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# Genetic variation in *Trithuria inconspicua* and *T. filamentosa* (Hydatellaceae): a new subspecies and a hypothesis of apomixis arising within a predominantly selfing lineage

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**Abstract.** While examining herbarium specimens of *Trithuria inconspicua* Cheeseman, we observed differences in the stigmatic hairs among plants from New Zealand's North and South Islands. This motivated us to assess genetic and morphological variation within this species and its sister *T. filamentosa* Rodway from Tasmania. Samples were collected from lakes in the three disjunct geographic areas where the two species occur. Genetic variation in both species was assessed with simple sequence-repeat (SSR, microsatellite) markers and analyses of genetic distances. We also compared the morphology of northern and southern New Zealand *T. inconspicua* using fresh material. Samples of each species clustered together in a minimum evolution tree built from genetic distances. *Trithuria filamentosa* had more genetic diversity than did *T. inconspicua*. Within *T. inconspicua*, plants from lakes in the North Island and the South Island formed discrete genetic groups diagnosable by subtle morphological differences. Low levels of heterozygosity in both species are consistent with a high level of selfing, as suggested for other co-sexual *Trithuria* species, but unusual for a putative apomict. On the basis of genetic and morphological variation, we propose recognition of the northern New Zealand and southern New Zealand lineages of *T. inconspicua* at subspecies rank.

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## Introduction

The recognition that *Trithuria* Hook.f. (including *Hydatella* Diels) is not a monocot but a highly reduced member of the Nymphaeales (Saarela *et al*. 2007), part of the Amborellales–Nymphaeales–Austrobaileyales (ANA) grade (often referred to as 'basal' or 'early diverging' angiosperms), has spurred interest in its biology. Recent studies into *Trithuria* phylogeny (Iles *et al*. 2012), reproductive biology (Sokoloff *et al*. 2008*a*; Rudall *et al*. 2009; Taylor *et al*. 2010; Friedman *et al*. 2012; Iles *et al*. 2012; Taylor and Williams 2012), cytology (Kynast *et al*. 2014), anatomy and morphology (Sokoloff *et al*. 2008*b*, 2009, 2013, 2014) and genome duplication (Marques *et al*. 2016) have been conducted.

*Trithuria* is represented in New Zealand solely by *T. inconspicua* Cheeseman. As its species epithet implies, *T. inconspicua* is an easily overlooked, tiny aquatic plant that grows submerged around the margins of lakes. The species is known from two disjunct areas ~1000 km apart, namely, coastal dune lakes in the north of New Zealand's North Island (Northland province; Edgar 1970), and glacial lakes in the south of the South Island (Southland and Otago provinces; Wells *et al*. 1998). Plants of both the North and South Island populations are typically found on moderately exposed shores, ranging from occasionally emergent (P. D. Champion, pers. obs.) to 2-m depth (Wells *et al*. 1998).

Until very recently, *Trithuria inconspicua* was classed as *Threatened–Nationally Endangered* under the New Zealand threat-classification system (de Lange *et al*. 2013), reflecting its decline, particularly in Northland. In 1998, 13 Northland lakes were reported to support populations of *T. inconspicua*, but it has since become extinct in seven of these. In the most recent listing (de Lange *et al*. 2018), *T. inconspicua* is categorised as *Threatened–Nationally Critical* and a new listing is provided for *T.* aff. *inconspicua* (CHR 502358; South Island), which is categorised as *Threatened–Nationally Vulnerable*. Although *T. inconspicua* has been grown for periods in aquaria (Pledge 1974) and readily germinates from seed (P. D. Champion, pers. obs.), it has not persisted in cultivation (P. J. de Lange, New Zealand Department of Conservation, pers. comm.).

Phylogenetic analyses (Iles *et al*. 2012, 2014) have suggested that *T. inconspicua* is sister to the Tasmanian endemic *T. filamentosa* Rodway, and that the two are part of a clade including two additional Australian species, namely, *T. austinensis* D.D.Sokoloff, Remizowa, T.D.Macfarl. & Rudall, and *T. australis* (Diels) D.D.Sokoloff, Remizowa, T.D.Macfarl. & Rudall. *Trithuria filamentosa* is found

'localized but sometimes abundant in marshes, roadside soaks or on the margins of lakes and lagoons especially in the midlands and north of [Tasmania]' (Duretto 2011. p. 2). It is not listed as threatened (Tasmanian Government, Department of Primary Industries, Parks, Water and Environment 2014).

Breeding systems vary among *Trithuria* species (Iles *et al*. 2012). Some species are dioecious (*T. cookeana* D.D. Sokoloff, Remizowa, T.D.Macfarl. & Rudall, *T. polybracteata* D.A.Cooke ex D.D.Sokoloff, Remizowa, T.D.Macfarl. & Rudall, *T. austinensis*, and *T. occidentalis* Benth.), others co-sexual (*T. bibracteata* Stapf ex D.A.Cooke, *T. submersa* Hook.f., *T. lanterna* D.A.Cooke, *T. konkanensis* S.R.Yadav & Janarth., *T. cowieana* D.D.Sokoloff, Remizowa, T.D.Macfarl. & Rudall, and *T. australis*). *Trithuria inconspicua* and *T. filamentosa* were provisionally coded as co-sexual by Iles *et al*. (2012), who noted that stamens are not always present; when present, the majority of pollen is sterile, embryo development occurs in the absence of pollen tubes in *T. filamentosa*, and seed is set underwater in *T. inconspicua*. All of these circumstances could be interpreted as indicating that *T. inconspicua* and *T. filamentosa* have a predominantly apomictic breeding system (Hamann 1976; Rudall *et al*. 2008). In *T. inconspicua*, male flowers are uncommon in North Island populations, with Edgar (1966) reporting 89.5% of reproductive units being female only, 8.5% male only, and 2% bisexual from Lake Waiparera, and Pledge (1974) reporting 97.8% being female only, 1.7% male only, and 0.5% bisexual reproductive units from Lake Kai Iwi. At the whole-plant level, individuals were either co-sexual or exclusively female. One of the authors (PC) collected over 1500 flowering plants from seven Northland lakes in 1991–1992, finding that 84.4% had only female reproductive units, 9.3% had both female and male reproductive units, 3.7% had only male reproductive units, and 3.6% had bisexual reproductive units. Several lake populations were exclusively female-flowered. However, the inflorescences in one population sampled over 2 years (Lake Rotoroa) contained a maximum of 44.4% plants ($n$ = 195) with male flowers present. Male flowers have not been observed in South Island Lakes (K. A. Ford, pers. obs.; but note that few collections from early summer are available). Precise figures are not available, but the reproductive units of *T. filamentosa* are most often female, less often male, and only rarely bisexual (Duretto 2011).

Chromosome numbers in *Trithuria* range from $2n$ = 14 to at least $2n$ = 56 (Iles *et al*. 2012), suggesting a base of $x$ = 7. *Trithuria inconspicua* has been reported to have $2n$ = ~24, with ambiguity being attributed to the extremely small size and poor staining of the chromosomes (de Lange *et al*. 2004). Nonetheless, this number suggests a possible tetraploid ancestry with subsequent aneuploidy. As far as we are aware, the chromosome number of *T. filamentosa* is unknown.

While examining herbarium specimens, differences in the uniseriate stigmatic hairs were detected between North and South Island populations of *T. inconspicua*. It appeared that specimens of the southern populations have greatly shortened stigmatic hairs. We sought to confirm this apparent difference by using fresh material, and otherwise compare the morphology of North and South Island *T. inconspicua* specimens.

We also characterised a set of 11 simple sequence-repeat (SSR, or microsatellite) DNA markers and applied them to samples of *T. inconspicua* from nine lakes in New Zealand (five in Northland, four in Southland and Otago) and to *T. filamentosa* samples from three lakes in Tasmania. We aimed to assess the amount and distribution of genetic diversity in *T. inconspicua* and compare it to that in *T. filamentosa*. We also sought to assess whether the distributions of genetic diversity in *T. inconspicua* and *T. filamentosa* are consistent with predominantly clonal breeding systems.

## Materials and methods

### Plant samples

Plants were collected from lake margins and either placed in silica gel (some North Island and Tasmanian samples) or returned to our laboratory packaged wet (South Island sites), in which case they were then frozen at −80°C for DNA analysis. For morphological study, herbarium specimens, including loaned material from other collections, were examined at CHR. Extensive field collections were made in northern and southern New Zealand and Tasmania and vouchers were deposited in CHR. Sample locations are shown in Fig. 1. Voucher specimens are as follows: North Auckland: Lake Ngatu, *Wells* (CHR 638453); Lake Waikare, *Champion* (CHR 638454); Lake Rotokawau (Pouto Peninsula), *Wells* (CHR 638452); Lake Kai Iwi, *Champion* (CHR 638451); and Lake Rotoroa (no voucher); Otago: Lake Sylvan, *Ford & Smissen* (CHR 638457); Southland: South Mavora Lake, *Ford & Smissen* (CHR 638458); Lake Hauroko, *Ford and Smissen* (CHR 638456); and Lake Poteriteri, *Champion* (CHR 638449); Tasmania: Lake Dobson, *Champion* (CHR 643733); Lake Dove, *Champion* (CHR 643732); and Lake St Clair, *Champion* (CHR 643821). The number of samples from each population is shown in Table 1. DNA was extracted from samples by CTAB method (Doyle and Dickson 1987, modified to incorporate a phenol–chloroform extraction and DNA recovery with spin columns).

### Simple sequence-repeat development

We produced a genomic DNA-sequence library by using a Roche (Basel, Switzerland) GS Junior sequencing platform and titanium emPCR and sequencing kits (Catalogue numbers 07138814001 and 07138822001) and Titanium PicoTiterPlate kit (Catalogue number 05996619001). Processed sequence output from the 454 sequencer (188 742 reads) was searched for SSR motifs by using Microsatellite Commander (Rozen and Skaletsky 2000; Faircloth 2008).

We screened 124 primer pairs by first subjecting them to gradient polymerase chain reaction (PCR; 45−65°C) on a single DNA extract and agarose-gel electrophoresis to select an appropriate annealing temperature. PCR reactions used Roche FastStart DNA polymerase with the buffer and nucleotides supplied with it. Reactions were performed in 20-μL volume using 1 unit of polymerase, 0.3 μL of dNTPs (10 mM each) and final concentration of 0.5 pmol of each primer. Primers showing promising products in gradient PCR were further tested against panels of 8–12 samples from New Zealand and Tasmania. Forward primers were tailed

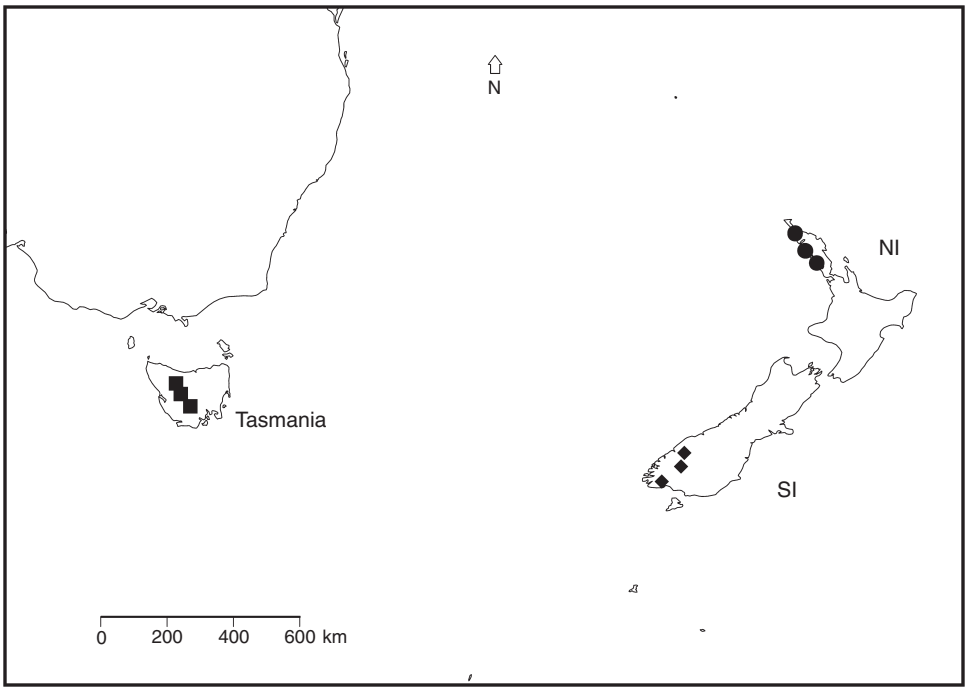


**Fig. 1.** Map of south-eastern Australia and New Zealand, showing sampled lake locations. Note that some locations in New Zealand are not resolved as separate points at this scale. NI, New Zealand North Island. SI, New Zealand South Island. Squares, *Trithuria filamentosa*. Circles, *T. inconspicua* subsp. *inconspicua*. Diamonds, *T. inconspicua* subsp. *brevistyla.* Numbers refer to populations in Table 1.

**Table 1. Genotypic variation for each lake and island (for lake location, see Fig. 1)**
Tas, Tasmania (*Trithuria filamentosa*); SI, South Island (*T. inconspicua*); NI, North Island (*T. inconspicua*). $H_E$ (expected heterozygosity) and $H_O$ (observed heterozygosity) have been calculated only from markers interpretable as disomic (see text). Total genotype number for NI is not the sum of the numbers of genotypes in each lake from the region since some genotypes were recovered from more than one lake. Number refers to Fig. 1

| Population | Number | Sample size | Number of genotypes | All samples | | Unique genotypes | |
|---|---|---|---|---|---|---|---|
| | | | | $H_E$ | $H_O$ | $H_E$ | $H_O$ |
| Lake Dove (Tas) | 1 | 6 | 5 | 0.267 | 0 | 0.304 | 0 |
| Lake Dobson (Tas) | 2 | 10 | 5 | 0.408 | 0 | 0.496 | 0 |
| Lake St Clair (Tas) | 3 | 8 | 6 | 0.275 | 0 | 0.2444 | 0 |
| All Tas | | 24 | 16 | 0.579 | 0 | 0.5832 | 0 |
| Lake Sylvan (SI) | 4 | 1 | 1 | 0 | 0 | 0 | 0 |
| South Mavora Lake (SI) | 5 | 5 | 2 | 0.064 | 0 | 0.1 | 0 |
| Lake Hauroko (SI) | 6 | 8 | 3 | 0.088 | 0 | 0.1778 | 0 |
| Lake Poteriteri (SI) | 7 | 7 | 1 | 0 | 0 | 0 | 0 |
| All SI | | 21 | 7 | 0.355 | 0 | 0.3814 | 0 |
| Lake Waikare (NI) | 8 | 9 | 3 | 0 | 0 | 0 | 0 |
| Lake Kai Iwi (NI) | 9 | 10 | 1 | 0 | 0 | 0 | 0 |
| Lake Rotokawau (NI) | 10 | 6 | 1 | 0 | 0 | 0 | 0 |
| Lake Ngatu (NI) | 11 | 7 | 2 | 0.0267 | 0.029 | 0.075 | 0.1 |
| Lake Rotoroa (NI) | 12 | 7 | 1 | 0 | 0 | 0 | 0 |
| All NI | | 39 | 4 | 0.098 | 0.005 | 0.1172 | 0.025 |

at the 5′ end with the M13f universal primer sequence (tgtaaaacgacggccagt), to allow incorporation of 6-FAM labelled M13f universal primer for screening (Boutin-Ganache *et al*. 2001). PCR reactions were as above, except that the amount of forward primer was reduced to 0.2 pmol, reverse primer to 0.4 pmol, and 0.4 pmol of dye labelled M13f primer. Capillary electrophoresis was conducted using an Applied Biosystems (Foster City, CA, USA) 3100-Avant Genetic Analyzer and was performed at Landcare Research, Auckland. Fragment-size files were examined using Genemarker (ver. 1.51 or ver. 2.4, SoftGenetics LLC, State College, PA, USA).

### *Genotyping*

After screening, we selected 11 primer pairs to use in genotyping our full set of 84 samples. Primer sequences and the annealing temperatures used for these are shown in Table 2. Forward primers were labelled with 6-FAM (9), VIC (73, 79, 110), NED (28, 34, 101, 103) or PET (49, 111, 116). The rejected candidate primers either failed to amplify a product from some or all samples, amplified a product too large to genotype in our facility, amplified complex products not amenable to scoring, or amplified products that were not polymorphic. Primer quantities added to each 20-μL reaction were reduced to 0.25 pmol each. Reaction products were pooled into two sets, such that each could be distinguished by size range or dye colour (Set 1: 9, 28, 34, 49 73, 79; Set 2: 101, 103, 110, 111, 116).

Of the 11 primer pairs selected, three (9, 73, 79) could be interpreted as polymorphic single-locus markers (i.e. the primers amplified no more than two distinct fragments from any individual interpreted as alleles at a single locus). Another primer pair (28) produced fragments in the size range of 186–194 bp, which were interpretable as a single locus, but some samples also produced another fragment ~25-bp shorter, which we interpreted as a different locus, including both in analyses as 28a and 28b. Primer pairs 111 and 116 produced fragments in two narrow size ranges each, which could be interpreted as two different loci, and were scored as 'a' and 'b' loci, as for primer pair 28. Four primer pairs produced fragments that appeared to be from two or more loci (Primer pairs 34, 49, 103, 110), in that more than three polymorphic fragments were amplified from at least some samples. One primer pair produced a single fragment of fixed size found in New Zealand samples (Primer pair 101) and was null in all Tasmanian samples. Our interpretation of these data is summarised in Table 3.

Because of the low levels of polymorphism encountered in screening, and because of the very low levels of heterozygosity encountered for those markers that were polymorphic and could be interpreted as single-locus or two-locus (see Results), we retained complex profiles and scored all the markers as dominant data (i.e. fragment presence or absence; data matrix, see Table S1, available as Supplementary material to this paper). Because of the very low levels of observed heterozygosity (see Results), we considered that there was no disadvantage in treating the data as dominant in estimating genetic distances.

**Table 2. Primer sequences for simple sequence-repeat (SSR markers developed and used in the study**

| Marker | Forward primer | Reverse primer | Annealing temperature (°C) |
|---|---|---|---|
| 9 | 5′-TGCTGCAAACAAACCAGGG-3′ | 5′-GCTCAATCAGAGTAACCGTCC-3′ | 65 |
| 28 | 5′-ACGCGGCGGTATGTTAATTC-3′ | 5′-ATCGGATCTTCTGCAGCG-3′ | 50 |
| 34 | 5′-ACAGCATTCACGTGTAGGAAC-3′ | 5′-AAGAAGGGTGGGACTTGGC-3′ | 60 |
| 49 | 5′-CGCCTAACCTGGAAACACG-3′ | 5′-GTTTCCGGCTGACATTCTG-3′ | 65 |
| 73 | 5′-GGCCCGCATTATTTGACCG-3′ | 5′-GTTACGGTGGCAGATGCTC-3′ | 65 |
| 79 | 5′-CCCGTGCATTCGAGGTTATC-3′ | 5′-AGTCGGTCAAGCTTTCATTGTC-3′ | 65 |
| 101 | 5′-CCGCAAACGACTTCCTCAC-3′ | 5′-GAGCACCAAGAGCAAATTGAAG-3′ | 65 |
| 103 | 5′-ACAGAATTCGGGAAACCCTC-3′ | 5′-TCCTCTAACGCATGATCTCC-3′ | 55 |
| 110 | 5′-GAGTAGGACGTGCAACCATTC-3′ | 5′-CTCCGGACATAGCAAACGG-3′ | 55 |
| 111 | 5′-TGGGCGTGATCAAGAACTC-3′ | 5′-GATTCACCGGTTTCAGGCG-3′ | 65 |
| 116 | 5′-GGACAGATTTGGGTCAAGGG-3′ | 5′-TTGAAGGGTGTGTCCGCTC-3′ | 65 |

**Table 3. Details of products amplified for each marker**

If two loci are inferred, this is based on fixed heterozygosity in the majority of lakes. Heterozygosity values have not been calculated for markers treated as dominant. For Primer pairs 28, 111 and 116, two figures are given for $H_E$ and $H_O$, being 'a' and 'b' loci scored separately. This was not conducted for Primer pair 103 because alleles at the two putative loci overlapped in size range, nor for primers inferred to amplify >2 loci, nor for 116b which was fixed in New Zealand and null in Tasmania. The number of alleles, or number of scored fragment-size classes if more than one locus inferred, is given

| Primer pair | Number of alleles | Maximum fragments in a single sample | Size range | $H_E$ | $H_O$ | Inferred number of loci |
|---|---|---|---|---|---|---|
| 9 | 9 | 1 | 309–405 | 0.711 | 0 | 1 |
| 28 | 6 | 3 | 165–196 | 0.558, – | 0.012, – | 2 |
| 34 | 8 | 5 | 302–405 | – | – | >2 |
| 49 | 12 | 7 | 234–254 | – | – | >2 |
| 73 | 5 | 1 | 243–260 | 0.627 | 0 | 1 |
| 79 | 8 | 1 | 324–359 | 0.713 | 0 | 1 |
| 101 | 2[A] | 1 | 237 | – | – | 1 |
| 103 | 5 | 2 | 148–158 | – | – | 2 |
| 110 | 14 | 7 | 301–327 | – | – | >2 |
| 111 | 4 | 2 | 431–457 | 0.1913, 0.455 | 0, 0 | 2 |
| 116 | 4 | 2 | 295–350 | 0.430, – | 0, – | 2 |

[A]Including null allele fixed in Tasmanian samples.

### *Analysis of genetic distances*

The binary data matrix coded fragment presence as 1 and fragment absence as 0. Samples with identical profiles were reduced to a single representative (see Table 1). A minimum evolution tree was built from mean character distances by using a heuristic search with 1000 replicate random addition sequences in PAUP4.0a163 (Sinauer Associates: Sunderland, MA, USA; Swofford 2003). Negative branch lengths were allowed and other settings were default. Bootstrap support was calculated from 1000 replicate heuristic searches with random addition sequences, negative branch lengths were allowed and other settings were default. Labels were added after tree building to represent diversity at each lake when identical profiles were recovered from more than one lake.

## Results

### *Genetic data*

We genotyped 60 plants from New Zealand and identified 13 unique genotypes. Despite genotyping fewer plants from Tasmania (24), we detected more unique genotypes (16). Primer pair 79 produced a single fragment from some Tasmanian samples but not from others, suggesting that null alleles were present; however, it amplified at least one fragment from all New Zealand samples. Using the scoring approach outlined in the 'Materials and methods' section, we assessed heterozygosity at five polymorphic loci (9, 28b, 73, 79, 111b), which amplified at least one allele from all the New Zealand samples (i.e. for which there was no strong evidence of null alleles). Expected and observed heterozygosities are shown in Table 1, with each lake being treated as a population and, separately, all lakes within an island being treated as a single population. In only one sample (Lake Ngatu 2) was any heterozygosity detected, and only at one locus (Locus 28b). Similarly, for Tasmanian plants, five polymorphic loci could be assessed for heterozygosity (9, 28b, 73, 111a, 116a); however, none showed any evidence of heterozygosity in any samples (Table 1). Any apparent heterozygosity in the raw data (other than in the Lake Ngatu sample mentioned above) was consistent with 'fixed heterozygosity' resulting from duplication of the locus, because homozygotes were absent in at least some populations (e.g. Loci 28a and 28b considered together, Locus 103).

Given this situation, we scored all fragments produced from all primer pairs as presence or absence characters producing a matrix with 76 characters (two of which were fixed in all samples). A heuristic search using minimum evolution as optimality criterion produced a single tree from pairwise percentage distances (Fig. 2). This tree illustrates distinct New Zealand and Tasmanian clusters of genotypes. Within the New Zealand cluster, there is separation into North and South Island groups. There was strong bootstrap support for Tasmanian

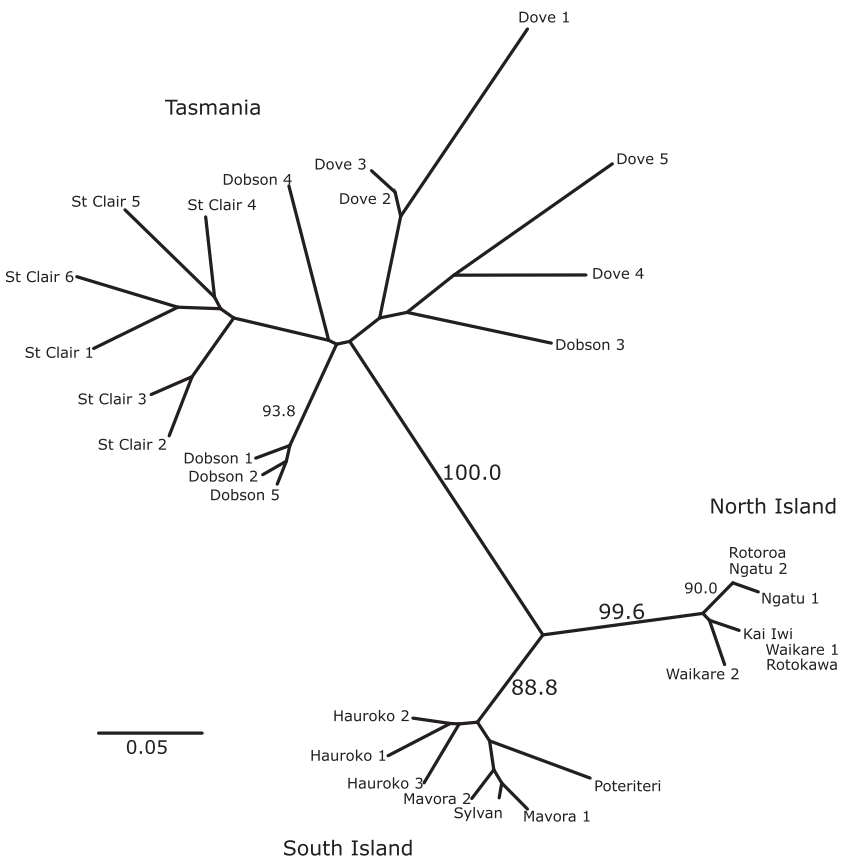


**Fig. 2.** Minimum evolution tree built from genetic distances among all distinct genotypes detected in this study. Branch labels are bootstrap percentages shown where they are >80%.

and North Island clusters (>99%) and moderate support for the South Island cluster (88.8%). There is some separation of Tasmanian clusters by lake; however, this is incomplete. There is greater genetic diversity among Tasmanian plants than among New Zealand plants, overall and within each lake, both in terms of the number of distinct genotypes (Table 1) and the mean differences among samples (Fig. 2).

### *Morphological characters*

Differences in the stigmatic hairs between northern and southern New Zealand populations indicated by examination of dried herbarium specimens were confirmed in fresh material collected from all four of the southern lakes from which we sampled reproductive material. In southern populations, the stigmatic hairs arising from the carpel apex, which are elongated in all other species of *Trithuria*, are reduced, forming a coalesced capitate head (Fig. 3D). The short hairs are not caused by collapse of expanded hair cell walls or senescence of hairs with maturation or drying. This is evident under a light microscope in freshly collected plants at different developmental stages. This shortening is consistent in all populations examined from Otago and Southland, and is absent from northern populations (Fig. 3). In the southern lakes, 100% seed set was observed on freshly collected plants and herbarium material at CHR, and no male flowers were observed, despite careful searching (K. A. Ford, unpubl. data). Southern plants of *T. inconspicua* were also observed to have scapes that do not elongate with maturation and fruits that are ovoid to globose, in contrast to northern plants, whose scapes elongate at maturity and have ellipsoid to ovoid fruits.

Morphological differences in reproductive-unit bract number, shape, length and the relative length of individual bracts subtending a reproductive unit in all southern populations were observed to be similar to those previously reported in northern populations of *T. inconspicua* and in other species of *Trithuria* (Rudall *et al*. 2007; Sokoloff *et al*. 2008*a*). We observed variation among plants from different lakes in the frequency of bract number, shape and length. Notably, plants in populations from Lake Hauroko commonly had two short, broad-ovate bracts (Fig. 3B), whereas South Mavora plants commonly had four long, narrow-ovate bracts (Fig. 3C); plants from all southern lakes exhibited variation within populations, with three, five, six, and seven bracts being recorded, as well as variation in bract shape, length, and whether individual bracts were of the same or varying lengths.

## Discussion

### *Allelic diversity, fixed heterozygosity and polyploidy*

Interpretation of SSR profiles in polyploid species can be complicated, because duplicated loci can result in more than two alleles per genotype and unambiguous genotypes cannot always be inferred (Pfeiffer *et al*. 2011). This is particularly true in autopolyploids with tetrasomic inheritance, but also applies to allopolyploids if homoeologous loci are not

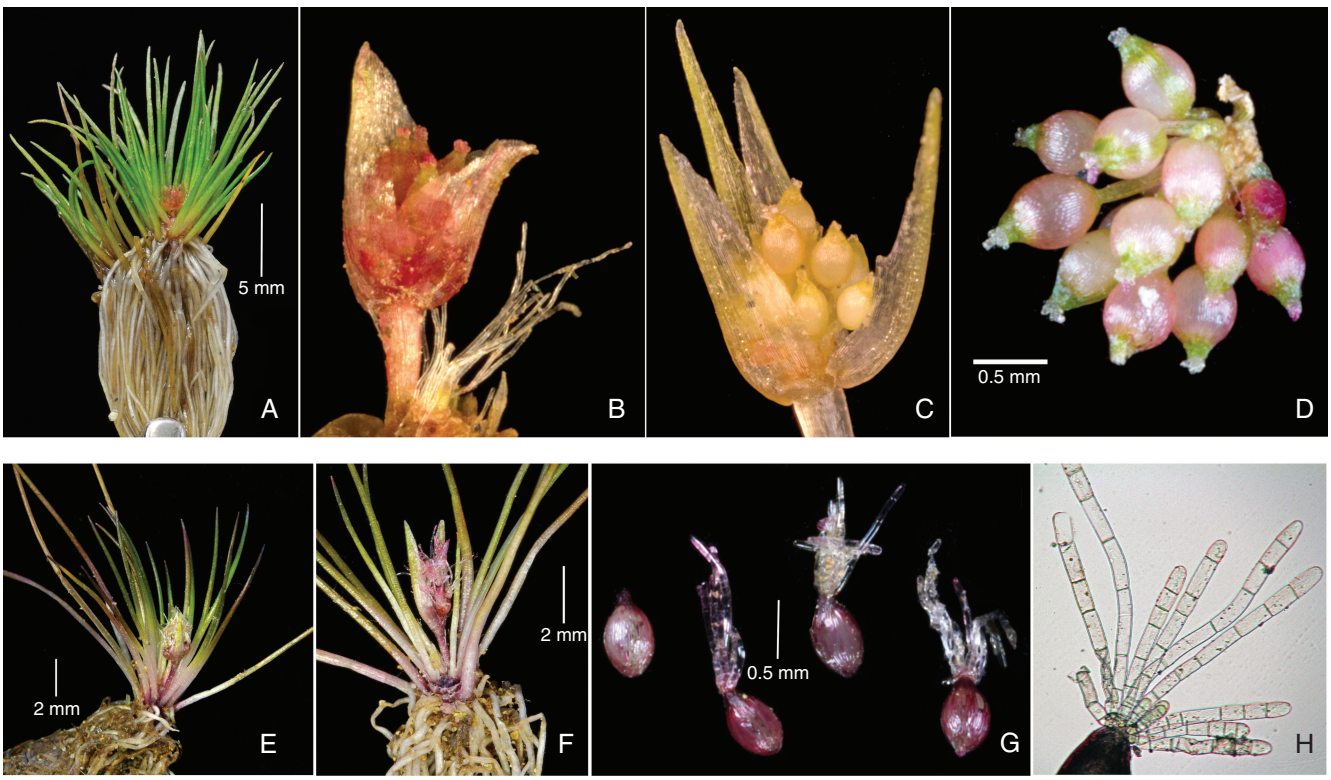


**Fig. 3.** A–D. *Trithuria inconspicua* subsp. *brevistyla*. A. Plant habit. Inflorescences, showing two (B) and four (C) bracts. D. Developing ovules showing short stigmatic hairs. Lake Hauroko (A, B and D; CHR 638456); South Mavora Lake (C; CHR 638458). E–H. *T. inconspicua* subsp. *inconspicua*. E. Plant habit. F. Inflorescence. G. Developing ovules showing long stigmatic hairs. H. Stigmatic hairs (light micrograph). Lake Waikere (E–H; CHR 638459).

sufficiently diverged to prevent their amplification by the same PCR primers. Over time, duplicated or homoeologous loci in polyploid species will diverge, provided that inheritance is disomic (Le Comber *et al*. 2010). Marques *et al*. (2016) reported analyses of SSR markers in the putatively octoploid ($2n = 56$) Australian species *T. submersa*. They observed fixed heterozygosity at many loci and interpreted this as consistent with *T. submersa* being a recent polyploid, with a high level of homozygosity resulting from a highly selfing mating system. In *T. inconspicua* and *T. filamentosa*, 4 of the 11 SSRs we assayed displayed patterns of allelic variation consistent with their representing a single locus, whereas another three showed patterns of fixed heterozygosity consistent with the presence of duplicated loci, each of them being homozygous in all individuals sampled. One interpretation of these data, together with the available chromosome counts for the species, is that *T. inconspicua* and *T. filamentosa* are older polyploids than is *T. submersa* and have undergone some degree of diploidisation. Alternatively, *T. inconspicua* and *T. filamentosa* might have allopolyploid origins where some pairs of homoeologous loci are sufficiently divergent so that only one amplifies with the primers we used. Because we did find some apparently unduplicated loci, it is also plausible that duplications of single genes or parts of chromosomes have caused the patterns of SSR variation we observed. However, it is more parsimonious to hypothesise that a single genome-wide duplication underlies the fixed heterozygosity.

### *An unusual case of apomixis evolving in a highly selfing plant?*

Regardless of their possible polyploid background, both *T. filamentosa* and *T. inconspicua* showed almost no heterozygosity at the loci we assayed (other than fixed heterozygosity we attribute to locus duplication). This is despite most of the markers showing polymorphism within lakes. This is particularly true for *T. filamentosa*, where multiple genotypes were present in all the lakes, yet no heterozygosity was observed. This is consistent with a high level of self-fertilisation in all the lakes, and at least suggests protracted and ongoing inbreeding. Our DNA data do not conform to the expected pattern for an apomict. Apomixis generally arises in outcrossing, often self-incompatible, plants (Bicknell and Koltunow 2004; Hörandl 2010). Consequently, typical apomictic lineages preserve the heterozygosity present in their ancestor, and will tend to acquire more through ongoing mutation in the absence of recombination. In contrast, some recombination must occur to maintain the high levels of homozygosity at the polymorphic loci that we observed in Tasmanian lakes (compare expected and observed heterozygosity in Table 1). Levels of polymorphism are lower in New Zealand, meaning that, for some lakes, expected-heterozygosity values were 0 because there was no polymorphism detected in the lake, or polymorphism was observed only in markers for which we could not infer allelic relationships (see Table 1). However, the same argument still applies; namely, in the absence of recombination, mutations should accumulate, resulting in heterozygosity. Sexual reproduction may be rarer in *T. inconspicua* than in *T. filamentosa*; because the amount of polymorphism detected within lakes is much lower, less sex would be required to maintain homozygosity and the single instance of heterozygosity we detected was in a *T. inconspicua* plant from Lake Ngatu. It is also possible that sexuality has been lost (or reduced) recently and that our small sampling of loci was insufficient to detect rising levels of heterozygosity. However, the data indicated that recombination continued until after the establishment of the North Island and South Island New Zealand lineages (because each exhibits unique and fixed alleles). The presence of separate plants displaying the same genotype within all the lakes is consistent with clonal reproduction, but could also result from self-fertilisation, given the low levels of heterozygosity.

*Trithuria filamentosa* and *T. inconspicua* are part of a clade within *Trithuria* recognised as *T.* section *Hydatella* (Diels) D.D. Sokoloff, Iles, Rudall & S.W.Graham (Iles *et al*. 2012). Also in this clade are their sister *T. australis* and the sister to those three species, *T. austinensis* (Iles *et al*. 2012). *Trithuria austinensis* is dioecious and, so, presumably highly outcrossing, whereas *T. australis* is likely to be self-compatible and habitually self-pollinating (Taylor 2011). Therefore, with current knowledge, it is most parsimonious to infer that the common ancestor of *T. filamentosa* and *T. inconspicua* was co-sexual and self-compatible.

Nonetheless, circumstantial evidence, particularly the production of abundant seed in South Island lakes underwater and in the absence of any observations of male flower organs, the observation that when present pollen is often abnormal in both species, the development of seed in *T. filamentosa* in the absence of pollen tubes, together with a generally high seed set in both species, strongly suggests that apomixis is habitual in at least some populations of *T. inconspicua* and also present in *T. filamentosa*. In the case of South Island *T. inconspicua*, we are forced to hypothesise either that sexual reproduction has only recently disappeared or that it continues at a level sufficient to prevent heterozygosity from accumulating at the loci we have assayed. Potentially, this could be achieved by irregular expression of male function, perhaps in response to environmental cues.

At least at face value, it appears that *T. filamentosa* and *T. inconspicua* represent an unprecedented example of a transition from habitual self-fertilisation to apomixis. Apomixis and selfing have often been considered alternative strategies for reproductive assurance (Hörandl 2010). Under most circumstances, there would, therefore, seem to be no selective pressure for the evolution of apomixis within a selfing population, because reproductive assurance is generally already achieved. Apomixis also allows the perpetuation of genotypes of odd ploidy level (e.g. Morgan-Richards *et al*. 2004) and of well adapted heterozygous genotypes, but these factors do not apply to a selfing race, because odd ploidy is unlikely if plants are fertile (self or otherwise), and plants in a selfing race will be highly homozygous, allowing adapted genotypes to effectively persist through meiosis. A plausible explanation for the putative evolution of apomixis within a selfing lineage of *Trithuria* is the breakdown of pollination as a result of a shift to more stable aquatic habitats where flowers are less often exposed (anthers of *T. submersa* only dehisce when exposed to desiccation; Taylor *et al*. 2010; Taylor 2011).

The stigmatic hairs of *Trithuria inconspicua* plants from southern New Zealand lakes are much shorter (<0.2 mm) than those in other Hydatellaceae, including *T. filamentosa* (~3.0 mm long) and northern populations of *T. inconspicua* (~1.0 mm long). The stigmatic hairs of Hydatellaceae are described as lacking a stylodium with specialised transmitting tissue and are homologous with stigmatic papillae, but functioning as an independent style and stigma, with pollen-tube growth within the cell walls of the unusually long stigmatic hair (Prychid *et al*. 2011). The disappearance of a recognisable hair to act as a conduit for male gametes appears to be correlated with loss of male function, less frequent emergence in more stable lake habitats, and, presumably, a greater reliance on apomixis.

## Taxonomy of *T. inconspicua*

*Trithuria inconspicua* is very similar morphologically to *T. filamentosa*, but, in the latter, the leaf-sheaths are more obviously dilated, the stigmatic hairs are longer, and there is a higher frequency of both female and male reproductive units. We maintain the current recognition of *T. inconspicua* and *T. filamentosa* at the rank of species.

North and South Island *T. inconspicua* populations form two distinct genetic clusters that are likely to represent diverged lineages. They also present some previously overlooked, subtle, but reliable morphological differences. We propose the recognition of these allopatric groups at subspecies rank and formally publish the name *T. inconspicua* subsp. *brevistyla* K.A.Ford below for the taxon from the South Island. Our choice to recognise the taxa at subspecies rather than species rank reflects their closer relationship to each other than to *T. filamentosa*, their allopatry, and their predominantly selfing or asexual reproduction. However, given these factors, choice of rank is inevitably somewhat arbitrary.

**Trithuria** *Hook.f.,* Fl. Tasman. *2(6): 78, 79; 2(7): pl. 138A (1858) ['1860']*

*Type*: *Trithuria submersa* Hook.f.

**Trithuria inconspicua** *Cheeseman,* Man. New Zealand fl. *756 (1906)*

*Hydatella inconspicua* (Cheeseman) Cheeseman, *Trans. & Proc. New Zealand Inst*. 39: 434 (1907).

*Type citation*: 'NORTH ISLAND: Auckland; Sandy shores of Lake Ngatu, near Ahipara, *H. Carse* and *R. H. Matthews*!'. *Type*: Sandy shores of Lake Ngatu, Waipapakauri, between Rangaunu Harbour and the West Coast, Feb. 1902, *H.Carse s.n.* (holo: AK 2889!).

Aquatic perennial herb, tufted 10–55 mm high, from a shortly branching erect rhizome, trichomes present; copious adventitious roots. Apomictic or sexual. Plants in populations often female only, or plants co-sexual with unisexual or bisexual reproductive units. Leaf-bases weakly dilated (not sheathing), hyaline, toothed auricles present or absent; leaves spreading, glabrous, 8.0–55 × 0.25–0.6 mm; lamina linear-filiform, adaxially faintly compressed below, terete above, apex rounded with a hydathode. Reproductive units (3.5–)4–5(–7) mm long, on glabrous scapes; involucral bracts 2–4(–7), sometimes dimorphic. Stamens 1–8, anthers 0.8–1.4 mm long, filaments 1–5 mm long (only subsp. *inconspicua*); pollination syndrome anemophilous or gravitational autogamy. Carpels (2–)8–25, stipitate, white–reddish, with multicellular, uniseriate stigmatic hairs of unequal length or reduced to a knobbly capitate head. Fruits 0.39–0.56 × 0.2–0.5 mm, beaked, deciduous from persistent stalks, pericarp thin and membraneous, smooth, indehiscent. Seed faintly reticulate, yellowish-brown to reddish-brown with a darker apical cap (formed by an operculum).

### *Notes*

The holotype specimen (AK 2889) is part of Cheeseman's herbarium and is, uniquely, labelled in his hand 'Sandy shores of Lake Ngatu'. Edgar (1970, p. 81) cited a specimen at CANTY, now transferred to CHR and numbered CHR 289027!, (Lake Ngatu, 1 Jan. 1902, *H.Carse s.n.*) as a type, but the visit of Carse and Matthews to Lake Ngatu was recorded as having occurred on 10 February 1902 (Godley 1998). There is no evidence that material from the January Carse gathering was seen by Cheeseman before he published the description of the species, no duplicate is currently held at AK in Cheeseman's herbarium, and CHR 289027 came from Carse's own herbarium, not that of Cheeseman. Likewise, duplicate specimens from this earlier gathering cited by Edgar (1970) as isotypes (CHR 289513 and K 794393) are not considered to be original material.

***Trithuria inconspicua*** Cheeseman subsp. ***inconspicua***

Tufted, 15–55 mm high. Apomictic or sexual, plants in populations often female only, or plants co-sexual with unisexual or bisexual reproductive units. Leaves 14–55 × 0.25–0.4 mm. Reproductive units 1–4 per tuft, on scapes 20–40 × 0.3–0.4 mm, terete, glabrous; involucral bracts 2–4 (–5), ovate to narrow-ovate. Male reproductive unit bracts 3.5–5.0 mm long; stamens (1–)3–8; anthers 0.8–1.4 mm long, bright red, filaments 1–5 mm long. Bisexual reproductive unit bracts 4–5 mm long; stamens 1–5; carpels 2–10. Female reproductive unit bracts 2.5–5.0 mm long; carpels 8–24, reddish, with 5–13 stigmatic hairs of unequal length, 0.3–1.0 mm long, red becoming hyaline. Fruits 0.4–0.56 × 0.2–0.4 mm, ellipsoid to ovoid. *Chromosome* $2n$ = ~24 (de Lange *et al*. 2004: AK 253948!). (Fig. 3E–H.)

### *Phenology*

Flowering October–January.

### *Distribution*

Known only from the North Island of New Zealand, Northland, in dune lakes behind coastal dunes of the western coast between 34.9 and 36.5° latitude.

### *Habitats*

Growing in sand and silt, occasionally in peaty sediment, often part of the aquatic-turf community and sometimes among sedges in the shallows; in dune lakes to a depth of ~1 m (occasionally exposed above the water in a dry season); 20–70 m ASL.

### Conservation status

On the basis of the criteria of Townsend *et al*. (2008), *T. inconspicua* subsp. *inconspicua* is *Nationally Critical B (3/1)* because of its low total area of occupancy (≤10 ha) and the predicted decline of 50–70%. *Trithuria inconspicua* subsp. *inconspicua* has declined since 1998, with lake-wide extinction in 7 of the 13 lakes reported to have supported populations of this plant (P. D. Champion, unpubl. data). This categorisation is unchanged from de Lange *et al*. (2018).

### Representative specimens

NORTH AUCKLAND: Northland, Lake Waiparera, 3 Feb. 1988, *Aquatic Plants Section-MAF Ruakura* (CHR 463864); Lake Waiparera, 17 Jan. 1967, *R.Cooper* (AK 115174); Lake Ngatu, 18 Dec. 1989, *C.C.Tanner s.n.* (WAIK 10945); Lake Ngatu, Sweetwater near Kaitaia, *P.D.Champion 201323* (CHR 638455); Lake Kai Iwi, 14 Nov. 1991, *P.D.Champion s.n.* (WAIK 13721); Lake Waikere, 7 May 2014, *K.A.Ford s.n.* (CHR 638459); Kai Iwi Lakes, 10 Oct. 2001, *P.B.Heenan & P.J. de Lange s.n.* (CHR 546283); Pouto Peninsula, Lake Rotokawau, 18 Nov. 1991, *P.J. de Lange 1146* (AK 207127).

## ***Trithuria inconspicua*** subsp. ***brevistyla*** K.A.Ford, subsp. nov.

*Type*: New Zealand: South Island, Southland, Lake Hauroko, Mary Bay, east side, 12 Mar. 2015, *K.A.Ford KF448 & R.D. Smissen* (holo: CHR 638456; iso: AK).

Tufted, 10–40 mm high. Apomictic, plants female only. Leaves 8–37 × 0.4–0.6 mm. Reproductive units 1–5 per tuft, on scapes 1–6 × 0.3–0.4 mm, terete, glabrous; involucral bracts 2–4(–7), ovate to broad-ovate or narrow-ovate. Female reproductive unit bracts 1.6–4.0 mm long; carpels 9–25, white–pinkish, with stigmatic hairs reduced to a knobbly capitate head (<0.2 mm long). Fruits 0.39–0.56 × 0.3–0.5 mm, ovoid to globose. (Fig. 3A–D.)

### Phenology

Flowering from at least late January to February; fruiting March–May.

### Distribution

Known only from the South Island of New Zealand. Southland, Lakes Poteriteri, Hauroko, Manapouri, Te Anau and South Mavora Lake; Otago, Lake Sylvan. Reported to be declining in Lake Manapouri and absent from the previously recorded locality of Brod Bay in Lake Te Anau.

### Habitats

Shallows of lakes (rarely exposed above the water in a dry season), between 35 and 600 m ASL. Growing in sand, silt and gravel, sometimes almost completely buried in muddy silt. Often part of the aquatic-turf community, particularly with short-growing shallow water-species (Wells *et al*. 1998); in glacial lakes to a depth of ~0.3–2 m.

### Conservation status

*Trithuria inconspicua* subsp. *brevistyla* was listed by de Lange *et al*. (2018), under the tag-name '*Trithuria* aff. *inconspicua* (CHR 502359; South Island)', as *Threatened–Nationally Vulnerable* (with qualifiers data-poor, partial decline). Following Townsend *et al*. (2008), the low area of occupancy of *T. inconspicua* subsp. *brevistyla* (≤10 ha) and the projected decline of 10–50% indicates a conservation status of *Nationally Endangered A (3/1)*, which we suggest is the appropriate category. The discrepancy may reflect differences in estimation of the area of occupancy.

### Etymology

From *brevis* (*L*.) brief and *stylus* (*L*.) pencil or pen, referring to the short stigmatic hairs of the carpels.

### Notes

*Trithuria inconspicua* subsp. *brevistyla* differs from *T. inconspicua* subsp. *inconspicua* in the shortened stigmatic hairs forming a knobbly capitate head, the fruit being ovoid to globose (rather than ellipsoid to ovoid), and scapes not elongating with maturity.

### Notes

The apparent absence of *T. inconspicua sensu lato* in Lake Monowai may be due to the artificial raising of the lake level by 2.8 m in 1926 for power generation, because it is present in other nearby lakes.

Recent surveys have not relocated populations of *T. inconspicua sensu lato* previously reported from Lake Moke near Queenstown in Otago and Lake Brunner in Westland. There are no specimens to verify whether these records represent *T. inconspicua* subsp. *brevistyla*.

### Representative specimens

SOUTHLAND: Lake Poteriteri, 13 Mar. 1993, *J.S.Clayton s.n.* (CHR 502297); Lake Poteriteri, 12 May 2015, *P.D.Champion s.n*. (CHR 638449); Lake Hauroko, 8 Mar. 1993, *J.S.Clayton & P.N.Johnson 1138* (CHR 480193); Lake Hauroko, 12 May 2015, *P.D.Champion s.n*. (CHR 638450); Lake Manapouri, 11 Mar. 1993, *R.D.S.Wells s.n.* (CHR 502337); Lake Te Anau, 10 Mar. 1993, *J.S.Clayton s.n.* (CHR 502317); Lake Te Anau N. of Brod Bay, 10 Mar. 1993, *J.S. Clayton 1145* (CHR 480200); South Mavora Lake, 13 Mar. 1993, *M.D.de Winton s.n*. (CHR 502395); South Mavora Lake, *K.A.Ford KF457 & R.D.Smissen* (CHR 638458). OTAGO: Lake Sylvan, near Glenorchy, *K.A.Ford KF456 & R.D.Smissen* (CHR 638457).

## Conflicts of interest

The authors declare that they have no conflicts of interest.

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