289 of the 353 targeted loci (147 and 305 in the case of the two samples of *C. alpina*). Resolution at shallower levels is often improved

by replicate sampling of five or more specimens per species (Schmidt-Lebuhn 2022), which is rarely feasible in higherlevel phylogenetic studies. However, our data do not provide strong evidence against the monophyly of the *filicula*-group, given this limited sampling.

Our results imply that the character traditionally used to delineate *Cotula*, namely, the absence of corollas in female florets, is in this case homoplasious. However, this is not without precedent, because Thompson (2007, p. 46) noted in one inverse case that 'the outer florets [of *Cotula*] are female and lack a corolla except for a weakly developed one in *C. bipinnata*'. Without more comprehensive sampling across the clade of *Cotula*, *Leptinella* and *Soliva*, it remains unclear whether absence or presence of corollas in female florets is the ancestral state, but either inference would require several gains or losses.

More generally, genetic analyses of the past two decades have repeatedly demonstrated that what might be called a total-evidence approach relying on a larger suite of characters including growth habit and branching pattern is more informative about evolutionary relationships than individual reproductive characters considered important *a priori*. Examples include the cases of *Odixia* Orchard and *Ozothamnus* R.Br., which differ only in the absence or presence of the pappus (Schmidt-Lebuhn and Constable 2013), and *Leucochrysum fitzgibbonii* (F.Muell.) Paul G.Wilson, which differs from members of *Waitzia* J.C.Wendl. only in the plesiomorphy of lacking a beak on the cypsela (Weber and Schmidt-Lebuhn 2015).

Because we confirmed the grouping of *Cotula alpina* with *Leptinella*, the question of a transfer to the latter genus arises. A counter-argument is the possible non-monophyly of *Cotula* in its current circumscription. The phylogeny of Himmelreich *et al.* (2012) showed *Cotula* as a clade comprising the type species *C. coronopifolia* L., *C. australis*, and *C. turbinata* L., but *C. mexicana* (DC.) Cabrera nested in *Soliva*, and *C. abyssinica* Sch.Bip. ex A.Rich. in an isolated position. Should these relationships be corroborated, sinking all three genera into a broadly circumscribed *Cotula* might be justified, rendering a name change for *C. alpina* unnecessary.

However, this is entirely speculative on current evidence and, given the monophyly of *Leptinella* and the placement of the type species of *Cotula* in a clade of at least several species (including also *C. bipinnata* Thunb. and *C. cotuloides* in our phylogeny), a less disruptive solution would be to transfer a few other species of *Cotula* as required to maintain the genus-level taxonomy mostly as it is now.

On the basis of the phylogenetic evidence presented in this study, the shared stoloniferous, scapose growth form of *Cotula alpina* and members of *Leptinella*, and the currently accepted circumscription of the two genera, we therefore transfer the species to the latter genus. We also take the opportunity to lectotypify the basionym *Ctenosperma alpinum* Hook.f.





Fig. 2. Maximum-likelihood phylogeny of concatenated Angiosperms353 target-capture data of Australian native and introduced Anthemideae species, rooted on three representatives of related tribes. Numbers above branches are UltraFast Bootstrap values. Identifiers starting with ERR and SRR are Sequence Read Archive accession numbers of data published by PAFTOL; the five-digit identifier 80092 is a Genomics for Australian Plants sample number, with sequence reads published in the Bioplatforms Australia Data Portal (data.bioplatforms.com); identifiers starting with CANB or CBG are herbarium accession numbers of specimens sequenced specifically for this study. Red text indicates current circumscription of Cotula, blue text is Leptinella, and bold text indicates species indigenous to Australia, including Macquarie Island. Scale bar indicates estimated substitutions per site.

# Taxonomy

# Leptinella alpina (Hook.f.) Schmidt-Leb., comb. nov.

Ctenosperma alpinum Hook.f., London J. Bot. 6: 115–116 (1847); Cotula alpina (Hook.f.) Hook.f., Fl. Tasman. 1(3): 192, t. 51 A (1856). Type:

Marlborough, Tasmania, Jan. 1841, R.Gunn 1155 (lecto, here designated: K000885266, image seen; isolecto: BM 810481, image seen).

We designate the duplicate at K as the lectotype because that herbarium was the workplace of Joseph Dalton Hooker, and the specimen includes labels and drawings indicating that it was used by Hooker to prepare the description. The two specimens cited above represent all the material of the original collection that is currently known. A third specimen at K is also labelled *R.Gunn 1155* (K00885265). However, it is dated 1844, suggesting that it is from a separate gathering. Gunn is known to have re-used numbers to refer to multiple collections that he believed to represent the same taxon, rather than using a series of unique collecting numbers (Buchanan 1988). We therefore do not consider K00885265 to be an isolectotype.

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Data availability. Raw reads of data generated for this project are available on the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) as accessions SRR24286507–SRR24286507–SRR24286534 and SRR26780972. The concatenated data matrix and phylogenetic tree are available on the CSIRO Data Access Portal at doi:10.25919/2z7m-5866.

Conflicts of interest. The authors declare that they have no conflicts of interest.

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# Appendix A1.

Voucher information and Sequence Read Archive (SRA) accession numbers for data newly generated for this study. Information is presented in the following order: taxon name, collector and collection number (herbarium code and, if already databased, herbarium accession number), SRA accession number.

Achillea distans Waldst. & Kit. ex Willd., J.R. Hosking 2660 (CANB 691395), SRR24286534; Achillea filipendulina Lam., M.E. Phillips 1 (CBG 9301876), SRR24286533; Achillea millefolium L., A.N. Schmidt-Lebuhn 1809 (CANB 898516), SRR24286522; Anthemis arvensis L., I. Crawford 5228 (CANB 611431), SRR24286513; Anthemis cotula L., I.C. Clarke 3038 (CANB 527765), SRR24286512; Argyranthemum frutescens subsp. foeniculaceum (Pit.) Humphries, R.W. Purdie 10316 (CANB 882281), SRR24286511; Artemisia verlotiorum Lamotte, J.R. Hosking 1575 (CANB 505113), SRR24286510; Artemisia vulgaris L., J. Zaplatilkova s.n. (CANB 833857), SRR24286509; Cotula alpina (Hook.f.) Hook.f., G.H. Flowers 593 (CANB 865364), SRR24286508; Cotula alpina (Hook.f.) Hook.f., A.N. Schmidt-Lebuhn 1256 (CANB 808804), SRR26780972; Cotula australis (Sieber ex Spreng.) Hook.f., A.N. Schmidt-Lebuhn 1071 (CANB 795939), SRR24286532; Cotula bipinnata Thunb., A.N. Schmidt-Lebuhn 1489 (CANB 812985), SRR24286531; Cotula coronopifolia L., A.N. Schmidt-Lebuhn 1486 (CANB 812981), SRR24286530; Cotula cotuloides (Steetz) Druce, A.N. Schmidt-Lebuhn 1492 (CANB 812988), SRR24286529; Cotula turbinata L., A.N. Schmidt-Lebuhn 1533 (CANB 813028), SRR24286528; Leptinella filicula (Hook.f.) Hook.f., A.N. Schmidt-Lebuhn 2048 (CANB 959490), SRR24286507; Leptinella longipes Hook.f., A.N. Schmidt-Lebuhn 1561 (CANB 867531), SRR24286527; Leptinella plumosa Hook.f., R.D. Seppelt 15431 (CANB 829906), SRR24286526; Leptinella reptans (Benth.) D.G.Lloyd & C.J.Webb, M.L. Baker 2679 (CANB 821785), SRR24286525; Leptinella reptans (Benth.) D.G.Lloyd & C.J.Webb, A.N. Schmidt-Lebuhn 2052 (CANB 959494), SRR24286524; Matricaria discoidea DC., A.N. Schmidt-Lebuhn 1379 (CANB 866377), SRR24286523; Soliva anthemifolia (Juss.) Sweet, A.N. Schmidt-Lebuhn 1964 (CANB 920410), SRR24286521; Soliva sessilis Ruiz & Pav., A.N. Schmidt-Lebuhn 1138 (CANB 796000), SRR24286520; Soliva stolonifera (Brot.) Sweet, J.R. Hosking 2757 (CANB 691119), SRR24286519; Soliva valdiviana Phil., H.I. Aston 2150 (CANB 557170), SRR24286518; Tanacetum cinerariifolium (Trevir.) Sch.Bip., A.M. Buchanan 16471 (CANB 722258), SRR24286517; Tanacetum parthenium (L.) Sch.Bip., A.N. Schmidt-Lebuhn 1368 (CANB 811573), SRR24286516; Tanacetum vulgare L., B.J. Lepschi 1567 (CANB 470980), SRR24286515; Tripleurospermum maritimum (L.) W.D.J.Koch, A.N. Schmidt-Lebuhn 1381 (CANB 866379), SRR24286514.