

## Original research

# Molecular characterisation and phylogenetic placement of the endemic species *Helichrysum nuratavicum*

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## Abstract

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*Helichrysum nuratavicum* Krasch. is a narrow endemic species, found only in the Nuratau Mountains of Uzbekistan. However, its molecular characteristics and phylogenetic position remain poorly understood. In this study, we sequenced and compared the internal transcribed spacer (ITS1–5.8S–ITS2) region of *Helichrysum nuratavicum* with ITS sequences from closely related *Helichrysum* species in Central Asia. Bayesian phylogenetic analysis resolved *Helichrysum nuratavicum* as the sister species of *Helichrysum maracandicum* Popov ex Kirp., as shown by pairwise genetic distance analysis. Comparative sequence analysis identified three diagnostic nucleotide positions (438 bp, 559 bp and 576 bp), two of which (559 bp and 576 bp) were unique to *Helichrysum nuratavicum*. Sliding-window analysis revealed nucleotide diversity values ranging from 0.0000 to 0.0142, with the highest variability detected within the 501–595 bp region, where these two species-specific substitutions were located. These findings show that the ITS region provides sufficient phylogenetic resolution and contains informative diagnostic characters for distinguishing *Helichrysum nuratavicum* from closely related Central Asian *Helichrysum* species. The molecular data generated in this study will be a valuable resource for future taxonomic, phylogenetic, DNA barcoding, and conservation studies of this endemic species.

**Keywords:** Central Asia, diagnostic nucleotide substitutions, DNA barcoding, endemic species, molecular identification, phylogenetic analysis.

## INTRODUCTION

The genus *Helichrysum* Mill. (Asteraceae) comprises 564 species distributed mainly in Africa, the Mediterranean region, and Asia (POWO, 2026). Forty-one taxa occur in the Mediterranean region

and western to Central Asia (Galbany-Casals et al., 2004, 2009). In Uzbekistan, the genus is represented by five species: *Helichrysum arenarium* (L.) Moench, *Helichrysum maracandicum* Popov ex Kirp., *Helichrysum mussae* Nevski, *Helichrysum nuratavicum* Krasch., and *Helichrysum thianschani-*

cum Regel (Tojibaev et al., 2014). Species of the genus *Helichrysum* have long been used worldwide in traditional medicine for their anti-inflammatory, antimicrobial, antioxidant, and hepatoprotective properties (Viegas et al., 2014). In Uzbekistan, people widely collect and trade *Helichrysum maracandicum* as a medicinal plant in local markets, particularly in the Takhtakaracha Pass between the Samarkand and Kashkadarya regions (Khojimatov et al., 2023). Other native species, including *Helichrysum mus-sae*, *Helichrysum nuratavicum*, and *Helichrysum arenarium*, are also used for similar medicinal purposes (Yuldashev et al., 2026). Among these species, *Helichrysum nuratavicum* is a narrow endemic species restricted to the Nuratau Mountains of Uzbekistan and is listed in the Red Book of Uzbekistan (Xasanov, 2019), reflecting its limited distribution and conservation significance.

The genus *Helichrysum* is taxonomically complex because many closely related species share overlapping morphological characteristics, making species delimitation and inference of taxonomic relationships challenging when based solely on morphological evidence (Galbany-Casals et al., 2004; Viegas et al., 2014). Therefore, molecular markers have become indispensable tools for resolving phylogenetic relationships within the genus. Despite its taxonomic and conservation importance, molecular data for *Helichrysum nuratavicum* remain unavailable, and its phylogenetic relationships with other Central Asian *Helichrysum* species have not yet been investigated.

The internal transcribed spacer (ITS) region of nuclear ribosomal DNA is a widely used informative molecular marker for phylogenetic reconstruction and species delimitation in plants because it amplifies well and evolves relatively rapidly (Álvarez and Wendel, 2003). Previous molecular studies of *Helichrysum* have used ITS sequences to infer phylogenetic relationships within the genus (Galbany-Casals et al., 2009); however, these analyses have not included *Helichrysum nuratavicum*. Therefore, this study aimed to characterise the ITS sequence of *Helichrysum nuratavicum*, determine its phylogenetic position among *Helichrysum* species, evaluate nucleotide diversity and pairwise genetic distances, and assess sequence variation within the ITS region.

## MATERIALS AND METHODS

### Plant material and sampling

*Helichrysum nuratavicum* is a narrow endemic species restricted to Nuratau Mountains (Uzbekistan) and is listed in the Red Book of Uzbekistan (Xasanov, 2019). It is one of the five *Helichrysum* species recorded in the flora of Uzbekistan (Tojibaev et al., 2014).

We collected fresh leaves from a single healthy individual of *Helichrysum nuratavicum* in the vicinity of Uhum village, Hayatsay district, Jizzakh Region, Uzbekistan (40.508159° N, 66.723779° E; elevation 1776 m above sea level) on 13 May 2024. We selected the sampled individual because it represented a typical, healthy, flowering plant growing in its natural habitat. N.Yu. Beshko identified the specimen morphologically, and deposited voucher material at the National Herbarium of Uzbekistan (TASH) at the Institute of Botany of the Academy of Sciences of the Republic of Uzbekistan (accession number TASH W1131) (Fig. 1).

Leaves were dried on silica gel immediately after collection and stored at −20°C before DNA extraction.

### DNA extraction

We extracted total genomic DNA containing the nuclear ribosomal ITS region from approximately 20 mg of dried leaf tissue using the cetyltrimethylammonium bromide (CTAB) method of Doyle & Doyle (1987), with minor modifications. Leaf tissue was ground to a fine powder in liquid nitrogen and suspended in 2× CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 1% polyvinylpyrrolidone, and 0.2% β-mercaptoethanol).

The homogenate was incubated at 65°C for 30 min to lyse cells and release nucleic acids, then extracted with a chloroform and isoamyl alcohol mixture (24: 1). After centrifugation at 12 000 × g for 10 min, the aqueous phase was collected and DNA was precipitated with cold isopropanol. The pellet was washed with 70% ethanol, air-dried, and dissolved in 50 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).



Fig. 1. Voucher specimen of *Helichrysum nuratavicum* (TASH W1131).

We evaluated DNA concentration and quality using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and confirmed it by 1.5% agarose gel electrophoresis.

We subjected high-quality genomic DNA to Illumina sequencing. We then recovered the ITS sequence of *Helichrysum nuratavicum* from Illumina sequencing reads generated during whole-genome sequencing. Raw FASTQ reads were assembled using NOVOPlasty v4.3.5 (Dierckxsens et al., 2017), and the assembled ITS sequence was verified in Geneious v9.0.2 (Kearse et al., 2012) before downstream analyses.

We deposited the assembled ITS sequence in the NCBI GenBank database under accession number PQ804169.

### Diagnostic nucleotide variations in the ITS region

We identified diagnostic nucleotide variations within the ITS region by manually comparing the multiple sequence alignment of six Central Asian *Helichrysum* species in Geneious v.9.0.2 (Kearse et al., 2012). Nucleotide positions showing species-specific substitutions were recorded and compared among the examined taxa. We then summarised di-

agnostic nucleotide positions unique to *Helichrysum nuratavicum* in a comparative table.

Phylogenetic analysis

To investigate the phylogenetic relationships of *Helichrysum nuratavicum*, ITS (ITS1–5.8S–ITS2) sequences of 20 *Helichrysum* species were analysed. We selected the outgroup from closely related genera within the tribe Gnaphalieae: *Pseudognaphalium affine* (D. Don) Anderb. and *Pseudognaphalium luteoalbum* (L.) Hilliard & B.L. Burtt. All ITS sequences, except the newly generated sequence of *Helichrysum nuratavicum*, were retrieved from the NCBI GenBank database (Appendix I). We performed multiple sequence alignment using the MAFFT algorithm (Katoh & Standley, 2013). The aligned ITS sequences were manually inspected in Geneious v9.0.2 (Kearse et al., 2012), and only the terminal regions were trimmed to obtain a uniform alignment length of 595 bp. No internal alignment positions were manually modified or excluded. The final alignment comprised 22 taxa and contained 176 variable sites, 39 parsimony-informative sites, 143 singleton variable sites, and 15 gap-containing positions. We selected the best-fit nucleotide substitution model using jModelTest v2.1.4 (Darriba et al., 2012) based on the Akaike Information Criterion (AIC), which identified the GTR+G model as the optimal substitution model. We conducted Bayesian phylogenetic analysis using MrBayes v3.2 (Ronquist et al., 2012), and visualised the resulting phylogenetic tree in FigTree v1.4.4 (Rambaut, 2018).

Nucleotide diversity analysis

Nucleotide diversity ( $\pi$ ) across the ITS region was estimated using DnaSP v6.12.03 (Rozas et al., 2017). Sliding-window analysis was performed with a window length of 100 bp and a step size of 25 bp to evaluate patterns of nucleotide variation among the ITS sequences of the examined *Helichrysum* species.

RESULTS

Diagnostic nucleotide variations in the ITS region

Comparative analysis of the ITS sequences from six Central Asian *Helichrysum* species identified

three diagnostic nucleotide positions (438, 559, and 576 bp) that distinguish *Helichrysum nuratavicum* from the examined species (Table 1). At position 438, *Helichrysum nuratavicum* and *Helichrysum maracandicum* shared cytosine (C), whereas thymine (T) was present in the remaining four species. In contrast, positions 559 and 576 were unique to *Helichrysum nuratavicum*, containing guanine (G) and adenine (A), respectively. At the same time, all other examined species possessed cytosine (C) at position 559 and guanine (G) at position 576. These species-specific nucleotide substitutions indicate that positions 559 and 576 may serve as candidate diagnostic molecular markers for rapid identification of *Helichrysum nuratavicum* and may support DNA barcoding-based species discrimination within Central Asian *Helichrysum*.

Table 1. Diagnostic nucleotide positions in the ITS region distinguishing *Helichrysum nuratavicum* from other Central Asian *Helichrysum* species

| Species                           | ITS position (bp) |     |     |
|-----------------------------------|-------------------|-----|-----|
|                                   | 438               | 559 | 576 |
| <i>Helichrysum nuratavicum</i>    | C                 | G   | A   |
| <i>Helichrysum maracandicum</i>   | C                 | C   | G   |
| <i>Helichrysum arenarium</i>      | T                 | C   | G   |
| <i>Helichrysum armenium</i>       | T                 | C   | G   |
| <i>Helichrysum pallasii</i>       | T                 | C   | G   |
| <i>Helichrysum thianschanicum</i> | T                 | C   | G   |

Phylogenetic analysis

The phylogenetic tree resolved the analysed *Helichrysum* species into two principal clades (Fig. 2). *Helichrysum nuratavicum* was nested within the Central Asian lineage together with *Helichrysum maracandicum*, *Helichrysum arenarium*, *Helichrysum armenium* DC., *Helichrysum pallasii* (Spreng.) Ledeb., *Helichrysum thianschanicum*, and *Helichrysum lacteum* Coss. & Durieu, receiving a posterior probability of 0.86. Within this lineage, *Helichrysum nuratavicum* was resolved as the sister species of *Helichrysum maracandicum*, with strong support (posterior probability = 0.88). The second clade comprised representative Mediterranean species, including *Helichrysum italicum* (Roth) G.Don, *Helichrysum stoechas* (L.) Moench, *Helichrysum serotinum* (DC.) Boiss., *Helichrysum litoreum* Guss., *Helichrysum crassifolium* (L.) D.Don ex G.Don, *Helichrysum heldreichii*

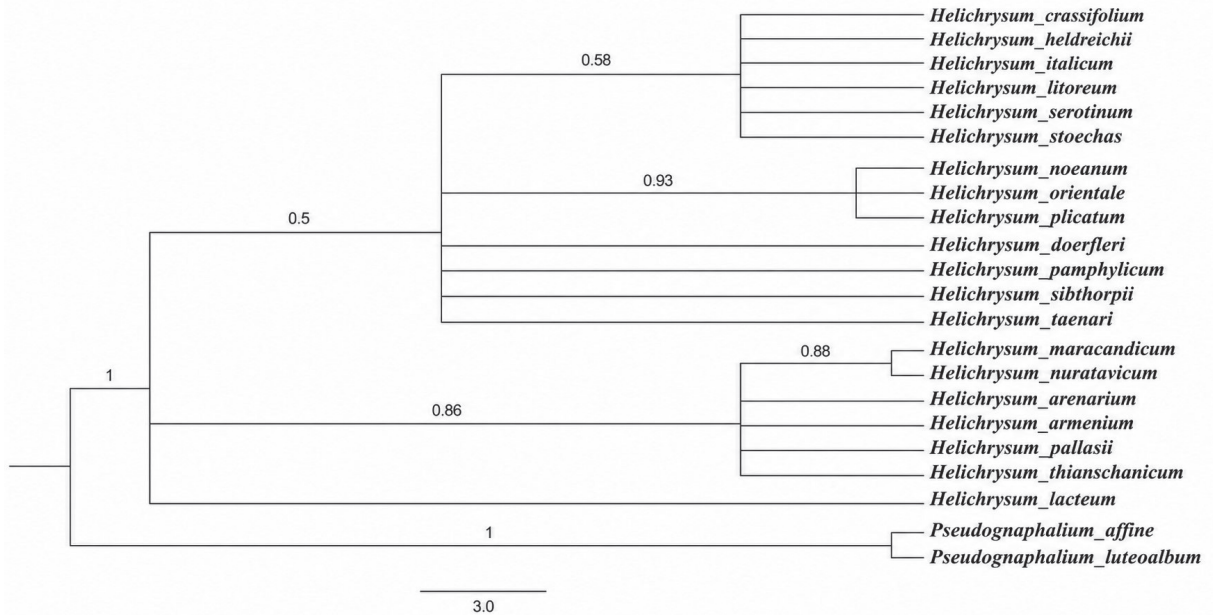


Fig. 2. Bayesian phylogenetic tree inferred from ITS (ITS1–5.8S–ITS2) sequences of *Helichrysum* species.

Boiss., *Helichrysum orientale* (L.) Gaertn., *Helichrysum plicatum* DC., *Helichrysum pamphylicum* P.H. Davis & Kupicha, *Helichrysum sibthorpii* Rouy, *Helichrysum tanari* Rothm., and *Helichrysum doerfleri* Rech.f. Relationships within this clade had posterior probabilities ranging from 0.58 to 1.00.

### Nucleotide diversity analysis

Sliding-window analysis of nucleotide diversity ( $\pi$ ) was performed using ITS sequences of six *Helichrysum* species (*Helichrysum thianschanicum*, *Helichrysum armenium*, *Helichrysum pallasii*, *Helichrysum maracandicum*, *Helichrysum arenarium*, and the newly generated *Helichrysum nuratavicum*) (Fig. 3). Nucleotide diversity values ranged from 0.0000 to 0.0142. No nucleotide variation was detected within the 76–175 bp and 201–350 bp regions ( $\pi = 0.0000$ ), indicating highly conserved segments. In contrast, the 501–595 bp region showed the highest nucleotide diversity ( $\pi = 0.0142$ ), identifying it as the most variable portion of the ITS sequence among the analysed *Helichrysum* species.

### Pairwise genetic distance analysis

Pairwise genetic distances among the ITS sequences of six Central Asian *Helichrysum* species

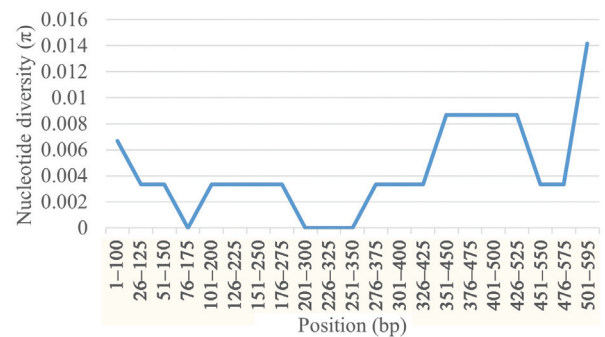


Fig. 3. Sliding-window analysis of nucleotide diversity ( $\pi$ ) across the ITS region of six *Helichrysum* species. The x-axis represents alignment position (bp), and the y-axis indicates nucleotide diversity ( $\pi$ ).

were calculated using MEGA 12 (Stecher et al., 2026). Evolutionary distances were estimated using the Kimura two-parameter (K2P) model after removing all ambiguous positions from the aligned dataset. We exported the resulting pairwise distance matrix and visualised it as a heatmap to illustrate genetic divergence among the examined species.

### Pairwise genetic distance analysis

Pairwise genetic distance analysis based on ITS sequences revealed genetic divergence values ranging from 0.0017 to 0.0102 among the six *Helichrysum*

species (Fig. 3). The newly generated ITS sequence of *Helichrysum nuratavicum* exhibited pairwise genetic distances of 0.0051 with *Helichrysum armenium* and *Helichrysum maracandicum*, 0.0068 with *Helichrysum pallasii*, 0.0084 with *Helichrysum thianschanicum*, and 0.0102 with *Helichrysum arenarium*. The smallest genetic distance among all analysed taxa was observed between *Helichrysum armenium* and *Helichrysum pallasii* (0.0017), whereas the greatest divergence was detected between *Helichrysum nuratavicum* and *Helichrysum arenarium* (0.0102). Overall, the heatmap demonstrates that the newly sequenced *Helichrysum nuratavicum* can be readily distinguished from the remaining species based on ITS sequence variation (Fig. 4).

DISCUSSION

Because this study was based on a single individual of this narrowly endemic species, the results should be interpreted within the limitations of the available sampling. Future studies including additional individuals, where available, will be valu-

able for assessing intraspecific genetic variation and evaluating the consistency of the molecular patterns observed in the present study.

The close phylogenetic relationship between *Helichrysum nuratavicum* and *Helichrysum maracandicum* aligns with their previously described morphological similarity (Yuldashev et al., 2026), indicating good agreement between molecular and morphological evidence. This finding is also supported by the pairwise genetic distance analysis, which revealed that *Helichrysum nuratavicum* exhibited the lowest genetic divergence from *Helichrysum maracandicum* among the examined Central Asian *Helichrysum* species. Furthermore, the clustering pattern observed in the present study agrees with previous phylogenetic analyses of the genus, in which Central Asian *Helichrysum* species formed a closely related lineage (Galbany-Casals et al., 2009). Collectively, these results demonstrate that the ITS region provides sufficient phylogenetic resolution to infer relationships among closely related Central Asian *Helichrysum* species.

In addition to resolving phylogenetic relationships, comparative analysis of the ITS sequences

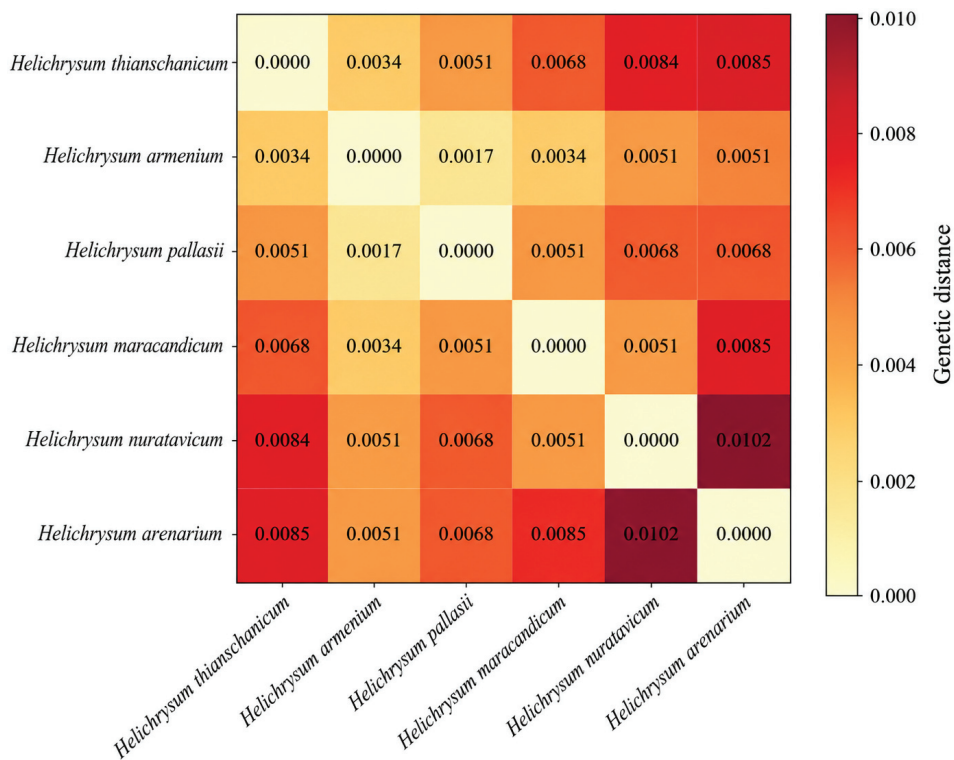


Fig. 4. Pairwise genetic distance heatmap based on ITS sequence data for six *Helichrysum* species. Numbers within the cells indicate pairwise genetic distance values, while the colour scale reflects the magnitude of sequence divergence.

identified three diagnostic nucleotide positions that distinguish *Helichrysum nuratavicum* from the examined Central Asian *Helichrysum* species. Notably, the two species-specific nucleotide substitutions identified in *Helichrysum nuratavicum* (positions 559 and 576) were located within the ITS region exhibiting the highest nucleotide diversity. Species-specific nucleotide substitutions within the ITS region have previously been recognised as valuable molecular characters for species delimitation and DNA-based identification in plants (Álvarez & Wendel, 2003). Similar observations have been reported in *Tetradium daniellii* (Benn.) T.G. Hartley, where comparative nucleotide sequence analyses revealed conserved and polymorphic regions within stress-related genetic markers, highlighting that even a limited number of informative nucleotide differences can provide valuable molecular information for sequence characterisation. Likewise, although only a limited number of diagnostic substitutions were identified in the ITS region of *Helichrysum nuratavicum*, these substitutions provided useful molecular characters for distinguishing this species from its closely related Central Asian congeners (Mamadjanova et al., 2024). Therefore, the species-specific nucleotide substitutions identified in this study provide an additional molecular tool for distinguishing *Helichrysum nuratavicum* from its closely related Central Asian congeners, thereby complementing traditional morphological identification. Integrating phylogenetic reconstruction with diagnostic nucleotide variation further strengthens the molecular evidence supporting the taxonomic distinctiveness of *Helichrysum nuratavicum* (Yuldashev et al., 2026).

Although the ITS region resolved phylogenetic relationships among the examined Central Asian *Helichrysum* species and identified diagnostic nucleotide substitutions, it has inherent limitations. The ITS marker may be affected by paralogy, incomplete concerted evolution, hybridisation, and relatively low sequence divergence among closely related taxa, which can complicate phylogenetic inference and species delimitation (Álvarez & Wendel, 2003). Future studies integrating additional nuclear and plastid markers or genome-wide datasets will provide a more comprehensive understanding of evolutionary relationships within the genus. Similar patterns have been reported in other closely related plant groups,

where the ITS region provides sufficient sequence variation for species delimitation despite relatively low sequence divergence among related taxa (China Plant BOL Group, 2011). Therefore, despite relatively low sequence divergence, the ITS region may be sufficiently informative to resolve phylogenetic relationships and identify diagnostic nucleotide substitutions that distinguish *Helichrysum nuratavicum* from closely related Central Asian *Helichrysum* species.

## CONCLUSION

This study expands molecular knowledge of the narrowly endemic species *Helichrysum nuratavicum* by providing its first ITS-based characterisation, clarifying its phylogenetic placement among closely related Central Asian *Helichrysum* species. The findings suggest that the ITS region is a valuable molecular marker for identifying and inferring the phylogeny of this group and provide further molecular evidence in support of recognising *Helichrysum nuratavicum* as a distinct taxon. While broader sampling and additional molecular markers are needed to further evaluate intraspecific variation and evolutionary relationships, the molecular data generated in this study provide a valuable foundation for future taxonomic, conservation, and DNA barcoding research on *Helichrysum* in Central Asia.

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**Author contributions.** Conceptualisation and literature analysis were performed by K.Y. and B.K. The first draft of the manuscript was prepared by K.Y., B.K., E.R. and M.M., who critically analysed

and gave suggestions to finalise the manuscript. Supervision was conducted by Z.Y. All authors read and approved the final version of the article.

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## APPENDIX I

*Helichrysum* species and outgroup taxa included in the phylogenetic and comparative analyses, together with their corresponding GenBank accession numbers

| Species                                                               | NCBI accession numbers |
|-----------------------------------------------------------------------|------------------------|
| <i>Helichrysum nuratavicum</i> Krasch                                 | PQ804169               |
| <i>Helichrysum maracandicum</i> Popov ex Kirp.                        | MT810473               |
| <i>Helichrysum arenarium</i> (L.) Moench                              | PP906953               |
| <i>Helichrysum thianschanicum</i> Regel                               | AY445200               |
| <i>Helichrysum crassifolium</i> (L.) D.Don ex G.Don                   | AY445190               |
| <i>Helichrysum pamphylicum</i> P.H.Davis & Kupicha                    | AY445194               |
| <i>Helichrysum serotinum</i> (DC.) Boiss.                             | AY445198               |
| <i>Helichrysum plicatum</i> DC.                                       | AY445201               |
| <i>Helichrysum lacteum</i> Coss. & Durieu                             | AY445202               |
| <i>Helichrysum sibthorpii</i> Rouy                                    | AY445203               |
| <i>Helichrysum noeanum</i> Boiss.                                     | AY445202               |
| <i>Helichrysum armenium</i> DC.                                       | AY445208               |
| <i>Helichrysum orientale</i> (L.) Gaertn.                             | HE611509               |
| <i>Helichrysum stoechas</i> (L.) Moench                               | HE611526               |
| <i>Helichrysum italicum</i> subsp. <i>microphyllum</i> (Willd.) Nyman | HM244710               |
| <i>Helichrysum pallasii</i> (Spreng.) Ledeb.                          | HM244733               |
| <i>Helichrysum italicum</i> (Roth) G.Don subsp. <i>italicum</i>       | KJ159126               |
| <i>Helichrysum litoreum</i> Guss.                                     | OQ330867               |
| <i>Helichrysum doerfleri</i> Rech.f.                                  | PX622882               |
| <i>Helichrysum heldreichii</i> Boiss.                                 | PX622883               |
| <i>Helichrysum taenari</i> Rothm.                                     | PX622884               |
| <i>Pseudognaphalium affine</i> (D.Don) Anderb.                        | OR700175               |
| <i>Pseudognaphalium luteoalbum</i> (L.) Hilliard & B.L.Burt           | OR700179               |