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The origins and evolution of the genus *Myosotis* L. (Boraginaceae)

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Abstract

Although morphologically well defined, the phylogeny and taxonomy of *Myosotis* has been uncertain. In particular it has been unclear whether the genus had a Northern Hemisphere or Australasian origin. However, separate analyses of the ITS and the 3′ region of *matK*, as well as a combined analysis of ITS, 3′ *matK*, the *psbA–trnA* spacer, and 3′ *ndhF* regions indicate that several distinct lineages exist within *Myosotis* and strongly support a Northern Hemisphere origin for the genus. Further, the observed transoceanic distributions and levels of genetic divergence between lineages indicate that long distance dispersal has been important for establishing the current geographic range expansion of *Myosotis*. Our molecular data also suggest that the diversification of Australasian *Myosotis* has occurred since the late Tertiary and is largely due to radiation within and from New Zealand. This inference is consistent with the findings of recent phylogenetic studies on other New Zealand alpine genera. Our results highlight the important role played by late Tertiary and Quaternary climate change in explaining current floristic diversity. The genetic relationships reported here also suggest that the current infrageneric taxonomy of *Myosotis* does not fully reflect the evolution of the genus.

1. Introduction

Myosotis consists of about 100 species distributed predominantly in the temperate zones of both hemispheres, with a few taxa occurring in alpine regions of the tropics. Within this broad distribution the genus has two centres of diversity—one in western Eurasia, where approximately 60 taxa occur (Al-Shehbaz, 1991), and the other in New Zealand where approximately 35 species are formally described (Moore, 1961; Moore and Simpson, 1973) and several remain undescribed (Druce, 1993). Outside these two regions the genus is poorly represented with fewer than 10 species restricted to North America, South America, Africa, New Guinea, and Australia (Al-Shehbaz, 1991).

Although *Myosotis* is clearly defined by both vegetative and reproductive morphology the genus is considered taxonomically complex, with little consensus on the limits, ranks, and infrageneric classification (Al-Shehbaz, 1991). Earlier taxonomic treatments (e.g., de Candolle, 1846; Stroh, 1941) relied heavily on the nature of the corolla scales and anther exsertion. However, both Moore (1961) and Grau and Leins (1968) have questioned the usefulness of these characters, in particular anther exsertion. The criticism is well illustrated in New Zealand where several species pairs, indistinguishable on the basis of vegetative characteristics, have been assigned to different infrageneric groups due to differences in the degree of anther exsertion (Moore, 1961), Most recently, Grau and co-authors (Grau and Leins, 1968; Grau and Schwab, 1982) have revised the infrageneric taxonomy of *Myosotis*. Based on their studies of pollen morphology and microscopic characters of the stigma and corolla scales, Grau and Schwab (1982) have proposed that the genus be divided into two sections—section *Myosotis* and section *Exarrhena*. The geographic distribution of these morphologically distinct groups

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Table 1
Outline of the infrageneric classification of *Myosotis* proposed by Grau and Schwab (1982)

Subgeneric group	Morphological characteristics	Distribution and content
Section *Myosotis*	Pollen grains small, constricted at equator and with fine perforations at poles; stigmas two lobed with papillae only slightly differentiated; corolla scales with long papillae	All Eurasian, African, and North American species except those belonging to the *discolor* group
Section *Exarrhena* austral group	Large pollen grains, surface conspicuously sculptured; simple stigmas with large club shaped papillae; corolla scales with short papillae	All Australasian and South American taxa
Section *Exarrhena discolor* group	Large pollen grains, but lacking surface sculpturing or perforations found in other groups; form of corolla scales and stigmas as in the austral group	A small group of taxa distributed in Eurasia, but with one species endemic to east Africa

corresponds to the two centers of *Myosotis* diversity. Section *Myosotis* has a Northern Hemisphere distribution, while section *Exarrhena* occurs in the Southern Hemisphere. The only important exception to this general geographical pattern is a small group of taxa related to the Eurasian annual *M. discolor* that were included in section *Exarrhena* because their pollen morphology suggested a close relationship with the Southern Hemisphere taxa (Table 1; Grau and Schwab, 1982).

The evolutionary relationships of the infrageneric groupings proposed by Grau and Schwab (1982) have remained unclear. Grau and Leins (1968) suggested that the greater diversity of pollen morphology between austral taxa indicated a Southern Hemisphere origin for the genus. Consistent with this interpretation, both Fleming (1962) and Wardle (1963, 1968) suggested that the ancestors of many New Zealand alpine lineages, including those of *Myosotis*, were already present in the Southern Hemisphere during the Tertiary period. An alternative hypothesis proposed by Raven (1973) was that the ancestors of the Australasian *Myosotis* arrived from the Northern Hemisphere only during the late Pliocene or Pleistocene.

We determined DNA sequences of the nuclear ITS and three chloroplast DNA markers (the 3′ region of the *matK* gene, the 3′ region of the *ndhF* gene, and the *trnK–psbA* intergenic spacer) for *Myosotis* species and representatives from possible outgroup genera within the Boraginaceae. Analyses of these data test hypotheses on the evolution and taxonomy of *Myosotis*.

2. Materials and methods

2.1. Plant material

Tissue samples for this investigation were either silica gel preserved field collections or were obtained from existing herbarium specimens. Appendix A provides details of the accessions used for this investigation.

2.2. DNA extraction, marker amplification, and DNA sequencing

Genomic DNA was extracted from dried tissue samples using a cetyltrimethylammonium bromide (CTAB) protocol, modified from Doyle and Doyle (1990). The marker loci were amplified in reaction volumes of 20 μL containing 1× Q solution (Qiagen), 1× PCR buffer (Qiagen), 250 μmol each dNTP (Boehringer Mannheim), 10 pmol each amplification primer, 1 U *Taq* DNA polymerase (5 U/μL; Qiagen), and 10–100 ng of total cellular DNA. Sequences and combinations of oligonucleotide primers used in PCR amplifications are detailed in Table 2. The reaction conditions for amplification of the *matK* and *ndhF* loci were: initial denaturation at 94 °C for 2 min, then 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C with a final extension at 72 °C for 5 min. These reaction conditions were modified for amplification of the remaining loci. For the *psbA–trnK* intergenic spacer the extension time was reduced to 1 min; for ITS a 1 min extension and 48 °C annealing temperature was used. PCR fragments were then purified using the QIAquick PCR Purification Kit (Qiagen). Both DNA strands of amplification products were characterized using a Perkin–Elmer ABI Prism 377 sequencing protocol. Oligonucleotide primers used for DNA sequencing are detailed in Table 2. For each taxon multiple DNA sequences were aligned by eye and any ambiguity checked against the corresponding electrophoretograms.

2.3. Phylogenetic analyses

2.3.1. Data alignment and tree building

Preliminary multiple alignments of DNA sequences were obtained using the progressive alignment procedure implemented in ClustalX (Thompson et al., 1994). These alignments were then inspected visually and checked for misalignment.

A conservative approach was taken towards the analysis of the aligned sequences—gapped sequence positions, variable positions immediately flanking gaps, and all ambiguous sequence positions were excluded from tree building analyses. Phylogenetic trees were

Table 2
Oligonucleotide primers used in the PCR amplification and sequencing of DNA marker loci

DNA locus	Primer name	Oligonucleotide sequence[a]	Used in
matK	trnK3R	5′ GATTCGAACCCGGAACTAGTCGG 3′	Amplification, sequencing
	trnK3AR	5′ CGTACASTACTTTTGTGTTTMCG 3′	Sequencing
	tK3MY1F	5′ CCAATTATGCCAATGATTGCATC 3′	Sequencing
	tK3MY1FB	5′ CGATACTCTTCTTCCAATTATG 3′	Sequencing (alternative to tK3MY1F)
	tK3MY2F	5′ CAATCAAAATCTTCTGGAATC 3′	Amplification, sequencing
ndhF	ND1318F	5′ GGATTAACYGCATTTTATATGTTTCG 3′	Amplification, sequencing
	ND1656F	5′ ACTTTGTTTGTTGGATGTTTA 3′	Sequencing
	ND1762R	5′ CCGAAATAAGCTATACTGACT 3′	Sequencing
	ND2110R	5′ CCCYABATATTTGATACCTTCKCC 3′	Amplification, sequencing
psbA–trnK	trnK3F	5′ CCGACTAGTTCCGGGTTCGAATC 3′	Amplification, sequencing
	PSBAR	5′ CGCGTCTCTCTAAAATTGCAGTCAT 3′	Amplification, sequencing
ITS	ITS5	5′ GGAAGTAAAAGTCGTAACAAGG 3′	Amplification, sequencing
	ITS3	5′ GCATCGATGAAGAACGTAGC 3′	Sequencing
	ITS2	5′ GCTACGTTCTTCATCGATGC 3′	Sequencing
	ITS4	5′ TCCTCCGCTTATTGATATGC 3′	Amplification, sequencing

[a] With the exception of the ITS primers (from White et al., 1990) and the *ndhF* amplification primers (ND1318F and ND2110R from Olmstead and Sweere, 1994) the oligonucleotide primers were designed during this study.

constructed for *Myosotis* with parsimony and quartet puzzling, the latter using a maximum likelihood (ML) optimality criterion, as implemented in PAUP*4.0b8 (Swofford, 2001). Parsimony analyses were performed using the heuristic search option and the "tree-bisection-reconnection" (TBR) swapping algorithm with accelerated transformation (ACCTRAN) optimization used to infer branch (edge) lengths. For quartet puzzling with ML, a HKY 85 invariable sites model (Swofford et al., 1996) was used in all analyses. Empirical base frequencies were assumed and other model parameters (e.g., the transition/tranversion ratio and proportions of invariable sites) were estimated on Neighbor Joining (NJ) trees.

2.3.2. Rooting the phylogeny

Evolutionary relationships within the Boraginaceae are not well understood (Al-Shehbaz, 1991). The uncertain taxonomy and current lack of a thoroughly sampled molecular phylogeny for the Boraginaceae made it unfeasible to a priori identify appropriate outgroups for *Myosotis*. For this reason, species from several genera of the Boraginaceae—*Borago*, *Echium*, *Eritrichium*, *Myosotidium*, *Plagiobothrys*, and *Symphytum* were sequenced (Appendix A) and phylogenetic analyses of these data used to infer the position of the root in ITS and *matK* phylogenies for *Myosotis*. For each potential outgroup all possible placements of the DNA sequence on to the most parsimonious unrooted *Myosotis* tree were considered. Pairwise comparisons of trees with different outgroup placements were then evaluated for significance using Kishino–Hasegawa (K–H; Kishino and Hasegawa, 1989) and Shimodaira–Hasegawa (S–H; Shimodaira and Hasegawa, 1999) sites tests as implemented in PAUP*4.0b8. Strictly, the K–H sites test compares topologies for two trees chosen without reference to the sequence data. In contrast, the more conservative S–H sites test compares two trees belonging to a set of possible trees that may have been chosen with reference to the sequence data (Goldman et al., 2000).

The placements of two potential outgroup taxa (*Echium vulgare* and *Plagiobothrys albiflorus*; see Appendix A) were also evaluated on a combined data set that included sequences from the ITS, *matK*, *ndhF*, and *psbA–trnK* regions for a representative sample of *Myosotis*. A preliminary phylogenetic analysis of these data appeared in Winkworth et al. (1999) and we expand on those here. Combining sequence data from different gene loci have been shown to improve phylogenetic resolution in some situations (Cunningham, 1997). However, the approach can be inappropriate when a common mechanism of sequence evolution between loci cannot be assumed and when rates of change are significantly different between data sets (Huelsenbeck et al., 1996; Lockhart and Cameron, 2001). Prior to testing outgroup placements with the combined data we tested for partition incongruence using the partition homogeneity test (Michevich and Farris, 1981) as implemented in PAUP*4.0b8.

2.3.3. Age of the austral lineage

The length of time that the austral lineage has been in the Southern Hemisphere was investigated using the simplified phylogenetic scheme shown in Fig. 1. An upper bound for this age was assumed to correspond to the distance between ancestral node III and extant taxa within the Southern Hemisphere group.

For our test, we built branch and bound ML trees (details of the model were the same as for quartet puzzling analyses). These contained the nine Eurasian taxa, inferred from the analyses shown in Figs. 2 and 3 to be most closely related to the Southern Hemisphere austral group. They also contained either *M. albo-sericea* or

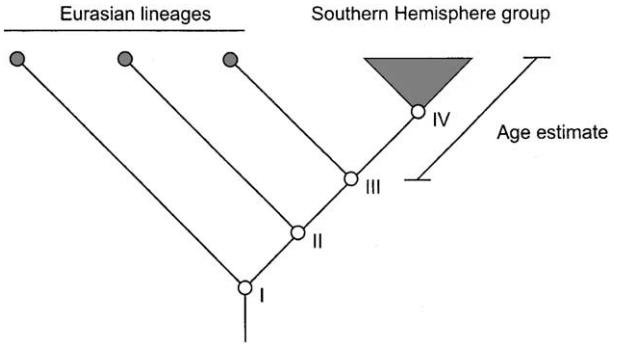

Fig. 1. Estimation of the possible age of the Southern Hemisphere universal common ancestor. Grey shading indicates extant taxa (circles) or groups of taxa (triangle) and open circles represent inferred ancestral taxa. Ancestors I and II are assumed to have had a Eurasian distribution. Ancestor III may have been present in either Eurasia or the Southern Hemisphere, while IV was the most recent common ancestor of the extant austral radiation. For estimates of the maximum possible age for the austral ancestor we assume the most parsimonious reconstruction—III had a Northern Hemisphere distribution and IV was Australasian.

M. exarrhena, both from the austral group. The topology of the ML tree and optimal branch lengths were calculated and constrained to these values, except for the length of the edge that led to the austral representative. The length of this branch was varied such that it corresponded to a calibrated time of between 0 and 100 million years (MY).

We used several rate calibrations for sequence divergence and time. The first made use of the New Zealand fossil pollen record and observed sequence diversity among the extant austral species shown in Fig. 3. That is, we assumed that the first appearance of *Myosotis* pollen in the New Zealand fossil record corresponded to the immediate ancestor (node IV in Fig. 1) of the extant radiation of Australasian species, and not the last common ancestor (node III in Fig. 1) of Southern Hemisphere and Eurasian species. The average genetic distance (path length) between Southern Hemisphere species was calculated for independent paths that crossed the node that corresponded to node IV in Fig. 1. This genetic distance was calibrated with the earliest record of *Myosotis* pollen in New Zealand (2 MY; Mildenhall, 1981).

A second calibration of sequence divergence with absolute time was made by comparison of ITS sequences from *Myosotis* with those from the Juan Fernandez Island endemic genus *Dendroseris* (Asteraceae). In an earlier study, Sang et al. (1994) reported pairwise comparisons for ITS sequences from all *Dendroseris* taxa, and by assuming an age of 4 MY for the Juan Fernandez Islands, estimated an evolutionary rate of 3.94×10^{-3} substitutions/site/MY for ITS. Since their approach used non-independent paths between taxa we also re-calculated the substitution rate for *Dendroseris* ITS sequences using independent paths from their data set.

In our analyses, we excluded sequences which did not meet requirements of rate constancy as inferred using a relative rates test (Steel et al., 1996) implemented in SplitsTree 3.1 (Huson, 1998).

3. Results

3.1. Aligned DNA sequences

The high degree of similarity between the DNA sequences characterized for each molecular marker resulted in data matrices that contained little alignment ambiguity. Statistics from the aligned data matrices for *Myosotis* sequences are presented in Table 3.

3.2. Phylogenetic gene trees

Nuclear ITS and chloroplast *matK* regions were analyzed under two evolutionary tree building methods. Under maximum parsimony a single most parsimonious tree of 75 steps was recovered in analyses of the *matK* data set; this tree had a consistency index (CI) of 0.933 and retention index (RI) of 0.956. ITS sequences produced three equally parsimonious trees of 194 steps with CI of 0.691 and RI of 0.855. These trees differed only in the relationships inferred between *Myosotis alpestris*, *M. lithospermifolia*, and *M. semiamplexicaulis*. Fig. 2 presents 50% majority rule consensus trees for both the ITS and *matK* data sets. These data were also analyzed using quartet puzzling with a ML optimality criterion; trees from these analyses are shown in Fig. 3. Note that although indels were removed from analyses, only one was incompatible with the phylogenetic reconstructions based on nucleotide substitutions. That is, only a single 1 bp indel in the ITS region shared by the *M. sylvatica* lineage and the North American species, *M. macrosperma*, and *M. verna* (sequence position 432) was not compatible with the evolutionary trees reported.

The topology of trees recovered with chloroplast and nuclear markers were highly congruent with each other. Congruence between trees built from these data sets is highlighted by the observation that sequences from only two taxa differ in their placement. In the ITS gene tree, the sequence from *M. persoonii* is closest to species from Southern Hemisphere, whereas in *matK* trees *M. persoonii* groups closely with several Eurasian taxa (compare Figs. 2 and 3A with 2 and 3B). The *matK* sequence for the African species *M. abysinnica* groups it with members of the "discolor" species group, however the ITS sequence places this species with representatives of section *Myosotis*.

The gene trees presented here suggest five distinct phylogenetic groups terminal to branches A–E in Figs. 2 and 3. Group A contains several Eurasian representatives and all Southern Hemisphere species. The

remaining four groups have species restricted to the Northern Hemisphere, with the exception of two southern African species that have been derived from two of these. Although our analyses indicate that genetic groups within *Myosotis* are distinct from each other, relationships between them are not resolved as bifurcating. Rather lineages are mostly separated from each other by polytomies or by weak bifurcations with low support values (e.g., Figs. 2 and 3).

3.3. Relative genetic diversity and outgroup rooting

The branch lengths in gene trees derived from *matK* and ITS sequences (Figs. 2 and 3) indicate that the greatest genetic diversity in *Myosotis* occurs amongst the Northern Hemisphere lineages—an observation consistent with a Northern Hemisphere origin for the genus. This hypothesis was tested by investigating the preferred placement of six potential outgroup sequences onto the optimal *matK* and ITS phylogenies for *Myosotis*. The sequences from these six taxa were found to be genetically divergent from their orthologs in *Myosotis* species, consistent with these species being outgroups to the genus *Myosotis*. In the tests made, hypotheses proposing a Northern Hemisphere origin for the genus (trees in which the branch to the outgroup joined an edge within a Eurasian lineage) were always favored over those suggesting that *Myosotis* arose in the Southern Hemisphere (trees in which the branch to the outgroup either joined within the austral radiation or the branch subtending this group). Arrows on Fig. 2 indicate the optimal rooting position for each of the outgroups. While a Northern Hemisphere origin is always favored, gene trees supporting a Southern Hemisphere origin were only significantly worse in 44% of the Kishino–Hasegawa tests at the $P < 0.05$ level, and were less significant under the more conservative Shimodaira–Hasegawa sites test.

To investigate whether significance could be obtained by using longer sequences, a combined data set comprising sequences from ITS, *matK*, *ndhF*, and *trnK–psbA* loci were jointly analyzed. These additional sequences were determined for the outgroup taxa *Echium vulgare* and *Plagiobothrys albiflorus* as well as a representative sample of *Myosotis* species. Again the optimal placement of the outgroup sequences were on edges within Northern Hemisphere lineages. More importantly, under Kishino–Hasegawa and Shimodaira–Hasegawa sites tests, all but one of the outgroup placements (i.e., 95.5% of tests) suggesting a Southern Hemisphere origin were significantly worse at the $P < 0.05$ level.

3.4. Dispersal in the Southern Hemisphere

Quartet puzzle and maximum parsimony trees for the ITS region (Fig. 2A, 3A, 4) all suggest New Zealand taxa are basal to the other species of the austral group (93% support under QP). Support for this inferred relationship is not significant under pairwise sites tests. However, there is less support for competing hypotheses. These include hypotheses that taxa from other Southern Hemisphere lands are more basal to the New Zealand species in outgroup rooted phylogenies (less than 1% support under QP; Fig. 4). As will be discussed, these results are consistent with New Zealand as the most likely center for the origin of the extant Southern Hemisphere species.

3.5. Age of the Australasian lineage in the Southern Hemisphere

The length of time that the austral lineage has been present in the Southern Hemisphere was investigated by examining sequence divergence and various rate calibrations for sequence diversity and absolute time. The first and most conservative calibration was derived from the earliest fossil record for *Myosotis* in New Zealand. This was 1.1×10^{-3}–3.4×10^{-3} substitutions/site/MY, and using these estimates our ML inference suggests that the earliest possible Southern Hemisphere ancestor existed 2.0–14.7 MYA (Table 4). A second rate calibration, derived from a study of *Dendroseris* ITS sequence diversity, suggested ITS sequence evolution at 3.3×10^{-3}–5.8×10^{-3} substitutions/site/MY (a range which included the point estimate of Sang et al., 1994). With this calibration method the austral *Myosotis* lineage is estimated to have existed in the Southern Hemisphere 1.2–4.9 MYA (Table 4).

The variance on these estimates was examined by comparing log-likelihood scores for trees constrained for topology and branch lengths. When *M. albo-sericea* was used as a representative of the austral group, trees suggesting the presence of a Southern Hemisphere lineage older than 16.4 MY (assuming the conservative pollen calibration) and 5.5 MY (assuming the conservative *Dendroseris* ITS calibration) were rejected ($P < 0.05$). When *M. exarrhena* was used as the Southern Hemisphere representative, the age of its lineage in the Southern Hemisphere was found to be less than 29.5 and 9.8 MY, respectively ($P < 0.05$). These results reject the possibility that the austral group has a relictual Gondwanan distribution.

4. Discussion

4.1. Myosotis phylogeny: the similarity between gene trees

Few incompatibilities exist between trees derived from the ITS and *matK* loci. This high degree of congruence between gene trees derived from different markers suggests that the inferred phylogenetic patterns reflect a common evolutionary history for *Myosotis*.

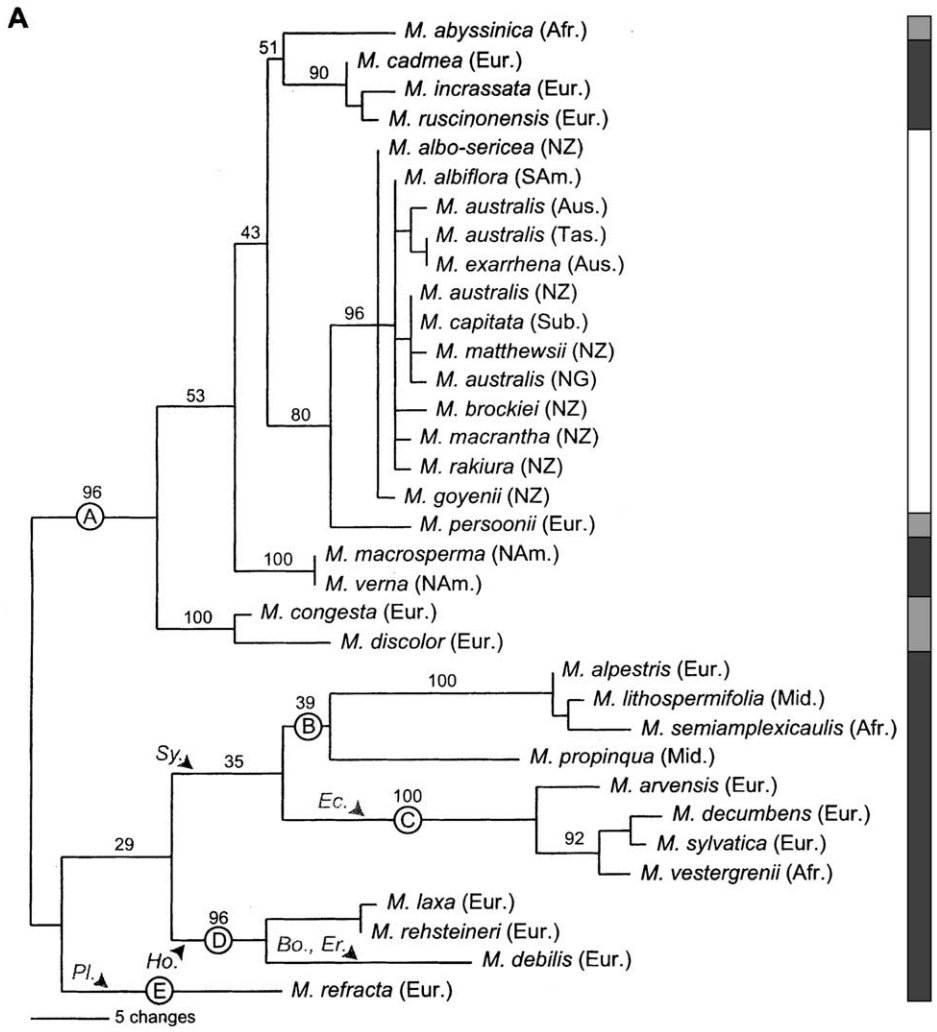

Fig. 2. Bootstrap majority rule consensus trees reconstructed using a parsimony criterion; branch lengths have been estimated under ACCTRAN (using PAUP 4.0b8). (A) Data set for 34 *Myosotis* taxa from the ITS region of the nrDNA (610 nucleotides). (B) Data set for 34 *Myosotis* taxa from the 3′ region of the chloroplast *matK* gene locus (867 nucleotides). Main genetic groupings have been indicated by a circled capital letter on the branch subtending each lineage. Subgeneric groups of Grau and Schwab (1982) are also indicated by different shadings in the rectangular box to the right of the figure—section *Exarrhena* austral group are white; section *Exarrhena* discolor group are light grey; section *Myosotis* are shown as dark grey. Preferred outgroup rooting positions are marked by arrows, outgroups are denoted by two letter code—Bo., *Borago officinalis*; Ec., *Echium vulgare*; Er., *Eritrichium nanum*; Ho., *Myosotidium hortensia*; Pl., *Plagiobothrys albiflorum*; Sy., *Symphytum* × *uplandicum*. Generalized distributions for taxa are indicated by abbreviations: Afr., Africa; Aus., mainland Australia; Eur., Europe; Mid., Middle East; NAm., North America; NZ, New Zealand; SAm., South America; Sub., Subantarctic Islands; Tas., Tasmania. Bootstrap values (250 replicates) for internal branches are given.

Differences between gene trees included the placement of *M. persoonii* and *M. abysinnica* (Figs. 3A and B). These incongruences are possibly explained by either homoplasy or ''chloroplast capture,'' since sequence analysis of independent PCR amplified products gave identical results. Characterization of further chloroplast and nuclear markers for populations of *M. persoonii* and *M. abysinnica* could help to distinguish between these possible explanations.

4.2. Origins and early evolution of *Myosotis*

Based on the greater diversity of pollen morphology in Australasian *Myosotis*, Grau and Leins (1968) proposed that the Eurasian lineages arose from taxa with long histories in the Southern Hemisphere. However, the greater genetic diversity between Northern Hemisphere lineages, relative to that observed among austral representatives, suggests that the genus arose in the Northern Hemisphere. Tests of outgroup placement on to *Myosotis* phylogenies support a Eurasian origin. In particular, analyses using a combined data set favored a Northern Hemisphere ancestry. Whilst our molecular results strongly suggest that *Myosotis* arose in the Northern Hemisphere it was not possible to identify the most ancestral lineage as no single optimal root position was identified.

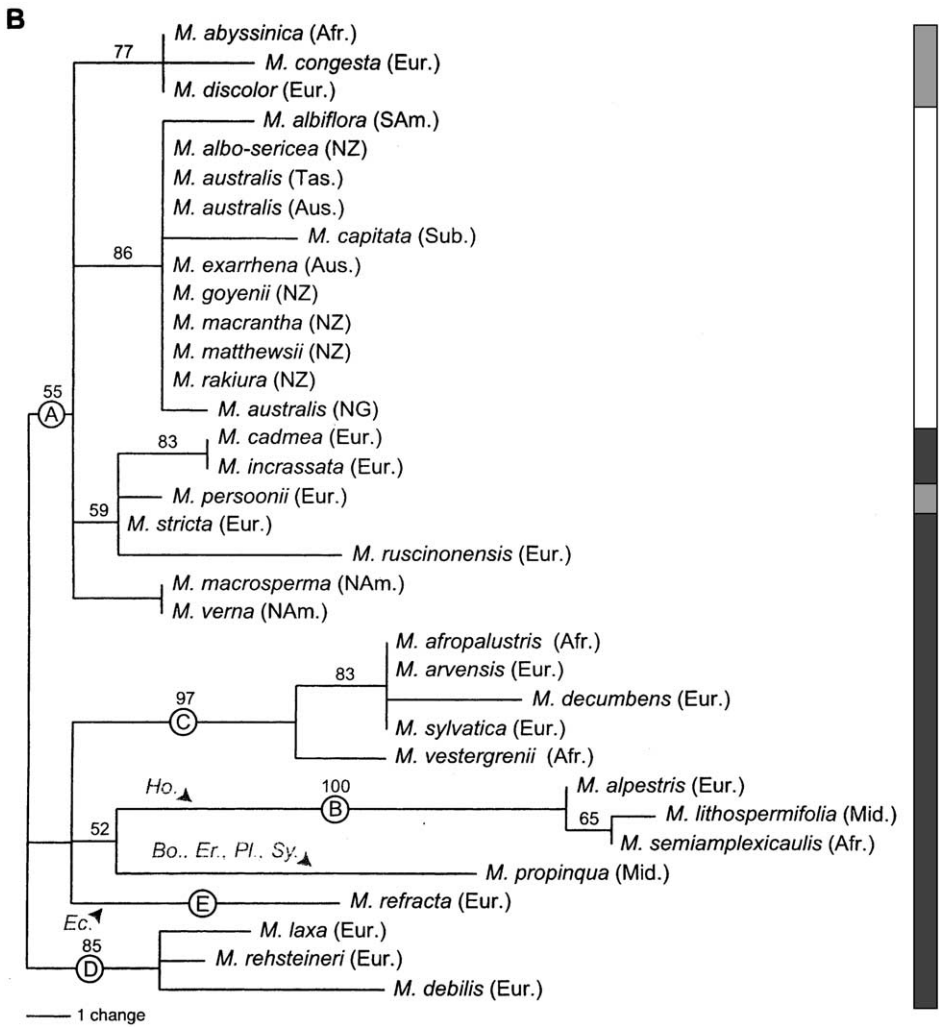

Fig. 2. (*continued*).

Our molecular analyses identified five distinct lineages within *Myosotis*; molecular characterization of additional species may reveal others. Although these major groups are genetically well differentiated, the relationships between them are poorly resolved (i.e., related to each other either by polytomies or short branches with low support values) suggesting that they may have originated during a period of rapid diversification, perhaps shortly after the genus arose.

4.3. Dispersal and the worldwide distribution of Myosotis

An important event in the evolution of *Myosotis* was the dispersal and establishment of the genus in Australasia. Consistent with the hypothesis of Raven (1973), our phylogenetic analyses suggest that a single, mid, or late Tertiary (<20 MYA) transoceanic dispersal event by a Northern Hemisphere ancestor established *Myosotis* in New Zealand. In ITS gene trees, the *discolor* group species *M. persoonii* is the closest Eurasian relative to austral species; a finding consistent with Grau and Schwab's (1982) suggestion that the *discolor* group is closely related to the austral species. The chloroplast data do not provide sufficient resolution to address this issue.

The extent of sequence divergence within the austral group suggests that *Myosotis* may have been in the Southern Hemisphere longer than is indicated by fossil deposits in New Zealand. In this case, species extinctions associated with Quaternary climate change cannot be discounted and consideration of this possibility may also have important implications for understanding plant dispersal routes into New Zealand. Raven (1973) suggested that dispersal of many alpine groups, including that of *Myosotis*, to New Zealand occurred via the Australian and New Guinean Alps. Our analyses do not support this hypothesis. However, if the isolated alpine zones of Australia and New Guinea were seriously restricted as climate change

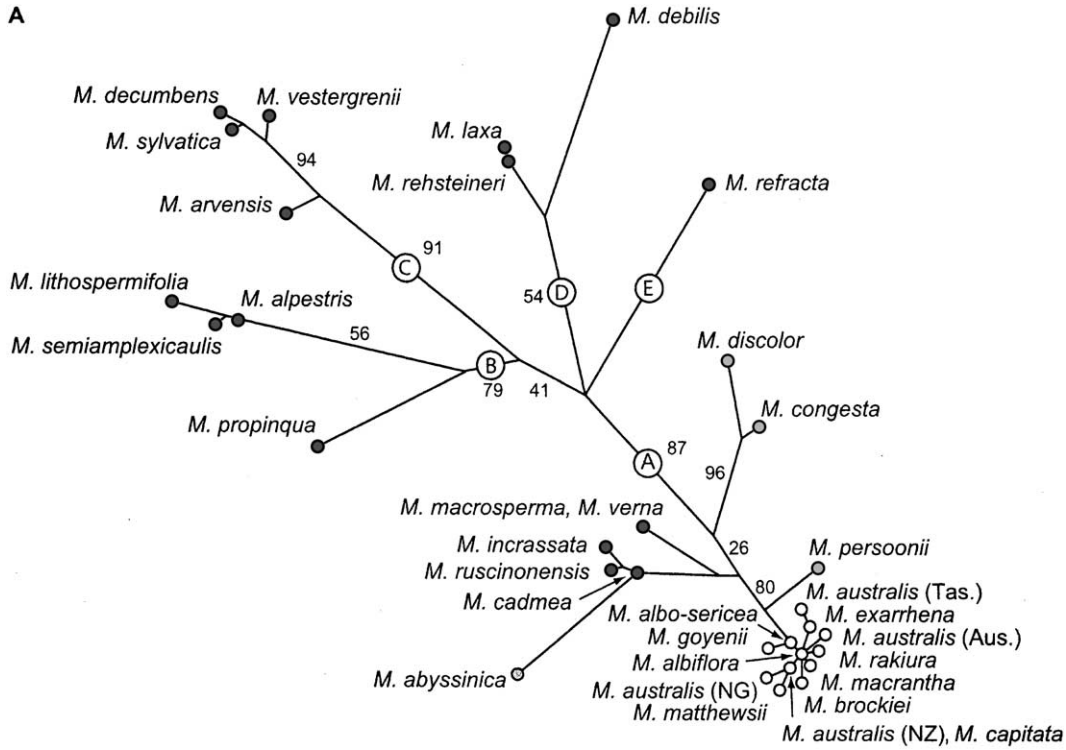

Fig. 3. Quartet puzzle trees with branch lengths estimated using ML (using PAUP 4.0b8). (A) Data set for 34 *Myosotis* taxa from the ITS region of the nrDNA (610 nucleotides; $p_{inv} = 0.803$ and ti/tv = 1.792). (B) Data set for 34 *Myosotis* taxa from the 3′ region of the chloroplast *matK* gene locus (867 nucleotides; $p_{inv} = 0.639$ and ti/tv = 1.412). Puzzle support values for internal branches are given. Genetic lineages, taxonomic groups, and the distribution of *M. australis* accessions are denoted as for Fig. 2.

during the late Tertiary/Quaternary as in New Zealand (Lee et al., 2001), such "stepping stones" may no longer contain the ancestral austral species. If so, then it may be problematic to test dispersal routes into New Zealand for plant groups using analyses of extant species alone.

Despite the concern that sequence analyses may be unable to pinpoint dispersal routes into New Zealand, our results do indicate transoceanic dispersals from New Zealand to other Southern Hemisphere lands (Fig. 4). The phylogenetic inference that some dispersal events have occurred in northerly and westerly directions is of particular interest. Previous authors have suggested the importance of west wind drift, the dominant meteorological pattern at high southern latitudes, in facilitating plant dispersal in an easterly direction (e.g., Fleming, 1979; McPhail, 1997; Mildenhall, 1981; Pole, 1994; Raven, 1973). While our results provide some evidence for the possibility of dispersal facilitated by west wind drift (i.e., from New Zealand to South America for *M. albiflora*), they also suggest that successful dispersal events have occurred to the north and west (i.e., from New Zealand to Australia for *M. australis*; and from New Zealand to New Guinea also for *M. australis*), movements that are counter to the direction of west wind drift.

Other instances of transoceanic dispersal by representatives of Eurasian lineages are also suggested by our phylogenetic graphs. Specifically analyses of both nuclear and chloroplast markers suggest that the establishment of *Myosotis* in Africa and North America has been the result of dispersal from Eurasian sources.

Few data are available on the dispersal mechanisms in *Myosotis* (e.g., Bresinsky, 1963; Ridley, 1930; Steyermark, 1963). Given that *Myosotis* nutlets appear to be poorly adapted for wind dispersal, this mechanism seems unlikely. Rather the trichomes found on the calyxes and pedicels of many *Myosotis* species (Al-Shehbaz, 1991; Heywood, 1993) suggest that animal vectors may facilitate dispersal. Indeed, bird-mediated dispersal has been favored as a mechanism for transoceanic dispersal of disseminules in several plant groups in the South Pacific (Carlquist, 1996; Godley, 1967; Swenson and Bremer, 1997).

4.4. Recent species diversification

In direct contrast to the reported morphological diversity of austral *Myosotis* species (Grau and Leins, 1968; Grau and Schwab, 1982; Winkworth et al., 1999), our molecular investigations show that this group is

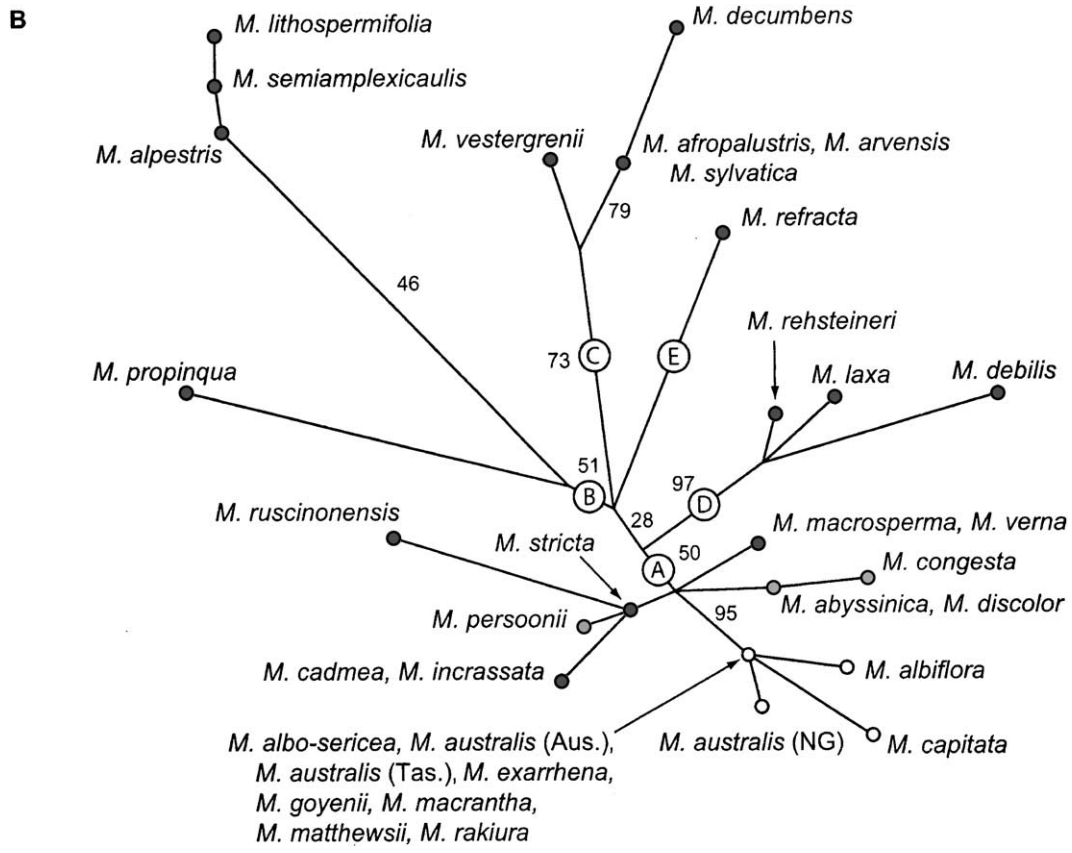

Fig. 3. (*continued*).

Table 3
Statistics from the aligned data matrices of *Myosotis* DNA sequences

	ITS	Chloroplast		
		matK	*ndhF*	*trnK–psbA*
No. of taxa sequenced	34	34	14	14
Sequence length range (bp)	646–649	882–923	725–731	223–250
Aligned length (bp)	659	925	731	250
No. of indels	11	11	1	6
No. of excluded sites	49	58	6	28
No. of constant sites	511	801	687	211
No. of varied sites	99	66	36	11
No. of parsimony informative sites	68	34	19	4
% GC content range (all sites included)	55.6–58.9	32.4–33.4	27.1–28.1	32.9–33.3
% GC content mean (all sites included)	57.2	33.0	27.4	32.9
% GC content range (varied sites only)	42.4–58.6	51.5–65.7	44.4–63.4	36.4–54.5
% GC content mean (varied sites only)	52.5	63.3	49.4	45.5

largely undifferentiated at several presumably neutrally evolving DNA loci. This pattern of low genetic and high morphological diversity suggests that *Myosotis* has radiated only recently in the Australasian region—an interpretation supported by molecular clock analyses on ITS sequences which suggest that the austral *Myosotis* species have radiated only since the Pliocene (Table 4). The ITS gene trees also suggest that considerable morphological diversification associated with this radiation has occurred in New Zealand (Fig. 4). The diversification of *Myosotis* in New Zealand correlates with the late Tertiary and Quaternary period—a time of considerable climatic fluctuation and geological upheaval (Raven, 1973; Wardle, 1968), Further, the branching patterns within the major Northern Hemisphere lineages may suggest that environmental changes have also been

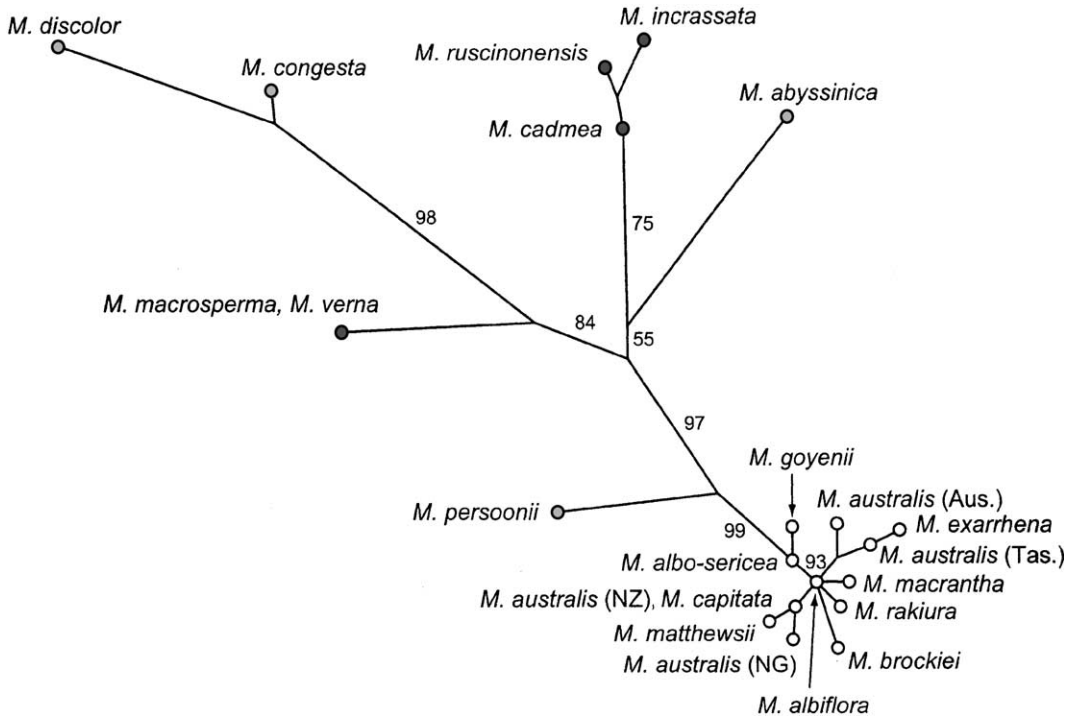

Fig. 4. Quartet-puzzle tree with branch lengths estimated using ML (PAUP 4.0b8), made using a dala set of 634 nucleotides from the ITS region of the nrDNA for 22 *Myosotis* taxa ($p_{inv} = 0.803$ and ti/tv = 1.792). Puzzle support values for internal branches are given. Subgeneric groups and the distribution of *M. australis* accessions as for Fig. 2.

Table 4
Age estimate for the origins of the austral lineage of *Myosotis*

Age estimate for	Divergence in optimal ML tree	Estimated ages (MY)	
		Re-estimated *Dendroseris* rate	Pollen based calibration
M. albo-sericea	0.00639	1.2–2.0	2.0–6.1
M.exarrhena	0.01563	2.8–4.9	4.8–14.7

important for establishing the extant species diversity of Eurasian *Myosotis*. Our molecular evidence for recent diversification in *Myosotis* is comparable with similar results reported for other New Zealand plant groups (e.g., see discussion in Winkworth et al., 1999) and from genetic studies on continental floras of Europe and North America. These investigations suggest that environmental change during the late Tertiary and Quaternary periods, both at global and local scales, is of great importance in understanding the development of the world's modern floristic biodiversity (Comes and Kadereit, 1998).

4.5. Implications of molecular analyses for the intrageneric classification of Myosotis

The issue of monophyly in taxonomy is well debated and it is widely accepted that only monophyletic groups should be formally recognized in taxonomic classification (Donoghue and Cantino, 1988). While the molecular results reported here support certain aspects of the intrageneric classification proposed by Grau and Schwab (1982), in particular the close relationship between the *discolor* and austral groups of sect. *Exarrhena*, they do not provide evidence for the monophyly of the currently recognized sections. Our molecular results may indicate the need for revised intrageneric classification.

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Appendix A. Details of accessions of *Myosotis* and outgroup taxa

Taxon	Location	Herbarium reference[a]	GenBank Accession
Borago officinalis L.	Palmerston North, North Island, New Zealand (naturalized introduction)	MPN 24675	ITS: AY092898 *matK*: AY092892
Echium vulgare L.	Lake Ohau, South Island, New Zealand (naturalized introduction)	MPN 24676	ITS: AY092900 *matK*: AY092893 *ndhF*: AY092890 *psbA-trnK*: AY092952
Eritrichium nanum (L.) Schrader ex Gaudin	Switzerland	Hertel 25764[b]	ITS: AY092901 *matK*: AY092894
Myosotidium hortensia (Decne) Baill.	Palmerston North, North Island, New Zealand	No voucher (nursery origin)	ITS: AY092902 *matK*: AY092895
Myosotis abyssincia Boiss. & Reuter	Ethiopia	De Wilde 6944[b]	ITS: AY092904 *matK*: AY092856
Myosotis afropalustris C.H.Wr.	Natal, South Africa	K. Balkwill & M.J. Balkwill 5260[b]	*matK*: AY092857
Myosotis albiflora Banks & Sol.	Punta Arenas, Chile	MPN 24679	ITS: AY092906 *matK*: AY092858 *ndhF*: AY092848 *psbA-trnK*: AY092938
Myosotis albo-sericea Hook. F.	Leaning Rock, South Island, New Zealand	Site voucher CHR 416092[c]	ITS: AY092905 *matK*: AY092859 *ndhF*: AY092842 *psbA-trnK*: AY092939
Myosotis alpestris F.W. Schmidt	Karten, Hochobir, Austria	"FE-M2"	ITS: AY092907 *matK*: AY092860 *ndhF*: AY092854 *psbA-trnK*: AY092951
Myosotis arvensis (L.) Hill	Bavaria, Germany	H. Gröger 1018[b]	ITS: AY092908 *matK*: AY092861 *ndhF*: AY092843 *psbA-trnK*: AY092949
Myosotis australis R. Br.	Tasmania, Australia	MPN 24677	ITS: AY092910 *matK*: AY092863 *ndhF*: AY092844 *psbA-trnK*: AY092944
Myosotis australis R. Br.	Mt. Kozciuscko, Australia	MPN 24678	ITS: AY092909 *matK*: AY092862
Myosotis australis R. Br. (identified as *M. saruwagedica*)	New Guinea	CHR 198545	ITS: AY092933 *matK*: AY092884
Myosotis australis R. Br. "Yellow"	Mt. Ben More, South Island, New Zealand	Site voucher CHR 365380[c]	ITS: AY092911
Myosotis brockiei L. Moore et M. Simpson	Cobb Gorge, South Island, New Zealand	Site voucher CHR 311717[c]	ITS: AY092912
Myosotis cadmea Boiss.	Greece	Stainton 7320[b]	ITS: AY092913 *matK*: AY092864
Myosotis capitata Hook. F.	Cultivated (B. Rance)	MPN 24680	ITS: AY092915 *matK*: AY09B866 *ndhF*: AY092853 *psbA-trnK*: AY092943

Appendix A. (*continued*)

Taxon	Location	Herbarium reference[a]	GenBank Accession
Myosotis congesta Shuttlew. Ex Alb. & Reynier	Greece	Phitos M-33[b]	ITS: AY092916 *matK*: AY092867
Myosotis debilis Pomel	Spain	Gomez Vigide317[b]	ITS: AY092917 *matK*: AY092868
Myosotis decumbens Host. ssp. *decumbens*	Alpi Maritime, Schönswetter & Tribsch	"FE-M"	ITS: AY092918 *matK*: AY092869
Myosotis discolor Pers.	Kahuterawa Valley, North Island, New Zealand (naturalized introduction)	MPN 11910	ITS: AY092919 *matK*: AY092870 *ndhF*: AY092852 *psbA-trnK*: AY092948
Myosotis exarrhena (R. Br.) F. Mueller	South-eastern NSW, Australia	CBG 9519354	ITS: AY092920 *matK*: AY092871
Myosotis goyenii Petrie	Broken River, South Island, New Zealand	Site voucher CHR 278306[c]	ITS: AY092921 *matK*: AY092872 *ndhF*: AY092846 *psbA-trnK*: AY092942
Myosotis incrassata Guss.	Greece	Merxtmüller & Wiedmann 20130[b]	ITS: AY092922 *matK*: AY092873
Myosotis laxa Lehm. ssp. *caespitosa* (C.F Schultz) Hyl.	Hopkins River, South Island, New Zealand	MPN 24681	ITS: AY092914 *matK*: AY092865 *ndhF*: AY092851 *psbA-trnK*: AY092946
Myosotis lithospermifolia Hornem.	Persia	Rechinger 6521[b]	ITS: AY092923 *matK*: AY092874
Myosotis macrantha (Hook, F.) Benth. Et Hook. F.	Hooker Valley, South Island, New Zealand	Site voucher CHR 252807[c]	ITS: AY092924 *matK*: AY092875 *ndhF*: AY092849 *psbA-trnK*: AY092940
Myosotis macrosperma Engelm.	USA	MPN 24682	ITS: AY092925 *matK*: AY092876 *ndhF*: AY092855 *psbA-trnK*: AY092947
Myosotis matthewsii L. Moore	Cultivated (Percy's Reserve, Wellington, New Zealand)	No voucher	ITS: AY092926 *matK*: AY092877 *ndhF*: AY092847 *psbA-trnK*: AY092941
Myosotis persoonii Rouy	Spain	Zubizarreta 5327[b]	ITS: AY092927 *matK*: AY092878
Myosotis propinqua Fisch. & Mey.	Persia	Rechinger 39832[b]	ITS: AY092928 *matK*: AY092879
Myosotis rakiura L. Moore	Curio Bay, South Island, New Zealand	MPN 24686	ITS: AY092929 *matK*: AY092880 *ndhF*: AY092845 *psbA-trnK*: AY092945
Myosotis refracta Boiss. ssp. *refracta*	Greece	Gröger 1463a[b]	ITS: AY092930 *matK*: AYQ92881
Myosotis rehsteineri Wartm.	Bavaria, Germany	Dörr[b]	ITS: AY092931 *matK*: AY092882
Myosotis ruscinonensis Rouy	France	Kunz & Reichstein M-310[b]	ITS: AY092932 *matK*: AY092883
Myosotis semiamplexicaulis DC.	South Africa	Acocks 21310[b]	ITS: AY092934 *matK*: AY092885

Appendix A. (*continued*)

Taxon	Location	Herbarium reference[a]	GenBank Accession
Myosotis stricta Link	Bavaria, Germany	Förther 7895[b]	*matK*: AY092886
Myosotis sylvatica Ehr. ex Hoffm. ssp. *Sylvatica*	Palmerston North, North Island New Zealand (naturalized introduction),	MPN 24687	ITS: AY092935 *matK*: AY092887 *ndhF*: AY092850 *psbA-trnK*: AY092950
Myosotis uniflora Hook. f.	Hopkins River, South Island, New Zealand	MPN 24688	
Myosotis verna Nutt.	USA	Taylor 3489[b]	ITS: AY092936 *matK*: AY092888
Myosotis vestergrenii Stroh	Ethiopia	MO 3808817	ITS: AY092937 *matK*: AY092889
Plagiobothrys albiflorus (Griseb.) R.L. Pérez-Mor.	Paso Cardenal Samore, Argentina	MPN 24689	ITS: AY092899 *matK*: AY092896 *ndhF*: AY092891 *psbA-trnK*: AY092953
Symphytum × *uplandicum* Nyman	Wellington, North Island, New Zealand (naturalized introduction)	MPN 24690	ITS: AY092903 *matK*: AY092897

[a] Herbarium codes. CBG—Australian National Botanic Gardens, Canberra, ACT, Australia; CHR—Landcare Research New Zealand, Christchurch, New Zealand; MO—Missouri Botanical Garden, Saint Louis, Missouri, USA; MPN—Massey University, Palmerston North, New Zealand.
[b] Herbanum accession lodged at the Staatsherbarium München [MSB].
[c] Due to the small size of these populations or rarity of the species in general no voucher was taken for this study. However, these populations are represented by herbarium collections made by previous workers.

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