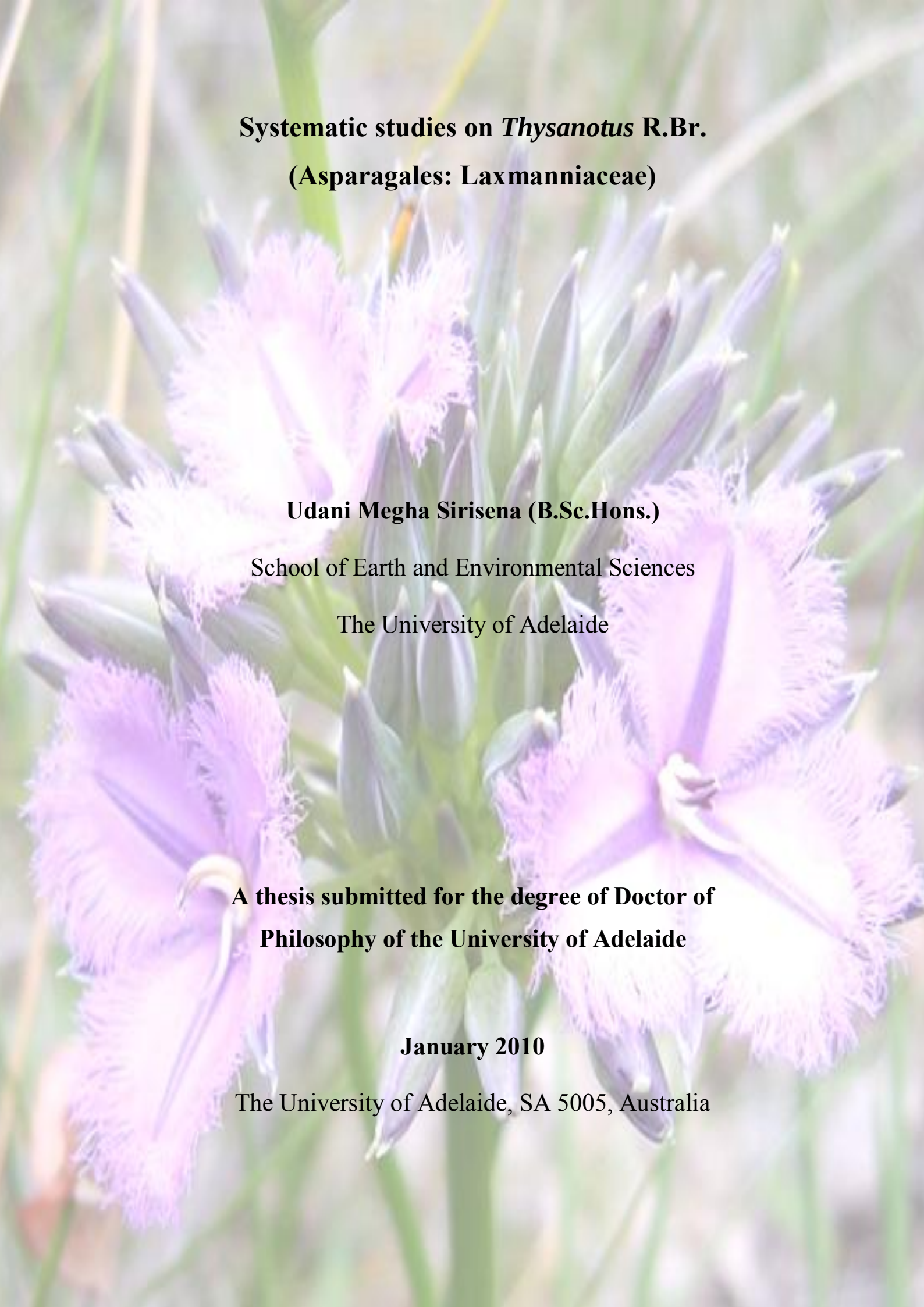

Systematic studies on *Thysanotus* R.Br. (Asparagales: Laxmanniaceae)

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General introduction and literature review: Systematic studies on *Thysanotus* R.Br.

Systematic studies and importance of phylogeny

Systematics is the study of biological diversity and its evolutionary history. According to Judd *et al.* (2002), building up the sequence of evolutionary events that took place in an organism is the construction of phylogeny. Classification of a particular organism or identification is possible only after developing its phylogeny. *Thysanotus* R.Br. (Fringe Lilies) is a genus of native plants widely distributed throughout Australia; however, its systematic relationships are poorly understood and affinities with other related genera are unresolved. Therefore, research on the genus in a phylogenetic context is necessary for a more comprehensive understanding of its relationships and character evolution.

Historical introduction

Early history of Thysanotus R.Br.

The genus *Thysanotus* (*Thysanos* means a fringe in Greek) was erected by Robert Brown (1810) based on specimens collected during his visit to Australia as naturalist aboard the *Investigator* under the command of Captain Mathew Flinders. In 1792, the French botanist Labillardière visited Western Australia and collected specimens which he referred to the genus *Ornithogalum* L. He later published descriptions and illustrations of the specimens in his *Novae Hollandiae Plantarum Specimen* (Labillardière 1804–1806), but it was later determined that *Ornithogalum* was not congeneric with *Thysanotus*. Although Salisbury (1808) described the genus *Chlamysporum* Salisb. for a species grown in England from seed collected in New South Wales, Brown (1810) deliberately ignored/rejected that name. In the protologue to his description of *Thysanotus* Brown commented that Salisbury was well aware of his (then) unpublished manuscript name for the genus and renamed Salisbury's *C. juncifolium* Salisb. as *Thysanotus junceus* R.Br. using his own specimens. Subsequently, the name *Thysanotus* R.Br. was conserved against

Chlamysporum Salisb. in the 'International Rules for Botanical Nomenclature' for 1906.

Additions to the species list

Brittan (1960, 1972) described new Western Australian species (8 and 10 species respectively), subsequently (Brittan 1971, 1978) describing a further two species endemic to South Australia: *T. fractiflexus* Brittan from Kangaroo Island and *T. wangariensis* Brittan on the Eyre Peninsula. In his subsequent revision (Brittan 1981) and treatment for the *Flora of Australia* (Brittan 1987), he listed 45 species of *Thysanotus*. As presently understood, *Thysanotus* includes about 49 species, all of them native, and of which 47 are endemic to Australia (CHAH 2008).

Geographical Distribution

All species of the genus are found in Australia (including Tasmania). One species, *T. chinensis* Benth., extends the range to the mainland of China, Hong Kong, Thailand, the Philippines, Lesser Sunda Islands, Celebes, Aru islands and New Guinea. A second species *T. banksii* R.Br. also extends into New Guinea and the islands of Torres Strait (Fig. 1).

Ecology

Ecology of the species varies, however most of the species are associated with *Eucalyptus*-dominated ecosystems. The common habitats are sand plain vegetation, *Eucalyptus* forests and mallee vegetation, coastal and near coastal vegetation and open grassy habitats (Brittan 1981, 1987).

Taxonomy of *Thysanotus*

Classification

Although variously placed in Liliaceae (Cronquist 1981) or Anthericaceae (Marchant *et al.* 1987), Chase *et al.* (1996) proposed a new circumscription and a new family Lomandraceae/Laxmanniaceae of Asparagoid Lilies which included *Thysanotus*. The circumscription was based on previous *rbc*L data (Chase *et al.* 1995) and characteristic nucellar data (Rudall 1997).

Fig. 1. Distribution map of *Thysanotus* across Australia, New Guinea and South East Asia. Figure from AVH

To date, the Laxmanniaceae comprise 15 genera and about 180 species. The accepted genera are *Acanthocarpus* Lehm., *Arthropodium* R.Br., *Chamaexeros* Benth., *Chamaescilla* F.Muell., *Cordyline* Comm. ex R.Br., *Dichopogon* Kunth, *Eustrephus* R.Br., *Laxmannia* R.Br., *Lomandra* Labill., *Murchisonia* Brittan, *Romnalda* P.F.Stevens, *Sowerbaea* Sm., *Thysanotus* R.Br., *Trichopetalum* Lindl. and *Xerolirion* A.S.George.

Thorne (1992) placed *Thysanotus* in Laxmanniaceae within the order Asparagales, suborder Asparagineae. This classification is as follows:

Kingdom: Plantae
Class: Angiospermae
Subclass: Liliidae
Superorder : Liliinae
Order: Asparagales
Suborder: Asparagineae
Family: Laxmanniaceae
Genus: *Thysanotus* R.Br.

Most recently, however, Laxmanniaceae were submerged (along with many other families) into an expanded Asparagaceae (APG II 2003; Mabberley 2008; APG III 2009), but they still form a discrete, monophyletic lineage within Asparagaceae as the subfamily Lomandroideae (Chase *et al.* 2009). Since subfamily Lomandroideae *sensu* Chase *et al.* (2009) is only recently circumscribed and lacks any of the previously recognized infra-familial taxa of Laxmanniaceae, the latter was retained for the purpose of this study.

Morphology

External morphological characters are almost always the first type of characters to be used in the taxonomic exploration of a plant group above the species level, although some of these morphological characters are discrete whereas others are quantitative and hard to define (Stuessy 1994; Sattler and Rutishauser 1997; Judd *et al.* 2002). External morphological characters are mostly preferred due to the ease of assessment, the large amount of variation, the availability of well established descriptive terminology and access to herbarium and fossil collections (Stuessy 1994). However, care should be taken when using morphological data as there is possible occurrence of high level of within species polymorphism and influence of environmental factors (Quick 1993). Furthermore, critical evaluation and comparison of morphological data may be subjective from one researcher to another (Stuessy 1990).

Vegetative morphology of Thysanotus (Fig. 2)

Perennial herbs, sometimes rhizomatous; roots fibrous, thickened or tuberous. Leaves annual, often withering early, perennial or absent (Brittan 1987). Stems/inflorescence axes, annual or perennial. Annual stems, dichotomously to paniculately branched or simple (Brittan 1981). Perennial stems either dichotomously or monopodially branched, bearing annually produced flowers (Brittan 1981).

Reproductive morphology of Thysanotus (Fig. 2)

Flowers bisexual, actinomorphic, pedicellate, and single or in 2–50 flowered umbels; flowers or umbels single and terminal, in paniculate or cymose inflorescences, or scattered on stem. Perianth segments 6, free, usually pale

purple. Sepals linear to lanceolate, membranous edged. Petals elliptic; margins fringed. Stamens 3 or 6; anthers of two whorls, equal–subequal or unequal in length, dehiscing by terminal pores or by full length slits, introrse or latrorse. Ovary superior, 3 locular; usually 3 ovules per locule; axile placentation; style straight or curved. Fruit loculicidal capsule enclosed within persistent perianth. Seeds black, arillate. Flowers each last a day, opening in early morning and usually closing by early afternoon (Brittan 1987).

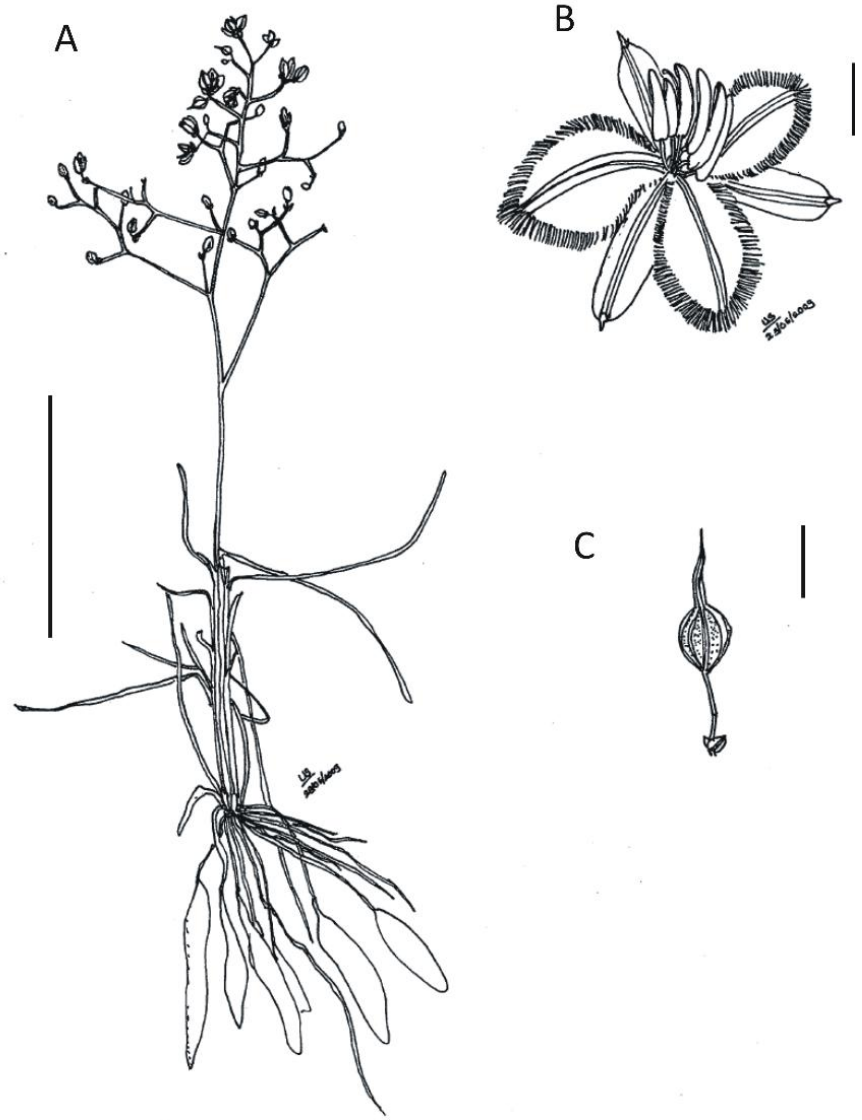

Fig. 2. Vegetative and reproductive morphology of *Thysanotus*. A. General habit of *T. exiliflorus* F. Muell. (TST 233) with tuberous roots, basal membranous bracts, terete linear leaves and a branched scape. B. Flower of *T. exiliflorus* with three oblong sepals and elliptic petals with fringed margins. C. Globose capsule with a basally articulated pedicel, ovate inflorescence bracts and a persistent perianth forming a tail extending beyond the capsule apex (Scale bars: A=7 cm; B=4 mm; C= 4 mm). Illustrations by Udani M. Sirisena

Previous morphological studies on *Thysanotus*

Bentham (1878) separated *Thysanotus* species into two series: *Triandrae* (stamens 3); and *Hexandrae* (stamens 6), depending on the number of stamens. Similarly, according to Conran (1998), there are a number of distinct forms within *Thysanotus*, with Thongpukdee (1989) also finding two clearly separated clades between the 3– and 6–staminate species.

Brittan (1970) defined four species groups in *Thysanotus*, each with distinct morphological characters (Table 1). However, these morphological groups were based only on a few morphological characters i.e. root form, lifespan of leaves and presence or absence of a separate scape. The grouping was also artificial and without any explicit evolutionary hypothesis or underlying analysis.

Stringer and Conran (1991) undertook micromorphological studies for *Arthropodium* R. Br. and *Dichopogon* Kunth., which are considered closely related to *Thysanotus*. Their study employed characters such as anther filament hairs and stamen appendages, seed cuticle and epidermal patterns, structures that have been proved useful for differentiating a number of lily species and genera from previous studies (e.g. Barthlott 1981, 1984; Ness 1989; Barthlott and Theisen 1995; Barthlott *et al.* 1998; Hassan *et al.* 2005). Therefore, we anticipate that seed micromorphology could offer a reliable source of characters for taxonomic differentiation and phylogenetic construction in *Thysanotus*.

Anatomy

Characteristics in relation to the internal structure of plants have been employed for over 150 years and are useful in both practical identification and determining phylogenetic relationships (Judd *et al.* 2002). However, the vegetative anatomy of *Thysanotus* is poorly studied and the systematic utility of anatomical characteristics is unknown.

a) Root Anatomy

Kauff *et al.* (2000) observed that Laxmanniaceae display a typical structure of monocotyledonous roots. They further observed that a dimorphic hypodermis

with rectangular, alternating long and short cells was present in almost all Asparagales, excluding a few lower asparagoids. In the cortex, a multilayered sclerenchymatous layer around the central cylinder was observed in some species of Laxmanniaceae, a feature which they regarded as a xeromorphic adaptation, but the phylogenetic utility of root anatomy is unknown for *Thysanotus*.

Root anatomy of *Thysanotus* shows variation in certain characters. Brittan (1970) sectioned roots from two species, *T. gageoides* Diels and *T. tuberosus* R.Br. Two large, centrally located lacunae with five surrounding xylem groups characterized the stele of *T. gageoides*, as well as a well-marked endodermis with radial and tangential thickenings. In contrast, *T. tuberosus* had central pith in place of lacunae and this was surrounded by five or six xylem vessels. The tuberous region of the root in *T. tuberosus* lacked the thickenings of the endodermis. Furthermore, McGee (1988) reported that in several field collections of *Thysanotus*, a fungal mantle develops on the surface of the cortex after the epidermis had been sloughed, but this feature is still to be explored across the genus.

b) Stem anatomy

According to Brittan (1970), the stem anatomy of *Thysanotus* was in general uniform; outside there was a ring of smaller bundles, closely associated with a ring of collenchyma or sclerenchyma and enclosed within a ring of endodermal cells. Another series of small bundles in an irregular circle occur towards the centre with larger bundles located at the centre. In addition, Rudall (1995) reported that secondary thickening meristems are present in *Thysanotus*, but her study examined only *T. spiniger* Brittan.

c) Epidermis of Leaf and Stem

The length-width ratio and the cell wall thickness of epidermal cells from leaves and stems of *Thysanotus* varies between species (Brittan 1970). However, those findings were only based on four species: *T. sabulosus* Brittan, *T. gageoides*, *T. chinensis* Benth. and *T. arbuscula* Baker.

Brittan (1970) also found that plants from arid environments had thicker cuticles, which he considered to be a xeromorphic adaptation. In addition, trichomes and hairs on both stems and leaves of *Thysanotus* varied in length and density, with variation within species. Therefore, he proposed that further work is necessary to determine whether these variations correlate with their habitats, or represent taxonomic distinctions.

Stomata in *Thysanotus* are anomocytic (Brittan 1970) and xeromorphic adaptations such as sunken stomata as well as variations in the symmetry of guard cells have been observed.

d) Calcium Oxalate Crystals

According to Prychid and Rudall (1999), calcium oxalate crystals are widespread in flowering plants with the three main types of crystals being raphides, styloids and druses. They reported the presence of raphides in family Laxmanniaceae and the distribution of these crystals within plants is known to be of systematic importance (Prychid and Rudall 1999).

Brittan (1970) observed that raphides in *Thysanotus* were present in canals, mostly in the chlorenchyma of stems and leaves. He observed inter-plant as well as intra-plant variation in raphide size. However, further work is necessary for clarification of the ecological, physiological and phylogenetic significance of these observations, as well as the distribution of the features across the genus.

Embryology

Embryological investigations are important in phylogenetics and the significance of certain ovular characters (e.g. number of integuments and nucellus type) has long been noted in higher-level systematics (Rudall and Cutler 1995; Rudall 1997; Conran 1999).

In monocotyledons, the plesiomorphic nucellar type is crassinucellate with the megasporophyte separated from the epidermis by one or more parietal layers, with this arrangement seen in *T. manglesianus* Kunth (Rudall 1997). A markedly enlarged proximal dermal layer, usually associated with large embryo sac nuclei (especially the antipodals) also occurs in Laxmanniaceae,

including *Thysanotus* (Chase *et al.* 1996) and *Thysanotus* displays successive microsporogenesis (Chase *et al.* 1996; Conran 1998).

Palynology

Palynology has been little studied for *Thysanotus,* but is of considerable importance in systematic studies generally. Furness and Rudall (2003) observed the presence of operculate pollen in Laxmanniaceae, with some zonasulcate and spiraperturate taxa. However, pollen of *Thysanotus* was not examined in that study. A ring-like aperture encircling the pollen grain, the so-called zona-aperturate condition was seen in Laxmanniaceae (as Lomandraceae) by Hesse and Zetter (2005), but tetrad types and aperture orientation are still unknown.

Karyology

Chromosome number is a useful systematic character and in *Thysanotus* the base chromosome number for diploid cells is 22 (Brittan 1962, 1981, 1987; Conran 1998).

Molecular level studies on *Thysanotus*

Molecular phylogeny of *Thysanotus* is poorly understood, with only a few studies including *Thysanotus* conducted, primarily to understand relationships at a higher level i.e. monocot and family level relationships (Chase *et al.* 1995; Chase *et al.* 1996; Fay *et al.* 2000). A gene tree of monocot classification was produced by Chase *et al.* (1995) using *rbc*L sequence data, including one for *Thysanotus*. The Asparagales tree showed that Anthericaceae were strongly polyphyletic, with *Thysanotus* grouped together with *Arthropodium*, *Eustrephus*, *Sowerbaea, Lomandra*, *Cordyline* and *Chamaescilla*; previously members of several polyphyletic families. In a subsequent study, Fay *et al.* (2000), found that Laxmanniaceae/Lomandraceae were again clarified as a definite group. The gene regions used in these studies were *atp*B, the *trn*L intron, *trn*L–F intergenic spacer, *rbc*L and *atp*A, but there were no obvious morphological synapomorphies to support the clade.

Present systematic status of *Thysanotus* and Laxmanniaceae

Continued work on Asparagales failed to clarify relationships within the order (Pires *et al.* 2006). As attempts are being made to recover higher-level relationships of the monocots, the demand for comprehensive studies on the lower level (generic and species level) increases. The genus *Thysanotus* is a relatively unexplored genus, with a lack of complete and detailed phylogenetic studies. To date, no detailed systematic study molecular or otherwise has been undertaken to determine species boundaries and/or phylogenetic relationships of *Thysanotus*. The present study therefore aims to do a detailed systematic study, based on both morphological and molecular data.

To understand the phylogenetic relationships of *Thysanotus*, construction of phylogenies using traditional and molecular data are proposed. Morphological (including seed micromorphology) and anatomical data will be used for phylogenetic reconstruction using traditional data. In addition, a molecular phylogeny will be constructed using *trn*L intron and *trn*L–F intergenic spacer and ITS2 nuclear DNA sequences.

Rudall and Chase (1996) and Conran (1998) recognised two informal groups within Lomandraceae/Laxmanniaceae: the *Lomandra* and the arthropodioid groups. The *Lomandra* group includes *Acanthocarpus*, *Chamaexeros*, *Lomandra*, *Romnalda* and *Xerolirion*, whereas the arthropodioid group contains *Arthropodium, Chamaescilla*, *Cordyline*, *Eustrephus*, *Laxmannia, Murchisonia, Sowerbaea, Thysanotus* and *Trichopetalum*. These groups were subsequently recognised as the Lomandroideae and Laxmannioideae by Thorne and Reveal (2007). The Lomandroideae are clearly morphologically recognisable, with generally distichous phyllotaxy, xeromorphic anatomy, and a lack of phytomelan in the seeds, tuberous storage roots or mucilage. In contrast, the Laxmannioideae are difficult to define clearly, with no obvious gross morphological synapomorphies (Conran 1998). In addition, the literature on the Laxmannioideae indicates a low reliability for vegetative or floral features to describe taxonomic relationships (Chase *et al.* 1995; 1996; Rudall and Chase 1996; Conran 1998).

Accordingly, this study proposes to use phylogenetic analyses of the *trn*L intron and *trn*L–*trn*F sequences and morphological data to reassess the

generic relationships of Laxmanniaceae and the status, if any, of the two generic groups. Simultaneously, the study will address phylogenetic relationships within *Thysanotus,* as well as any morphological synapomorphies to define the species groups within the genus and their patterns of character evolution.

Gene regions and molecular techniques

Chloroplast DNA

Many phylogenetic studies in plants have used chloroplast DNA (Olmstead and Palmer 1994), as the genome varies little in size, structure and gene content among angiosperms. It offers distinct advantages in phylogenetic studies at species level and above: first, the larger size and greater number of protein-coding provide much larger databases for restriction site studies and sequence comparisons; second, it displays relatively slow rate of silent substitution; third, structural rearrangements are relatively common, with many inversions and deletions (Olmstead and Palmer 1994). Chase (1995) used *rbc*L data to obtain the gene tree for monocotyledons and Fay *et al.* (2000) subsequently used four plastid DNA regions (including *rbc*L) to elucidate Asparagales phylogeny.

In finding relationships among closely related genera, other cpDNA genes have also been useful (Judd *et al.* 2002). Such regions include: the gene encoding subunit F of NADP dehydrogenase (*ndh*F); those that encode the α and β subunits of RNA polymerase II (*rpo*A and *rpo*C2); and a maturase gene in the intron that separates the coding region of *trn*K (*mat*K).

Gielly and Taberlet (1994) found that analysis of non-coding regions such as the *trn*L intron and *trn*L–F intergenic spacer regions (Fig.3) could further extend the utility of cpDNA at lower taxonomic levels. These regions are also relatively small, with the *trn*L intron ranging from 350–600 bp and the *trn*L–F intergenic spacer ranging from roughly 120–350 bp. This combined with the presence of universal primer regions makes them easy to amplify (Taberlet *et al.* 1991; Gielly and Taberlet 1994) and they also appear to be evolving faster than the *rbc*L gene (Soltis and Soltis 1998).

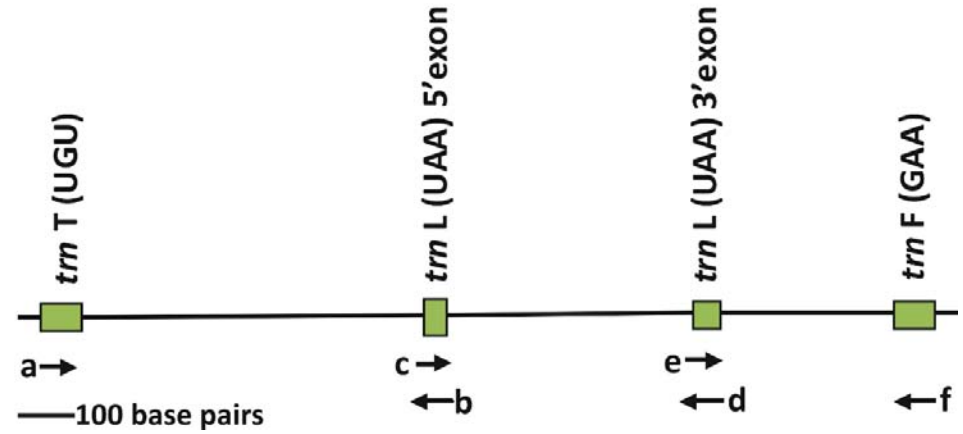

Fig. 3. *trn*L intron and *trn*L–F intergenic spacer regions of cpDNA. *trn*L intron ranging from 350–600 bp and the *trn*L–F intergenic spacer ranging from roughly 120–350 bp. Figure modified from Taberlet *et al.* (1991).

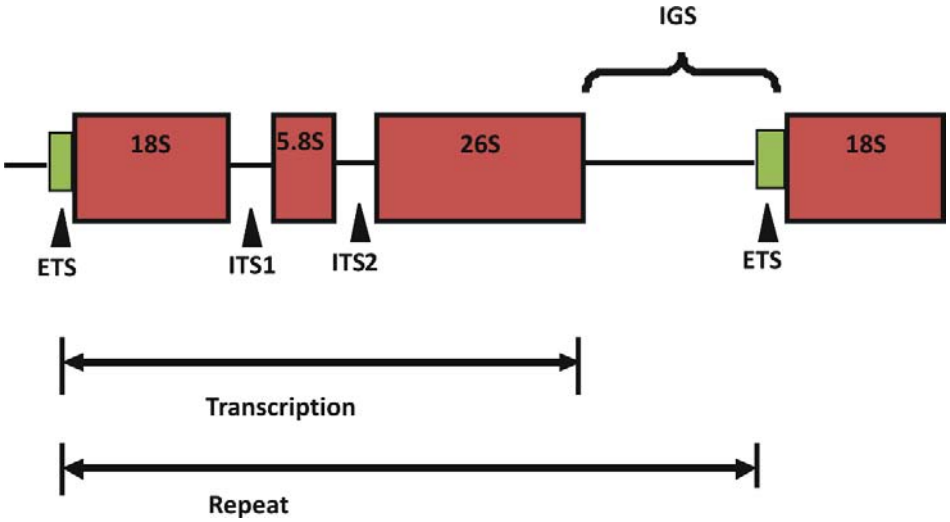

Fig. 4. The total length of the ITS regions plus the intervening 5.8S gene which is fairly short and relatively uniform (600–700 bp). Figure modified from Soltis and Soltis (1998).

Nuclear Sequences

Despite the larger size of the nuclear genome and the large number and diversity of genes that it includes, most attempts to infer phylogeny with nuclear gene sequences have involved the nuclear ribosomal DNA cistron (rDNA) (Soltis and Soltis 1998).

ITS (Internal Transcribed Spacer) (Fig.4)

Highly conserved coding regions (18S, 26S rDNA) are useful at the family level and above, whereas rapidly evolving regions such as ITS have become a

major focus of comparative sequencing at the specific and generic levels in angiosperms (Crawford *et al.* 2001; Pepper and Norwood 2001; Zomlefer *et al.* 2001). They appear to play a role in the maturation of nuclear rRNAs, bringing the large and small subunits into close proximity within a processing domain (Soltis and Soltis 1998). The ITS regions are easily amplified and in most angiosperms, due in part to their small size and the fact that the PCR primers use highly conserved flanking sequences from the adjacent rRNA genes (Soltis and Soltis 1998). The total length of the ITS regions plus the intervening 5.8S gene is fairly short and relatively uniform (600–700 bp) across angiosperms. Baldwin *et al.*(1995) described the characteristics and the possible utility of ITS region in constructing a phylogeny, whereas Álvarez and Wendel (2003) detailed the processes and possible pitfalls of its use.

Computer algorithms

The present study will use the PAUP* (Phylogenetic Analysis Using Parsimony) program (Swofford 2002), with the data sets edited using MacClade (Maddison and Maddison 2000) and molecular sequences aligned using Clustal X version 2.0 (Larkin *et al.* 2007). The program WinClada version 1.00.08 (Nixon 2002) will be used to map morphological character states onto resulting trees to explore character evolution.(Soltis and Soltis 1998)

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Generic relationships within Laxmanniaceae inferred from non-coding chloroplast DNA and morphology

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Statement of contribution

Ms. Udani M. Sirisena carried out the experimental work, data analysis and preparation of the manuscript. Dr. John Conran collected specimens used in this paper (unless otherwise stated) and helped in data analysis and manuscript preparation. Dr. Terry Macfarlane provided useful discussions in manuscript preparation.

Udani M. Sirisena **(**January 2010)

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Generic relationships within Laxmanniaceae inferred from non-coding chloroplast DNA and morphology

Abstract. A phylogenetic analysis using *trn*L intron and *trn*L–F intergenic spacer sequence data and morphological data was carried out in order to reassess the generic relationships of Laxmanniaceae and to properly redefine the two putative informal groups. Also, the phylogenetic position of *Thysanotus* R.Br. within Laxmanniaceae is established and morphological synapomorphies to define the family and putative groups are detected.

Our molecular and combined data strongly support the monophyly of Laxmanniaceae. Tentatively placed Laxmanniaceae taxa such as *Murchisonia* Brittan and *Trichopetalum* Lindl. were nested inside the family with strong bootstrap support. In all analyses, three groups: the Lomandroideae and the arthropodioid Laxmannioideae were recovered as separate clades and *Laxmannia* R.Br. and *Sowerbaea* Sm. (Laxmannioideae *s. str.*) were resolved as a separate clade from the arthropodioids, sister to the Lomandroideae in both analyses. The Lomandroideae were well supported and clearly defined by characters including: the absence of tubers and phytomelanic seeds and the presence of long-lived flowers with few ovules. Laxmannioideae *s. str.* possess bracteate, pedunculate flower heads and ligulate leaf bases. In contrast, the only synapomorphy to define the arthropodioids was multi-ovulate locules, but this character also occurs in at least some species of both *Laxmannia* and *Sowerbaea*.

Introduction

A proper understanding of phylogenetic relationships within any monocot group is essential for the establishment of a natural classification. *Thysanotus* is a diverse Australian monocot genus of which the phylogenetic relationships are poorly understood and family circumscription has been subjected to a number of changes.

Although variously placed in Liliaceae (Cronquist 1981) or Anthericaceae (Marchant *et al.* 1987), *Thysanotus* along with *Arthropodium* R.Br., *Eustrephus* R.Br., *Sowerbaea, Lomandra* Labill., *Cordyline* Comm. ex

R.Br. and *Chamaescilla* F.Muell. formed a single monophyletic clade in the first gene tree offered by Chase *et al.* (1995) for monocots. The tree also reflected that the old circumscription of Anthericaceae is strongly polyphyletic and Fay *et al.* (2000) also supported the monophyly Laxmanniaceae.

The woody genus *Cordyline* is traditionally associated with Agavaceae, or with the arborescent members of Dracaenaceae/Convallariaceae (Simpson 2000), but the genus was placed as sister to *Chamaescilla* within the Laxmanniaceae (=Lomandraceae) based on *rbc*L data; possibly representing a separate subfamily from the remainder (Chase *et al.* 1996). Based on climbing habit and similarities in leaf morphology, Brummitt (1992) placed *Eustrephus* in an expanded Philesiaceae; it had been placed previously in Luzuriagaceae by Dahlgren *et al.* (1985). Nevertheless, those families represent unnatural and heterogeneous assemblages (Chase *et al.* 1996).

Brummitt (1992) listed *Acanthocarpu*s Lehm., *Baxteria* R.Br. ex Hook., *Chamaexeros* Benth., *Lomandra*, *Romnalda* P.F.Stevens and *Xerolirion* A.S.George within the Xanthorrhoeaceae, following Cronquist (1981) and Bedford *et al.* (1986) in the *Flora of Australia*. Chase *et al.* (1996) suggested that *Baxteria* should be excluded from Lomandraceae due to its possession of cell wall ferrulates and silica, relating it to the Dasypogonaceae in the Commelinoid monocot clade rather than the Asparagoid clade and they proposed a new circumscription and new family of Asparagoid lilies. The circumscription was based on previous *rbc*L data (Chase *et al.* 1995) and characteristic nucellar data and the newly circumscribed families were Anthericaceae and Lomandraceae. The Lomandraceae included the genera: *Acanthocarpus*, *Chamaexeros*, *Lomandra*, *Romnalda*, *Xerolirion*, *Arthropodium, Chamaescilla*, *Cordyline*, *Eustrephus*, *Laxmannia, Sowerbaea* and *Thysanotus*. *Murchisonia* and *Trichopetalum* (of which *rbc*L and ovule structures are unknown) may probably belong to newly circumscribed Lomandraceae, thus placement of these two genera within the family was tentative (Chase *et al.* 1996). However, the inclusion of *Laxmannia* within the family made Laxmanniaceae the earliest valid name for the clade (Lotsy 1911).

The Laxmanniaceae are tropical to temperate and a remarkably large number of species (ca. 180) is from Australasia, SE Asia, the Mascarenes, New Caledonia, New Guinea, and New Zealand, South and North America and the

Pacific Islands (Conran 1998). Nevertheless, the relationships of most genera within Laxmanniaceae are still uncertain and require further work (Chase *et al.* 1995; Rudall and Chase 1996; Conran 1998; Fay *et al.* 2000). Although relationships within the *Lomandra* generic group have been investigated recently (Donnon 2009), the overall structure of the family, generic relationships within it and the morphological patterns reflected in these lineages have not been studied, hence this study.

Vegetative morphology

The plants are generally rhizomatous or tufted-caespitose, with single or branched aerial stems, however, in *Cordyline*, the plants are tall woody shrubs or small trees (Conran 1998). The roots are fleshy, tuberous, or fibrous to wiry, and stilt roots occur in most *Laxmannia* species (Pate *et al.* 1984).

Alternate leaves are present in all Laxmanniaceae but in many *Lomandra* species, leaves appear distichous or spirodistichous (Conran 1998). Keighery (1984) included *Laxmannia* and *Sowerbaea* in a tribe Sowerbeae within the Anthericaceae. Subsequently, Thongpukdee (1989) described the presence of short ligule like projections above the leaf sheath of the two genera.

Vegetative anatomy

Kauff *et al.* (2000) observed that Laxmanniaceae species possess typical monocotyledonous roots. Secondary thickened stem meristems were reported from at least some species of *Lomandra*, *Acanthocarpus*, *Cordyline* (Waterhouse 1987 [1986]), *Thysanotus* (Rudall and Cutler 1995), and have also been observed in some *Arthropodium* species (Conran unpubl. obs.). In the leaves, characteristic sclerenchyma girders of inner bundle sheath cells and enlarged parenchymatous outer bundle sheath cells which extend to the epidermis on both surfaces are reported from the Lomandroideae (Rudall and Chase 1996) and *Cordyline* (Conran 1998). Raphides occur in all genera (Stevens 1978; Prychid and Rudall 1999). Brittan (1970) discovered that the raphides in *Thysanotus* were present inside canals (i.e. the raphides are enclosed in sheaths), mostly in the chlorenchyma of the stems and leaves, observing inter- as well as intra-plant variation in raphide size.

Inflorescence and floral morphology

The majority of Laxmanniaceae have paniculate, contracted racemose or cymose inflorescences (Conran 1998). *Thysanotus* and *Murchisonia* have umbellate inflorescences (Brittan 1981), while Keighery (1984) observed that *Laxmannia* and *Sowerbaea* both possess contracted umbel-like inflorescences covered by imbricate bracts, at least in bud.

The two tepal whorls are either similar, or those of the inner whorl are broader, These inner tepals also bear prominently fimbriate margins in *Eustrephus*, *Thysanotus* and *Trichopetalum* (Conran 1998). *Arthropodium* also has hairy or papillate anther appendages which are sometimes fused to the filaments (Stringer and Conran 1991). This character, which is similar to the condition reported from *Tricoryne* and *Stypandra* (Hemerocallidaceae) is usually thought be associated with buzz pollination (Buchmann 1983) and therefore could be convergent, even within Laxmanniaceae (Chase *et al.* 1996).

Embryology

Anther wall formation in Laxmanniaceae is of the monocotyledonous type with successive microsporogenesis. The binucleate tapetum shows glandular secretory degeneration (Conran 1998).

The enlarged dermal region represents the most consistent apomorphy for the Laxmanniaceae, however embryo characters for *Cordyline* and *Chamaescilla* differ slightly from the other examined genera, although the dermal cells are still enlarged (Chase *et al.* 1996; Rudall and Chase 1996; Rudall 1997).

Karyology

Within the family there are taxa with chromosome numbers based on n=4 (*Sowerbaea* and *Laxmannia*); 6 (*Chamaescilla*); 7 (*Lomandra* and *Chamaexeros*); 8 (*Lomandra*, *Laxmannia* and *Acanthocarpus*); 10 (*Eustrephus*); 11 (*Chamaescilla*, *Arthropodium*, *Murchisonia* and *Thysanotus*) and 19 (*Cordyline*). Within many of these genera, there are also diploid, and tetraploid, hexaploid and octoploid species, as well as taxa with infraspecific polyploidy (Bedford *et al.* 1986; Briggs 1986; Brittan 1987; Conran 1998).

Fruit and seed

Fruits in most of the taxa are capsular with few taxa having berries, with few to numerous seeds (Conran 1998). The seeds of arthropodioid genera are black in colour with some genera possessing arils while the Lomandroideae of genera have brown to cream coloured (Rudall and Chase 1996; Conran 1998; Sirisena 2010). The endosperm is thick walled and pitted, storing aleurone, hemicelluloses and fats and the embryo is usually vertical, centrally positioned and straight with a micropyle basally positioned in the seed (Rudall and Chase 1996; Conran 1998).

Affinities

Thorne (1992) separated the Laxmanniaceae into two subfamilies: Lomandroideae and Laxmannioideae, and then Rudall and Chase (1996) recognised two informal groups within Laxmanniaceae (as Lomandraceae): the Lomandroideae and the arthropodioids. These were subsequently formalised as the subfamilies Lomandroideae and Laxmannioideae by Thorne and Reveal (2007). Nevertheless, the arthropodoioids are a separate clade from the Lomandroideae and the Laxmannioideae therefore need redefining. The Lomandroideae contained *Acanthocarpus*, *Chamaexeros*, *Lomandra*, *Romnalda* and *Xerolirion* while Laxmannioideae represented *Arthropodium, Chamaescilla*, *Cordyline*, *Eustrephus*, *Laxmannia, Murchisonia, Sowerbaea, Thysanotus* and *Trichopetalum*.

The Lomandroideae are morphologically distinct and readily recognisable, with generally distichous phyllotaxy, xeromorphic anatomy, and a lack of phytomelan in the seeds, storage roots or mucilage. In contrast, the Laxmannioideae are morphologically difficult to define clearly and are without obvious synapomorphies (Conran 1998). Furthermore, literature (Chase *et al.* 1996; Rudall and Chase 1996; Conran 1998) suggests that morphological data alone are unreliable in describing relationships within this unusual aggregation of Laxmanniaceae genera; hence molecular and combined molecular and morphological analyses seem appropriate to understand generic relationships.

The present study uses phylogenetic analysis using *trn*L intron and *trn*L–F intergenic spacer sequence data and combined molecular and morphological data to reassess the generic relationships of Laxmanniaceae and the monophyly

and circumscription of the subfamilies. As part of this, the study aims to determine the phylogenetic position of *Thysanotus* within Laxmanniaceae, as well as to identify any morphological synapomorphies for any resulting clades within the family.

Materials and methods

The taxa selected for this study included representatives of all currently recognised genera of Laxmanniaceae (Chase *et al.* 1996) with *Asparagus officinalis* L. used as the out group taxon (Table 1). For DNA extractions, live specimens were collected into silica gel.

Table 1. List of specimens used in the study and their voucher information

Species	Country of origin	Voucher source and specimen location
Acanthocarpus preissii Lehm.	Australia	*JGC 1948* (ADU)
Arthropodium milleflorum (DC.) J.F.Macbr.	Australia	Kuranga Nursery Victoria (ADU)
Chamaexeros fimbriata (F.Muell.) Benth.	Australia	*JGC 1156* (ADU)
Chamaescilla corymbosa var. *latifolia* (F.Muell.) R.J.F.Hend.	Australia	*JGC 1139* (ADU)
Cordyline rubra Otto & A.Dietr.	Australia	*JGC 1478* (ADU)
Eustrephus latifolius R.Br. ex. Ker.Gawl.	Australia	Adelaide Botanic Gardens *880587*
Laxmannia squarrosa Lindl.	Australia	*JGC 1062* (ADU)
Lomandra hystrix (R.Br.) L.R.Fraser & Vickery	Australia	Bunnings Nursery, Adelaide (ADU)
Murchisonia volubilis Brittan	Australia	*JGC 1186* (ADU)
Romnalda grallata R.J.F.Hend.	Australia	Bunnings Nursery, Adelaide (ADU)
Sowerbaea laxiflora Lindl.	Australia	*JGC 1235* (ADU)
Thysanotus fastigiatus Brittan	Australia	*JGC 1432* (ADU)
Trichopetalum plumosum J.F.Macbr.	South America (as seeds from Italy)	Orto Botanico, Facoltà di Scienze MM.FF.NN., Università degli Studi di Napoli Federico II (ADU)
Asparagus officinalis L.	Australia	*US 46* (ADU)

A total of 17 morphological characters were also scored for the 15 ingroup taxa and the outgroup using data from the *Flora of Australia* (George 1986, 1987) and Conran (1998). The characters and their respective states are presented in Table 2, with the matrix of the morphological characters given in Table 3.

DNA extraction

Total DNA was extracted from fresh, silica gel dried and herbarium material (leaves and stems) following the standard protocol for the DNeasy® Plant mini kit (Quiagen: Germantown, Maryland).

PCR and sequencing

PCR reactions were carried out using Failsafe PCR Premix Selection Kit® (Epicentre Biotechnologies: Madison, Wisconsin) following the standard protocol given by the manufacturers. Failsafe enzyme mix and Premix „E" provided by the manufacturers were used in these reactions. PCR reactions were carried out using 25 µl reactions (Table 4).

Table 2. List of morphological characters with respective states.

1. Habit: herbs=0, shrubs=1, trees=2
2. Tufted herbs: absent=0, present=1
3. Tuberous roots: absent=0, present=1
4. Rhizomes: absent=0, present=1
5. Wiry roots: absent=0, present=1
6. Perennial leaves: absent=0, present=1
7. Ligule-like structures above the leaf sheath: absent=0, present=1
8. Dioecy: absent=0, present=1
9. Umbellate inflorescences: absent=0, present=1
10. Umbel-like heads: absent=0, present=1
11. Pedicel articulation: absent=0, present=1
12. Fringed tepals: absent=0, present=1
13. Hairy/papillate anther appendages: absent=0, present=1
14. No of ovules per locule: 1=0, 2=1, numerous=2
15. Seed colour: black=0, not black=1
16. Arils: absent=0, present=1
17. Dermal layer in embryo: not enlarged=0, slightly enlarged=1, strongly enlarged=2

Amplification profile

The gene regions were amplified separately using the relevant primers. The amplification profile for 35 cycles was 94^{o}C for 1 min to denature the DNA, 60^{o}C to 1 min to anneal the primers, and 72^{o}C for 2 min for polymerization of the new strand. At the end of 35 cycles, temperature was held at 20^{o}C for 1 min. The universal primers used in (Taberlet *et al.* 1991) were used in all reactions (Table 5).

PCR purification

The PCR products were cleaned with QIAquick PCR purification kit following the manufacturers" protocol.

Sequencing

More or less a standard sequencing protocol was applied using a total volume of 20 µl, which contained 3 µl BigDye terminator v3.1, 5 µl of 3 pmol primer, 10 µl of water with 2 µl of template concentration adjusted depending on the quality of PCR product were used. Thermocycling included 25 cycles of 96°C for 30 secs, 50°C for 15 secs, and 60°C for 4 mins.

Table 3. Morphological data matrix

Taxa	Characters																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Acanthocarpus	0	1	0	1	1	1	0	0	0	0	1	0	0	0	1	0	2
Arthropodium	0	0	1	1	0	0&1	0	0	0	0	1	0	1	2	0	0	2
Asparagus	0	0	1	1	0	1	0	0	0	0	1	0	0	1	0	0	0
Chamaescilla	0	0	0&1	0	0	0	0	0	0	0	0	0	0	2	0	0	1
Chamaexeros	0	1	0	1	1	1	0	0	0	0	1	0	0	1	1	0	2
Cordyline	1&2	0	0	1	0	1	0	0	0	0	0	0	0	0&1&2	0	1	1
Eustrephus	1	0	1	1	0	0	0	0	0	0	1	1	0	2	0	1	2
Laxmannia	0	1	0	1	0	1	1	0	0	1	0	0	0	0&1&2	0	0	2
Lomandra	0&1	0	0	1	0	1	0	1	0	0	1	0	0	0	1	0	2
Murchisonia	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	1	?
Romnalda	0	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0	2
Sowerbaea	0	1	0&1	0	0	0	1	0	0	1	0	0	0	0&1&2	0	0	2
Thysanotus	0	0	0&1	0&1	0	0&1	0	0	1	0	1	1	0	1&2	0	1	2
Trichopetalum	0	0	1	1	0	0	0	0	0	0	1	1	1	2	0	0	?
Xerolirion	0	1	0	1	1	0	0	1	0	0	0	0	0	0	1	0	2

Table 4. Reaction volumes used in PCR reactions

Reagents	Quantities (µl)
PCR water	0.25
Premix E	12.5
Template	10
Enzyme	0.25
Primers(Forward and Reverse)	2
Total volume	25

Table 5. Forward and reverse primers used (Taberlet *et al.* 1991)

Primer	Sequence 5‟–3‟
*trn*L forward (C)	CGAAATCGGTAGACGCTACG
*trn*L reverse (D)	GGGGATAGAGGGACTTGAAC
*trn*L–F spacer (E)	GGTTCAAGTCCCTCTATCCC
*trn*L–F spacer (F)	ATTTGAACTGGTGACACGAG

Phylogenetic analyses

DNA sequences were aligned using CLUSTAL X version 2.0 (Larkin *et al.* 2007) and edited in MacClade version 4.0 (Maddison and Maddison 2000). The molecular matrix and the combined matrix were analysed using PAUP version 4.0b10 (Swofford 2002). Both data matrices were analysed with heuristic search option with tree-bisection-reconnection (TBR) branch swapping with MULTREES option in effect. 100 random stepwise addition replicates were performed. All characters were given equal weight and considered unordered. Further analyses were performed after applying successive weighting of all characters according to the retention index. After constancy was reached strict consensus and 50% majority rule consensus trees were calculated. Bootstrap analyses were carried out using 100 bootstrap replicates, and 10 random stepwise addition replicates.

Results

*trn*L and *trn*L–F intergenic spacer region sequences were obtained for all Laxmanniaceae genera and the outgroup. In the ingroup, the *trn*L intron length ranged from 530–560 bp and *trn*L–F intergenic spacer varied from 261–392 bp. Aligned combined gene regions were 1,084 bp long, with 212 variable characters of which 163 were informative.

Phylogenetic analysis based on this data set yielded 4 equally parsimonious trees of 329 steps, CI: 0.635, RI: 0.718 and RC: 0.456 and a strict consensus tree was calculated (Fig. 1).

Genera such as *Murchisonia* and *Trichopetalum* which were placed tentatively into Laxmanniaceae by Chase *et al.* (1996) were nested among the other genera of the family with strong bootstrap support (100 and 96 respectively), supporting their inclusion. Three major clades can be recognised from the strict consensus tree, all showing strong 96–100% bootstrap support. Clade 1 represents the genera of the Lomandroideae: *Acanthocarpus*, *Chamaexeros*, *Lomandra*, *Romnalda* and *Xerolirion* and this was placed as sister to *Laxmannia* and *Sowerbaea* (Clade 2: Laxmannioideae *s. str.*). Sister to Clades 1&2 was the arthropodioid Laxmannioideae (Clade 3): *Arthropodium*, *Cordyline*, *Eustrephus*, *Murchisonia, Thysanotus* and *Trichopetalum*. *Chamaescilla* was placed at the base of the family, below the Lomandroideae,

Laxmannioideae and arthropodioid clades. Within the arthropodioid clade, *Cordyline* was sister to rest of the family, below *Trichopetalum* and then two terminal pairs: *Arthropodium* & *Eustrephus* and *Thysanotus* & *Murchisonia*. In contrast, *Laxmannia* was again sister to *Sowerbaea* and more closely related to the Lomandroideae than to the arthropodioids. Within the Lomandroideae clade, *Lomandra* was paired with *Xerolirion*, sister to a *Romnalda*, *Acanthocarpus* and *Chamaexeros* clade.

Combined analysis

The combined analysis included 1,106 characters, of which 215 were variable and 180 parsimony informative. Phylogenetic analysis based on this data set yielded a single most parsimonious tree with 368 steps long, CI: 0.620, RI: 0.701 and RC: 0.434 (Fig. 2).

The topology of the combined tree is similar to the molecular tree and the three clades from the molecular tree were recovered in the combined tree; however, *Chamaescilla* now formed a clade with *Cordyline* (62% support), sister to the other arthropodioids. Apart from this, taxon positions were unchanged from the molecular analysis.

When morphological characters were plotted on the combined analysis tree (Fig. 3), no character specifically defined Laxmanniaceae. The Lomandroideae were well supported within Laxmanniaceae (100%) and clearly defined by the character: non-phytomelanic seeds (15/1). Within the lomandroids, dioecy (8/1) was a synapomorphy for the *Lomandra*/*Xerolirion* pair, but the presence of wiry roots in *Xerolirion* (5/1), absence of large, strap-like perennial leaves (6/0) and absence of pedicel articulation (11/0) clearly distinguished it from *Lomandra* in this analysis.

The arthropodioid Laxmannioideae shared the absence of tufted herbs, presence of root tubers, absence of perennial leaves (albeit mixed in *Arthropodium* and *Thysanotus*), numerous ovules per locule and black, phytomelanic seeds. Nevertheless, numerous ovules per locule was the only mapped synapomorphy for the clade (14/2).

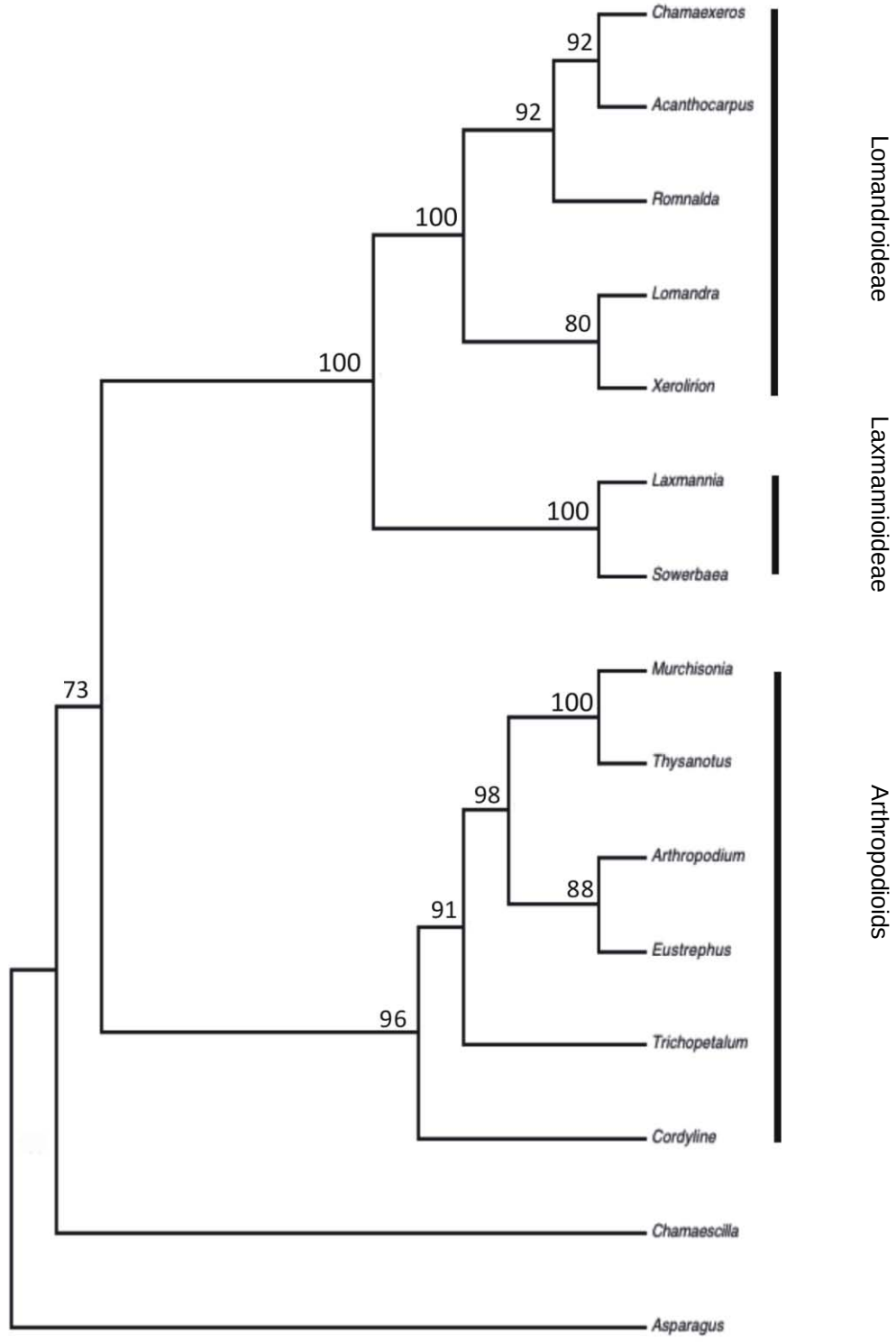

Fig. 1. Strict consensus tree of the 4 most parsimonious trees resulting from the phylogenetic analysis of combined *trn*L and *trn*L–F intergenic spacer sequence data sets (163 parsimony informative characters). 329 steps, CI: 0.635, RI: 0.718, RC: 0.456. Bootstrap values are indicated above the branches.

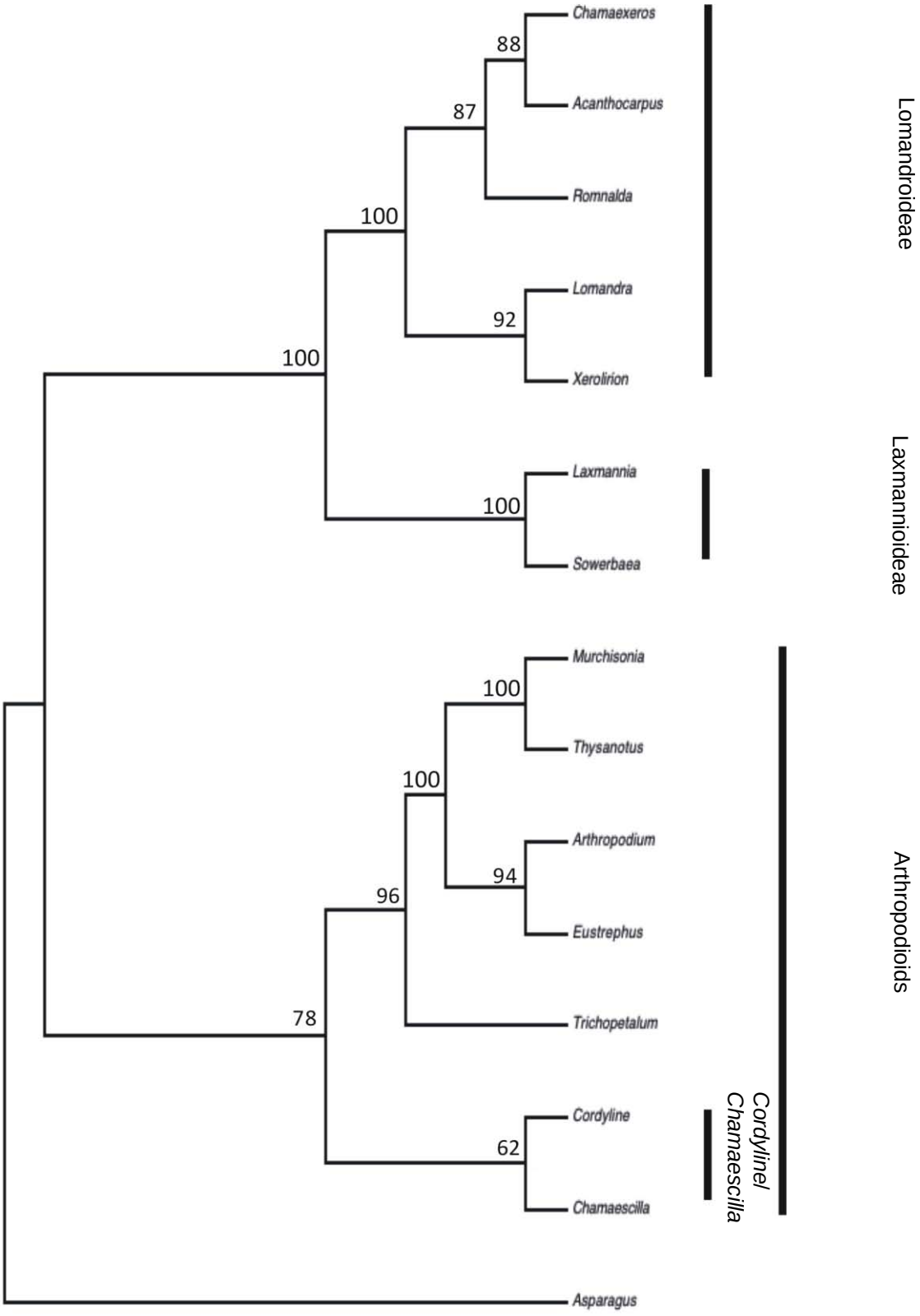

Fig. 2. Most parsimonious tree resulting from the combined morphological and molecular data analysis (180 parsimony informative characters). 368 steps, CI: 0.620, RI: 0.701, RC: 0.434. Bootstrap values are indicated above the branches.

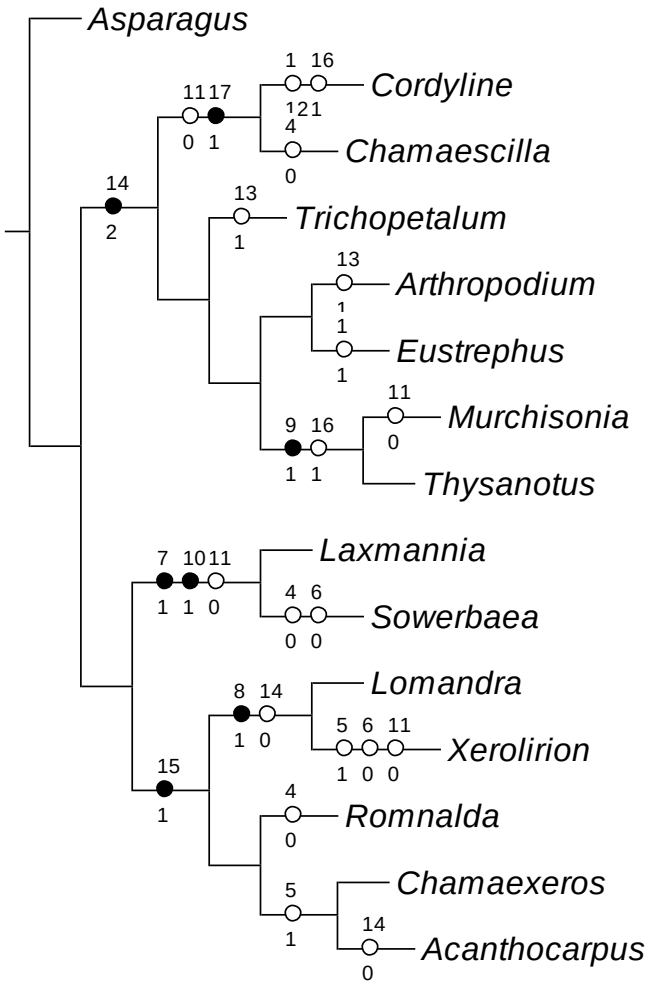

Fig. 3. Distribution of different characters and their states in Laxmanniaceae. Open circles indicate homoplasies, closed circles are synapomorphies.

Thysanotus and *Murchisonia* were sister to each other and the pair was then itself sister to *Eustrephus* and *Arthropodium*. *Thysanotus* and *Murchisonia* share umbellate inflorescences (9/1) as a synapomorphy and show similar seed features (16/1; see Chapter 5), however they are distinguished by the absence of articulated pedicels and fringed inner tepal margins in *Murchisonia* (11/0).

The *Cordyline*/*Chamaescilla* clade was defined by the absence of articulated pedicels (11/0) and the presence of slightly enlarged dermal layer in embryo (17/1), whereas the *Laxmannia*/*Sowerbaea* clade was defined by the presence of an unusual ligule-like structure above the leaf sheath (7/1) and bracteate umbel-like flowering heads (10/1).

Discussion

The *trn*L intron and *trn*L–F intergenic spacer sequence and combined analysis results were congruent with the circumscriptions for Laxmanniaceae (=Lomandraceae) by Chase *et al* (1995), Fay *et al.* (2000) and Thorne and Reveal (2007). Tentatively included genera in Laxmanniaceae such as *Murchisonia* and *Trichopetalum* (Chase *et al.* 1996) were also supported as members of the family. The absence of any clear morphological synapomorphy for Laxmanniaceae in the character mapping is unexpected. Nevertheless, the previously recognised embryological feature of a relatively enlarged proximal dermal layer (Chase *et al.* 1996) is still probably the best feature which could be used to support the molecular results.

The Lomandroideae were consistently grouped as a clade, both in this study and that of Donnon (2009), and have been shown previously to share distinctive seed and floral characteristics (Conran 1998). The genera within the Lomandroideae are considered to be closely allied and Rudall and Chase (1996) argued that Lomandraceae (if recognised as a distinct family) should at least include *Acanthocarpus*, *Chamaexeros*, *Lomandra* and *Romnalda*. Although *Acanthocarpus*, *Chamaexeros*, *Lomandra* and *Romnalda* also share similarities in leaf anatomy, such as enlarged outer bundle sheath cells and sclerenchyma girders from the inner bundle sheath, these characters occur elsewhere in Asparagales and are therefore homoplasious (Rudall and Chase 1996). Although the relationships of *Xerolirion*, a monotypic genus from south-west Western Australia have always been problematical, based on *rbc*L data it has been placed with *Lomandra* (Rudall and Chase 1996). Our molecular analysis and combined data analysis also placed *Xerolirion* within Lomandroideae, sister to *Lomandra* reflecting their closer relationship.

A close relationship between *Thysanotus*, *Murchisonia*, *Arthropodium*, *Eustrephus* and *Trichopetalum* is supported further by the presence of fringed tepals. Especially as although *Murchisonia* and *Arthropodium* are generally considered to lack fringed tepals, some *Arthropodium* species possess finely fimbriate inner tepal margins (Conran 1998) and in *Murchisonia fragrans* Brittan the inner tepals are also fringed towards the apex (Brittan 1987), indicating at least partial expression of this feature in these genera (Fig. 4), making fringed inner tepals a synapomorphy for this crown lineage.

Cordyline and *Chamaescilla* are at first glance an unlikely generic pairing, mainly because *Cordyline* consists of woody shrubs or trees with flowers borne in racemes or panicles while *Chamaescilla* consists of geophytic rosette herbs with flowers borne in scapose, paniculate cymes. However, both genera can be distinguished from other arthropodioids by the absence of articulated pedicels, fimbriate tepals or hairy anther appendages. *Chamaescilla* and *Cordyline* appear to represent a separate lineage from other arthropodioids, as suggested previously by Chase *et al.* (1996). The large, woody habit of *Cordyline* seems to be convergent, as anomalous secondary monocotyledonous growth occurs in a range of other monocot families (Waterhouse 1987 [1986]).

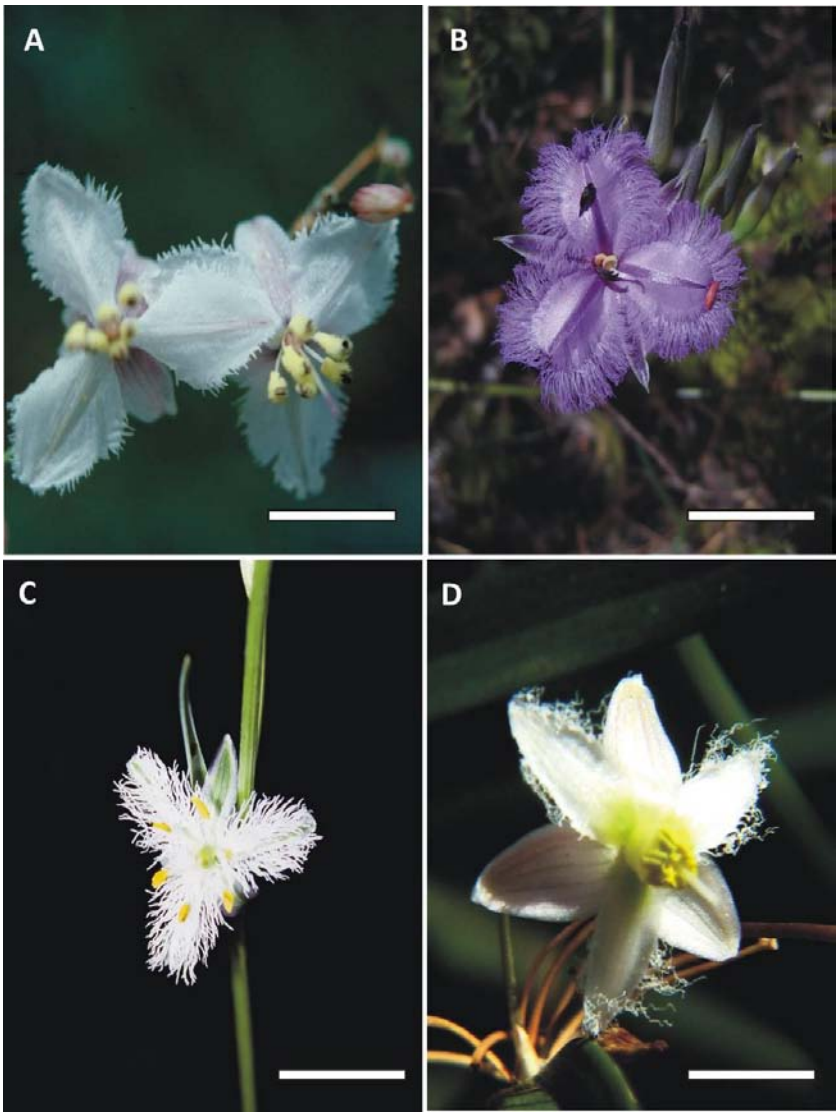

Fig. 4. Fringed tepal margins of A. *Arthropodium sp.* 'Mt Kosciusko". B. *Thysanotus multiflorus* (Western Australia). C. *Trichopetalum plumosum*. D. *Eustrephus latifolius*. (scale bars all ABC = c.15 mm; D = c.10 mm)

The relatively large number of characters shared between *Laxmannia* and *Sowerbaea* (the Laxmannioideae *s. str.* clade) led Keighery (1984) to include them in a tribe Sowerbeae within the family Anthericaceae. Our study leads us to support the grouping of these two taxa into subfamily Laxmannioideae *s. str.,* separate from both the Lomandroideae and the arthropodioids (these latter now requiring a separate name at subfamily rank). *Laxmannia* and *Sowerbaea* are more closely related to Lomandroideae than to the arthropodioids and many features are shared together such as presence of tufted herbs, perennial leaves and two ovules per locule however, none of these features seem to be apomorphic to the whole taxa clade.

Morphologically, the only supported synapomorphy for the family is the relatively enlarged dermal layer in the embryo which seems to be the only character that is common to all Laxmanniaceae so far examined (Chase *et al.* 1996). In all analyses in the present study, three clades were recovered within Laxmanniaceae: the Lomandroideae; the arthropodioids; and *Laxmannia*/*Sowerbaea* (Laxmannioideae *s. str.*), this last pair resolved as a separate clade, sister to the Lomandroideae. Our results are highly congruent with previous Lomandraceae circumscriptions and recognition of separate groups within the family (Lomandroideae, arthropodioid group and *Laxmannia*/*Sowerbaea* clade), and these would represent subfamilies within Laxmanniaceae (or tribes within Lomandroideae if a broader family concept placing them in Asparagaceae is adopted). The lack of obvious morphological synapomorphies in the Laxmanniaceae and the relatively high levels of homoplasy for morphological characters seem to represent a combination of divergence between genera growing in different habitats, as well as convergence towards particular lifestyles and life-history strategies.

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Phylogenetic relationships and major lineages within the genus *Thysanotus* R. Br. (Laxmanniaceae) determined from combined morphological data and anatomical data

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Statement of contribution

Ms. Udani M. Sirisena carried out the field collections, experimental work, data analysis and preparation of the manuscript. Dr. John Conran collected specimens and helped in data analysis and manuscript preparation. Dr. Terry Macfarlane collected specimens and helped in manuscript preparation.

Udani M. Sirisena **(**January 2010)

John G. Conran **(**January 2010)

Terry D. Macfarlane **(**January 2010)

Phylogenetic relationships and major lineages within the genus *Thysanotus* R. Br. (Laxmanniaceae) determined from combined morphological data and anatomical data

Abstract. A detailed morphological and anatomical systematic study was carried out to assess the phylogenetic relationships, taxon boundaries and subgroups/sections within *Thysanotus* using as many of the species and known variant forms as possible.

Two distinct species clades were recovered, mostly based on features such as plant habit, presence/absence of tubers and rhizomes, presence/absence of leaves at flowering and branching of the flowering stem. Characters such as absence of pendent flowers, presence of sessile arils and absence of irregular shaped epidermal cells in stem TS are the most obvious synapomorphies for *Thysanotus* and *Murchisonia* Brittan. Insights to intraspecific relationships and possible new species of *Thysanotus* are also given using combined morphological and anatomical data for species such as *T. patersonii* R.Br. and *T. juncifolius* (Salisb.) Willis & Court.

Introduction

Thysanotus R.Br. (Fringe lilies) is a widespread genus in Australia with *T. chinensis* Benth., extending the range to the mainland of China, Hong Kong, Thailand, the Philippines, Lesser Sunda Islands, Celebes, Aru islands and New Guinea. A second species *T. banksii* R.Br. also extends into New Guinea and the islands of the Torres Strait (Brittan 1981). Brittan (1981, 1987b) described 49 *Thysanotus* species with the highest diversity (42) concentrated in south-west Western Australia.

Recent molecular analyses of Asparagales (Chase *et al.* 1995; Fay *et al.* 2000) placed *Thysanotus* in a new grouping of genera (*Arthropodium* R.Br., *Chamaescilla* F. Muell., *Cordyline* Comm. ex R.Br., *Eustrephus* R.Br., *Lomandra* Labill. and *Sowerbaea* Sm.) which were previously members of several polyphyletic families. The group was described initially as Lomandraceae with several new additions (*Acanthocarpus* Lehm., *Chamaexeros* Benth., *Romnalda* P.F. Stevens, *Xerolirion* A.S. George and

Laxmannia R.Br.) and tentative placements of *Murchisonia* Brittan and *Trichopetalum* Lindl. (Chase *et al.* 1996). Subsequently, Thorne (1992) noted that the earliest family name for the group was Laxmanniaceae, later recognising two subfamilies: Laxmannioideae and Lomandroideae (Thorne and Reveal 2007). However, relationships within the Laxmanniaceae are still poorly understood and require further work.

Hutchinson (1959) considered *Thysanotus* to be close to *Trichopetalum* as both share absence of post flowering spiral twisting of the perianth and possess basifixed anthers and fimbriate inner tepal margins. Huber (1969) subsequently regarded the possession of dissimilar inner and outer series of perianth segments and campylotropous seeds as indications of a close relationship between *Thysanotus*, *Arthropodium* and *Dichopogon* Kunth. In addition, *Eustrephus* shares elongate, porate anthers, tuberous roots and fringed tepals with *Thysanotus* and, to a lesser extent, some *Arthropodium* species (Conran 1998). *Thysanotus* is generally characterised by its fimbriate inner tepals and flowers usually borne on umbels, while various vegetative features help to distinguish the other genera.

Despite research carried out to determine molecular generic relationships in Asparagales (Chase *et al.* 1995; Fay *et al.* 2000), to date there is no detailed systematic study to assess the phylogenetic relationships, taxon boundaries and subgroups/sections within *Thysanotus*. According to Conran (1998), there are a number of distinct forms within *Thysanotus*, with Thongpukdee (1989) finding two clearly separated clades representing 3- and 6-staminate species respectively. This agreed with Bentham (1878) who also separated *Thysanotus* into two series, *Triandrae* (stamens 3) and *Hexandrae* (stamens 6). Nevertheless, *Thysanotus* currently lacks formal subgenera or sections, unlike some of the other large Laxmanniaceae genera such as *Lomandra* (Lee and Macfarlane 1986)

Brittan (1970) did define four species groups within *Thysanotus* each with distinct morphological characters. However, these morphological groups were based on only a few characters (root form, lifespan of leaves and presence or absence of a separate scape) and were erected without any explicit evolutionary hypotheses**.** Therefore a phylogenetic evaluation is necessary for

an improved understanding of species relationships and the recognition of subgenera/sections as well as to understand the character evolution within the genus.

As a result of its wide distribution, *Thysanotus* occurs in many different ecological situations. In addition, some very widespread species have become isolated into small, disjunct populations, increasing the chance of developing intra-specific taxa or even into new species (Brittan 1962), but this variability requires proper systematic study. For example, widespread and/or variable species such as *T. patersonii* R.Br, *T. multiflorus* R.Br. and *T. triandrus* (Labill.) R.Br. have already been suspected to contain intra-specific taxa, but no studies have been undertaken to make a case for their separation/recognition.

Accordingly, a detailed systematic study including an explicit phylogenetic analysis is necessary for the genus and this study was undertaken to present a comprehensive phylogenetic reconstruction of *Thysanotus* based on morphological and anatomical data using as many of the known species and variant forms as possible in order to:

(1) Recognise any distinct phylogenetic lineages within the genus.
(2) Provide insights to any intra-specific categories or any new species of the genus *Thysanotus* based on the position of variant forms in the analyses.
(3) Explore character evolution of any major clade which results.

Materials and Methods

Taxon sampling

Fifty *Thysanotus* species and/or variant forms were included in the study. Where possible, live specimens were used (Table 1), but where live specimens were unavailable, herbarium specimens and literature were utilised.

A total of 148 morphological and anatomical characters were coded into discrete states (Table 2), with some characters coded as binary, while most were multistate due to intra-specific polymorphic variation.

Preparation of material

Morphological studies

Herbarium samples were reconstituted by soaking in boiling water with a drop of detergent for 10–15 minutes. Fresh flowers were preserved in 70% alcohol. Observations were made using a dissecting microscope.

Seed micromorphology

Seeds were obtained from field collections and herbarium specimens, with seeds of at least two samples examined for each species, depending on availability. The seeds were air dried on aluminium stubs with carbon tops, sputter coated with carbon and gold, viewed with a Phillips XL 20 SEM at an acceleration voltage of 10Kv and photographed. Seed morphological characters were derived from those of Barthlott (1981), Ness (1989) and Hassan *et al.* (2005).

Anatomical studies

Fresh materials of the specimens were collected, fixed in 70% FAA (5% formalin: 10% acetic acid: 50% ethanol) and stored in 70% ethanol. Herbarium material was reconstituted in boiling water with a drop of detergent. The material was sectioned using a razor blade and stained with 0.05% aqueous Toluidine Blue. The stained sections were mounted in glycerine, observed under a light microscope and photographed using a digital camera.

Outgroup

The genera *Eustrephus* and *Arthropodium* were selected as the outgroups based on the monocot gene tree of Chase *et al.* (1995). *Murchisonia volubilis* Brittan was also included as an outgroup as it had been previously named as *T. patersonii* var. *exfimbriatum* J.M.Black (1943) and was considered to be closely related to *Thysanotus*. Both share common vegetative features; however flowers of *Murchisonia* lack fringed inner perianth margins (Brittan 1987a).

Eustrephus and *Thysanotus* share fimbriate inner tepal margins and poricidal dehiscence of anthers (Conran 1998), but there are also some *Arthropodium* species with finely fimbriate inner tepal margins (Conran 1998)

and the species formerly placed into *Dichopogon* also possess poricidal anther dehiscence (Brittan 1987b).

Table 1. List of specimens observed with their voucher information

Specimen	Collector/source	Herbarium	Accession no
Arthropodium milleflorum R.Br.	Flora of Australia	NA	NA
Eustrephus latifolius R.Br. ex. Ker.Gawl.	Flora of Australia	NA	NA
Murchisonia volubilis Brittan	Flora of Australia	NA	NA
T. acerosifolius Brittan	N. H. Brittan	PERTH	1053604
T. anceps Lindl.	N. H. Brittan	PERTH	1122398
T. arbuscula Baker	Flora of Australia	NA	NA
T. arenarius Brittan 1	T. E. H. Aplin	PERTH	1978411
T. arenarius Brittan 2	T. A. Halliday	PERTH	3016099
T. asper Lindl. 1	F. Lullfitz	PERTH	1978365
T. asper Lindl. 2	C. A. Gardner	PERTH	1978381
T. banksii R.Br.	Flora of Australia	NA	NA
T. baueri R.Br. 1	T. R. N. Lothian	AD	96126149
T. baueri R.Br. 2	G. Jones	AD	97451186
T. baueri R.Br. 3	F. J. Badman	AD	99106157
T. sp. aff. *baueri*	F. J. Badman	AD	99324113
T. brachiatus Brittan	N.H. Brittan	PERTH	3001172
T. brachyantherus Brittan	A.S. George	PERTH	1041444
T. brevifolius Brittan	Flora of Australia	NA	NA
T. britanii H.R. White & T.D. Macfarlane	A. Chant	PERTH	6417728
T. chinensis Benth.	W.R. Barker	AD	9792103
T. cymosus Brittan	Flora of Australia	NA	NA
T. dichotomus (Labill.) R.Br.	C.A. Gardner	PERTH	3015513
T. exiliflorus F.Muell. 1 NT form	H. P. Vonow	AD	199674
T. exiliflorus F.Muell. 2 WA	K.F. Kenneally & D.J. Edinger	PERTH	6705286
T. exiliflorus F.Muell. 3 SA form	T. S. Te	AD	233
T. exiliflorus F.Muell. 4 WA form	N.H. Brittan	AD	98841035
T. fastigiatus Brittan	B. Nyanatusita	PERTH	6745008
T. formosus Brittan	R.J. Cranfield	PERTH	6032745
T. fractiflexus Brittan 1	G. Jackson	AD	96449063
T. fractiflexus Brittan 2	Hj. Eichler	AD	96650163
T. gageoides Diels 1	K.R. Newbey	PERTH	1041814
T. gageoides Diels 2	J.W. Wrigley	PERTH	1041916
T. glaucus Endl.	Brunonia (1981)	NA	NA
T. glaucifolius Brittan 1	N.H. Brittan	PERTH	1221477
T. glaucifolius Brittan 2	N.H. Brittan	PERTH	6265405
T. gracilis R.Br. 1	A.S. George	PERTH	1743953
T. gracilis R.Br. 2	S.P. Pfeiffer	PERTH	2638134
T. isantherus Benth.	Brunonia (1981)	NA	NA
T. juncifolius (Salisb.) Willis & Court - NSW	Dalby *et al.*	PERTH	94/09
T. juncifolius (Salisb.) Willis & Court-SA	Udani M. Sirisena & C. Trujillo	ADU	1
T. juncifolius (Salisb.) Willis & Court-SA-EP	B. Saunders	ADU	*s.n.*
T. lavanduliforus Brittan	Brunonia (1981)	NA	NA
T. manglesianus Kunth. 1	D.J.E. Whibley, P.E. Trezise	AD	97447210
T. manglesianus Kunth. 2	N.H. Brittan	AD	97447004
T. manglesianus Kunth. 3	N.H. Brittan	AD	97746471
T. manglesianus Kunth. 4	John G. Conran	ADU	2210
T. multiflorus R.Br. 41	Udani M. Sirisena and T. D. Macfarlane	DEC/PERTH	41
T. multiflorus R.Br. 41b	Udani M. Sirisena and T. D. Macfarlane	DEC/PERTH	41 b
T. multiflorus R.Br. 2	Udani M. Sirisena and T.	DEC/PERTH	8

	D. Macfarlane		
T. nudicaulis Brittan	C.R. Alcock	AD	96718043
	B.J. Lepschi & B.A. Fuhrer	PERTH	5015017
T. parviflorus Brittan			
T. patersonii R.Br. 1	John G. Conran	ADU	2119
T. patersonii R.Br. 2	Hj. Eichler	AD	96310272
T. patersonii R.Br. 3	D.E. Symon	AD	98666182
	R. Bates Gammon Ranges Survey 1993	AD	99416206
T. patersonii R.Br. 4			
T. patersonii R.Br. 5	Udani M. Sirisena	ADU	1
T. patersonii R.Br. 6	Udani M. Sirisena	ADU	4
T. patersonii R.Br. 7	John G. Conran	ADU	2255
T. pauciflorus R.Br.	N. Hoyle	PERTH	1743937
T. pseudojunceus Brittan	N.H. Brittan	PERTH	1224093
T. pyramidalis Brittan	D. & N. McFarland	PERTH	1978500
	Peter G. Wilson & R. Rowe	PERTH	6033318
T. ramulosus Brittan			
T. rectantherus Brittan 1	C.A. Gardner	PERTH	1978454
T. rectantherus Brittan 2	R.D. Royce	PERTH	3017591
T. sabulosus Brittan 1	N.H. Brittan	PERTH	4339266
T. sabulosus Brittan 2	J.S. Beard	PERTH	1053507
T. scaber Endl.	N.H. Brittan	PERTH	2979764
T. sparteus R.Br.	Brunonia (1981)	NA	NA
T. speckii Brittan	A.S. George	PERTH	2638096
T. spiniger Brittan	Brunonia 1981	NA	NA
T. tenellus Endl. 1	E.C. Black, J.M. Black	AD	96021091
T. tenellus Endl. 2	B. Copley	AD	96702146
	Udani M. Sirisena and T. D. Macfarlane	PERTH	12
T. tenellus Endl. 3			
	Udani M. Sirisena and T. D. Macfarlane	PERTH	44
T. tenellus Endl. 4			
T. tenuis Lindl.	Brunonia 1981	NA	NA
	B. Nordenstam & A. Anderberg	PERTH	1880152
T. teretifolius Brittan			
	Udani M. Sirisena and T. D. Macfarlane	PERTH	14
T. thyrsoideus Baker			
T. triandrus (Labill.) R.Br. 1	A.E. Orchard	AD	96429052
T. triandrus (Labill.) R.Br. 2	A.E. Orchard	AD	96846103
	Udani M. Sirisena and T. D. Macfarlane	PERTH	17
T. triandrus (Labill.) R.Br. 3			
	Udani M. Sirisena and T. D. Macfarlane	PERTH	6
T. triandrus (Labill.) R.Br. 4			
	Udani M. Sirisena and T. D. Macfarlane	PERTH	32
T. triandrus (Labill.) R.Br. 5			
T. tuberosus R.Br. *ssp. tuberosus*	Brunonia 1981	NA	NA
T. tuberosus ssp. parviflorus (Benth.) Brittan	Brunonia 1981	NA	NA
T. tuberosus R Br.	T. S. Te	PERTH	274
T. unicupensis Sirisena, T.Macfarlane & Conran	Udani M. Sirisena and T. D. Macfarlane	PERTH	13
T. vernalis Brittan	Brunonia 1981	NA	NA
T. virgatus Brittan	Brunonia 1981	NA	NA
T. wangariensis Brittan	C.R. Alcock	AD	96807209
T. wangariensis Brittan (WA)	A. S. George	PERTH	2980568

Table 2. List of morphological and anatomical characters together with their character states used in the study

1. *Habit of inflorescence: erect = 0, twining = 2
2. *Tuberous roots: absent = 0, present = 1
3. *Fleshy roots: absent = 0, present = 1
4. *Rhizomes: absent = 0, present = 1
5. *Stalked, distant tubers: absent = 0, present = 0

6. *Sessile tubers: absent = 0, present = 0
7. Tuber shape: NA = 0, cylindrical/bottle shaped = 1, elliptical = 2, oblong = 3, irregular = 4
8. *Leaves at flowering time: absent = 0, present = 1
9. *Leaves at fruiting: absent = 0, present = 1
10. *No of leaves: < 5 = 0, 6–10 = 1, 11–15 = 2, 16–20 = 3, >20 = 4
11. *Filiform leaves: absent = 0, present = 1
12. Leaf blade length: <10cm = 0, 10.1–20cm = 1, 20.1–30cm = 2, >30.1cm = 3
13. Leaf blade width: < 1mm = 0, 1.1mm–2mm = 1, 2.1–3mm = 2, >3.1 = 3
14. Leaf surface ridges: absent = 0, present = 1
15. *Leaf surface indumentum: absent = 0, present = 1
16. Persistent leaves: absent = 0, present = 1
17. *Leaves during plant life: absent = 0, present = 1
18. Basal wings on leaves: absent = 0, present = 1
19. Leaf sheath length: <1cm = 0, 1–3cm = 1, 3.1–5cm = 2, >5.1cm = 3
20. Leaf sheath width: <1 = 0, 1.1–3mm = 1, 3.1–5mm = 2, >5.1mm = 3
21. *Flat leaves: absent = 0, present = 1
22. *Terete leaves : absent = 0, present = 1
23. Length of aerial axes: 1–10cm = 0, 10.1–20cm = 1, 20.1–30cm = 2, 30.1–40cm = 3, 40.1–50cm = 4, >50.1 = 5
24. Width of aerial axes: < 1mm = 0, 1.1–2mm = 1, 2.1–3mm = 2, 3.1–4mm = 3, 4.1–5mm = 4, >5.1mm = 5
25. *Ridges on aerial axes (Upper part): absent = 0, present = 1,
26. *Aerial axes indumentums (Upper part): absent = 0, present = 1
27. Hairy indumentum (Upper part): absent = 0, present = 1
28. Warty indumentum (Upper part): absent = 0, present = 1
29. *Ridges on aerial axes (Lower part): absent = 0, present = 1,
30. *Aerial axes indumentum(Lower part): absent = 0, present = 1
31. Hairy indumentum (Lower part): absent = 0, present = 1
32. Warty indumentum (Lower part): absent = 0, present = 1
33. *Bracts along the stem/scape: absent = 0, present = 1
34. *Branches on aerial axes: absent = 0, present = 1
35. *Zigzag branches: absent: 0, present = 1
36. Nodes without branches: absent = 0, present = 1
37. More than one branch from nodes: absent = 0, present = 1
38. *Dichotomous branching pattern: absent = 0, present = 1
39. Branches restricted to upper 2cm of stem: absent = 0, present = 1
40. Branching starts from middle or above: absent = 0, present = 1
41. Branches from base of stem: absent = 0, present = 1
42. *Umbels: absent = 0, present = 1
43. *Pendent/downward facing flowers: absent = 0, present = 1
44. No of flowers per umbel/cluster: 1–3 = 0, 4–6 = 1, 7–9 = 2, 10–12 = 3, >12 = 4
45. *Single terminal umbels: absent = 0, present = 1
46. *Sessile umbels: absent = 0, present = 1
47. Pedicellate umbels: absent = 0, present = 1
48. Lanceolate inflorescence bracts: absent = 0, present = 1
49. Ovate inflorescence bracts: absent = 0, present = 1
50. Elliptical inflorescence bracts: absent = 0, present = 1
51. Deltoid inflorescence bracts: absent = 0, present = 1
52. Oblong inflorescence bracts: absent = 0, present = 1
53. Acuminate tip in inflorescence bract: absent = 0, present = 1
54. Inflorescence bract length: <1mm = 0, 1.1–3mm = 1, 3.1mm–5mm = 2, >5.1mm = 3
55. Inflorescence bract width: <1mm = 0, 1.1–2mm = 1, >2.1mm = 2
56. Transparency of inflorescence bract: non transparent = 0, <50% transparent = 1, >50% transparent = 2, 50% transparent = 3
57. *Annual inflorescence: absent = 0, present = 1
58. Articulations of flowering pedicels: absent = 0, present = 1
59. Basal articulation of flowering pedicels: absent = 0, present = 1
60. Middle articulation of flowering pedicels: absent = 0, present = 1

61. Flowering pedicels articulated at all positions: absent = 0, present = 1
62. *Fimbriae in tepals: absent = 0, present = 1
63. *Habit of flowering pedicels: erect = 0, nutant = 1
64. *Petal length: <10mm = 0, 10.1–20mm = 1, >20.1 = 2
65. *Petal width: 1–3mm = 0, 3.1–5mm = 1, >5.1mm = 2
66. *Petal shape: elliptical = 0, broadly elliptical = 1, obovate = 2, oblong = 3, ovate = 4
67. *Petal colour: purple = 0, dark purple = 1, bluish purple = 2, pink purple = 3, pale purple(almost white) = 4
68. *Sepal length: <10mm = 0, 10.1–20mm = 1, >20.1mm = 2
69. *Sepal width: 1–2mm = 0, 2.1–3mm = 1, 3.1–4mm = 2, 4.1–5mm = 3, >5.1mm = 4
70. Sepal shape: linear = 0, oblong = 1, elliptical = 2, lanceolate = 3, ovate = 4
71. Fimbriae length:<0.9 = 0, 1–2mm = 1, 2.1–3mm = 2, 3.1–4mm = 3, 4.1–5mm = 4, >5.1mm = 5,
72. *No of stamens: 3 = 0, 6 = 1
73. *Anther dehiscence: terminal pore = 0, longitudinal slits = 1
74. *Outer anther length: <3mm = 0, 3.1–5mm = 1, 5.1–7 = 2, 7.1–9 = 3, >9.1 = 4
75. *Straight outer anthers: absent = 0, present = 1
76. *Curved outer anthers: absent = 0, present = 1
77. *Twisted outer anthers: absent = 0, present = 1
78. *Outer anthers colour: all yellow = 0, all purple = 1, yellow above purple below = 2, purple above yellow below = 3
79. *Posteriorly extended outer anther pore lips: absent = 0, present = 1
80. *Anteriorly extended outer anther pore lips: absent = 0, present = 1
81. *Even outer anther pores: absent = 0, present = 1
82. *Outer anther pore length: <0.1mm = 0, 0.11–0.3mm = 1, 0.31–0.5mm = 2, 0.51–0.7 = 3, 0.71–0.9mm = 4
83. *Inner anther length: <3mm = 0, 3.1–5mm = 1, 5.1–7 = 2, 7.1–9 = 3, >9.1 = 4
84. *Straight inner anthers: absent = 0, present = 1
85. *Curved inner anthers: absent = 0, present = 1
86. *Twisted inner anthers: absent = 0, present = 1
87. *Inner anthers colour: all yellow = 0, all purple = 1, yellow distally, purple proximally = 2, purple distally yellow proximally = 3
88. *Posteriorly extended inner anther pore lips: absent = 0, present = 1
89. *Anteriorly extended inner anther pore lips: absent = 0, present = 1
90. *Even inner anther pores: absent = 0, present = 1
91. *Inner anther pore length: < 0.1mm = 0, 0.11–0.2mm = 1, 0.21–0.3mm = 2, 0.31–0.4mm = 3, >0.4mm = 4
92. *Tail formed by perianth in fruit: absent = 0, present = 1
93. Fruit length:1–3mm = 0, 3.1–5mm = 1, >5mm = 2
94. Fruit width: 1–3mm = 0, 3.1–5mm = 1, >5mm = 2
95. *Fruit shape: globose = 0, elliptical = 1, ovate = 2, oblong = 3
96. *Habit of fruiting pedicels: erect = 0, nutant = 1
97. *Seed shape: orbicular/globose = 0, elliptical/ovoid = 1, elongated = 2, angular = 3
98. *Median ridge: absent = 0, present = 1
99. *Aril/arillate appendage: absent = 0, present = 1
100. *Aril stalk with aril positioning: sessile with aril positioned close = 0, long stalk with aril positioned distant = 1, aril absent = 2
101. Aril shape: elongated = 0, flat = 1, aril absent = 2
102. Shiny seed coat in naked eye: absent = 0, present = 1
103. Seed surface texture: smooth = 0, rough = 1
104. Seed length: ≤ 2mm = 0, > 2mm = 1
105. Outer epidermal cell shape: polygonal = 0, rounded = 1, irregular = 2
106. *Outer periclinal wall shape: strongly convex = 0, slightly convex = 1, flat = 2
107. *Convex periclinal wall surface shape: flat = 0, rounded = 1, uneven = 2
108. *Cell surface microsculpturing: absent = 0, present = 1
109. Verrucose microsculpturing: absent = 0, present = 1
110. Striated microsculpturing: absent = 0, present = 1
111. *Anticlinal cell wall boundary: raised = 0, channelled = 1
112. Shallowly channelled anticlinal boundaries: absent = 0, present = 1

113. *Shape of transverse section: terete = 0, elliptical = 1, polygonal = 2, other = 3
114. *Cuticle surface: even = 0, ferrulate = 1
115. *Cuticle thickness: 0–10 µm = 0, 10.1–20 µm = 1, 20.1–30 µm = 2, >30 µm = 3
116. Ridges on the surface: absent = 0, present = 1
117. Furrows on the surface: absent = 0, present = 1
118. Projections on surface: absent = 0, present = 1
119. Hairy projections: absent = 0, present = 1
120. Projections on ridges: absent = 0, present = 1
121. Projections distributed outside ridges: absent = 0, present = 1
122. *Length of projections: NA = 0, ≤ 200 µm = 1, >200 µm = 2
123. *Projections of highly variable lengths: absent = 0, present = 1
124. *Tuberculate hairs: absent = 0, present = 1
125. *Epidermal cells h/w ratio: <1 = 0, 1 = 1, >1 = 2
126. Rectangular epidermal cells: absent = 0, present = 1
127. Square epidermal cells: absent = 0, present = 1
128. Columnar epidermal cells: absent = 0, present = 1
129. Irregular shaped epidermal cells: absent = 0, present = 1
130. Chloroplasts in epidermal cells: absent = 0, present = 1
131. No of chlorenchyma layers: <3 = 0, >4 = 1
132. *Elliptical chlorenchyma: absent = 0, present = 1
133. *Globose chlorenchyma: absent = 0, present = 1
134. *Elongated chlorenchyma: absent = 0, present = 1
135. *Irregular shaped chlorenchyma: absent = 0, present = 1
136. *Raphide canals in chlorenchyma: absent = 0, present = 1
137. Parenchyma/collenchyma layer below chlorenchyma: absent = 0, present = 1
138. *Sclerenchyma below chlorenchyma: absent = 0, present = 1
139. Vascular bundles adjacent to Chlorenchyma: absent = 0, present = 1
140. *Raphide canals around sclerenchyma: absent = 0, present = 1
141. No of central vascular bundles: 1–10 = 0, 11–20 = 1, >21 = 2
142. No of central vascular bundle rings:1 = 0, 2 = 1, >2 = 2
143. *Shape of xylem: irregular = 0, v–shaped = 1, half circular = 2, half square shaped = 3, straight = 4
144. *Average no of vessels in a bundle: <4 = 0, 5–10 = 1, >10 = 2
145. Parenchymatous pith: absent = 0, present = 1
146. *Lignified pith: absent = 0, present = 1
147. *Raphide canals in pith: absent = 0, present = 1
148. Diameter of the transverse section: <1mm = 0, 1.1mm–2mm = 1, 2.1 = 2

Cladistic analysis

To determine phylogenetic relationships, major lineages and to understand the variability within a species (taxon boundaries) inside a phylogenetic framework, cladistic analysis was carried out using PAUP version 4.0b10 (Swofford 2002) on a PowerMac G3 computer. The morphological matrix was constructed using MacClade version 4.0 and some morphological characters were mapped after analysis using the same program (Maddison and Maddison 2000).

Character mapping

The characters and character states were further mapped using WinClada ver. 1.00.08 (Nixon 2002). Certain characters were not mapped due to high intra-specific variation and variability. Mapped characters are indicated in asterisks.

Results

Heuristic search using PAUP yielded four equally parsimonious trees with a Tree Length: 1608 steps, Consistency Index: 0. 139, Retention Index: 0. 600 and Rescaled Consistency Index: 0. 083. After four rounds of successive weighting, stability was achieved and three trees were recovered (TL: 121.24, CI: 0.220, RI: 0.710, RCI: 0.156). A strict consensus tree is illustrated in Fig 1.

Thysanotus (if expanded to include *Murchisonia*) is monophyletic with a relatively strong bootstrap support of 94%. The strict consensus tree consisted of two major clades, both of which were poorly supported. The *Thysanotus sens.lat.* clade could be defined by a number of characters (Fig 2): absence of flat leaves (21/0); ridges present on upper part of aerial axis (25/1); presence of umbels (42/1); 0.11–0.3 mm long outer anther pores (82/1); 0.21–0.3mm long inner anther pores (91/2); and the synapomorphy; absence of pendent flowers (43/0). None of the investigated anatomical characters defined *Thysanotus*.

Within *Thysanotus*, Clade 1 had only 2% BS support but could be defined by the characters absence of tubers (2/0); absence of long-stalked, distant tubers (5/0) and presence of ridges on the lower part of aerial axis (29/1) all homoplasious, losing their overall reliability. Within Clade 1, *T. juncifolius, T. brachiatus, T. pseudojunceus, T. sparteus, T. anceps, T. virgatus, T. fractiflexus, T. asper, T. wangariensis* and *T. arenarius* formed a distinct lineage that was defined by a single synapomorphy: half square shaped xylem (143/3) and by the more homoplasious characters; absence of dichotomous branching (38/0) and 10.1–20 mm long petals and sepals (64/1, 68/1).

Thysanotus juncifolius accessions from South Australia (south-east South Australia and the Eyre Peninsula) were closely related but distant from the New South Wales specimen of that species (see Table 3 for differences). The *T. juncifolius* (SA) plants were sister to *T. sparteus* from WA, with the pairing

defined by the synapomorphy of >30 μm thick cuticles (115/3) and homoplasies >9.1 mm long inner anthers (83/4) and >1 epidermal cells h/w ratio (125/2). In contrast, *T. juncifolius* (NSW) was placed more proximally on the tree and related to *T. brachiatus* and *T. pseudojunceus*.

Table 3. Differences in *T. juncifolius* from South Australia and New South Wales

Character	*T. juncifolius* SA	*T. juncifolius* NSW
Sessile umbels	Present	Absent
Inner anther length	>7 mm	<7 mm
Inner anther pore lenth	0.11 mm–0.20 mm	0.31 mm–0.40 mm

Thysanotus anceps, T. virgatus, T. fractiflexus, T. asper, T. wangariensis and *T. arenarius* were all closely related to each other, sharing homoplasious characters such as indumentums on the upper part of the aerial axis (26/1); inner anther length 5.1–7 mm (83/2) and presence of >200 μm long projections on the stem (122/2). The unique zigzag branches of *T. fractiflexus* were a distinctive autapomorphy for the species (35/1).

The species of Clade 2 could not be defined clearly by any of the selected characters. Nevertheless, within Clade 2, *T. baueri* and *T. nudicaulis* formed a distinct proximal lineage: Clade 2i, united by the presence of sessile umbels (46/1) (Figs 3–4); twisted outer anthers (77/1); absence of straight inner anthers (84/0); presence of cell surface microsculpturing (108/1) (Figs 5–6); and epidermal cells h/w ratio <1 (125/0). Both species occur in South Australia and these results suggest that they are closely related.

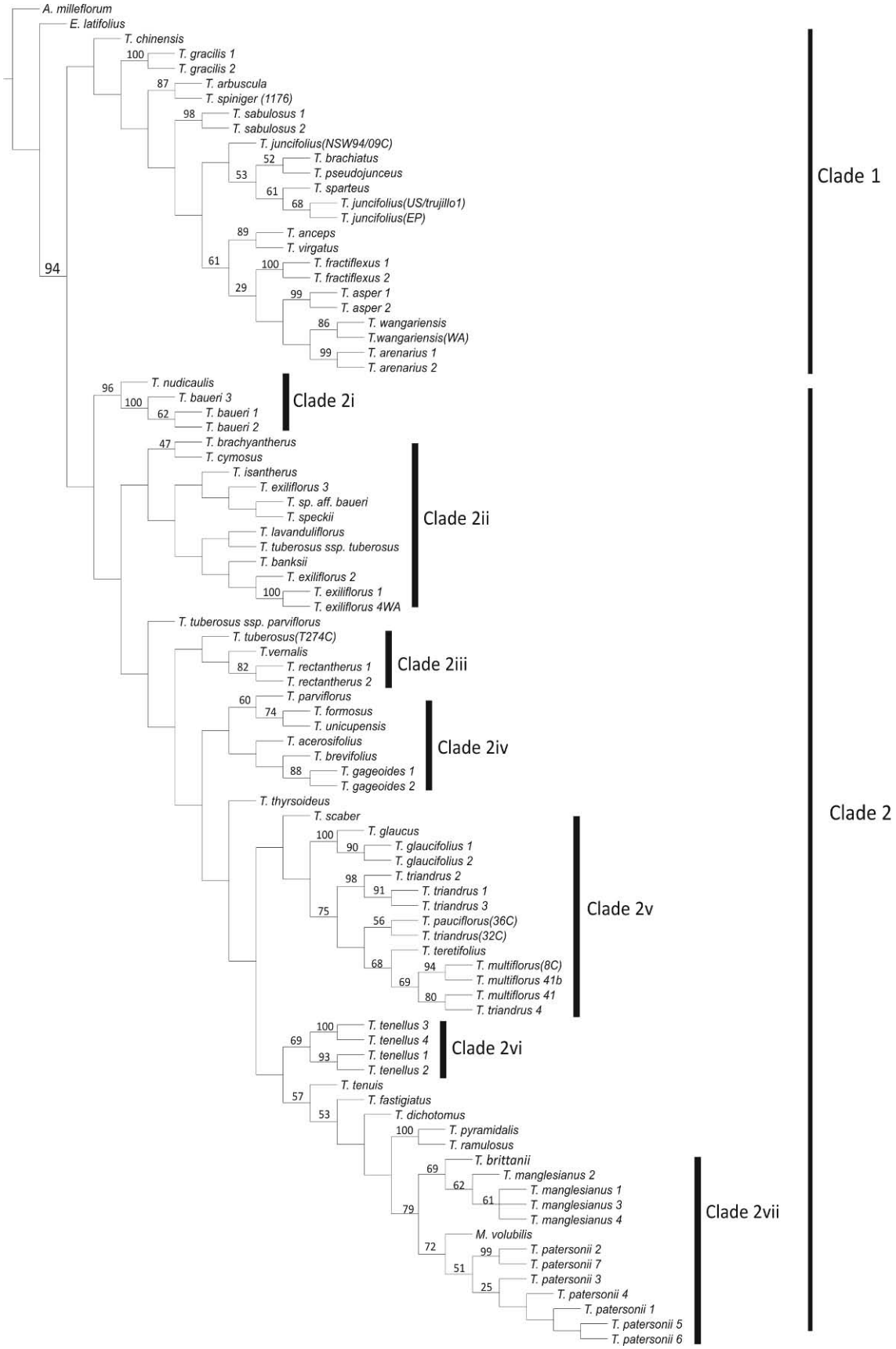


Fig. 1. Strict consensus tree with TL: 121.24, CI: 0.220, RI: 0.710, RCI: 0.156. Bootstrap values are indicated above the branches.

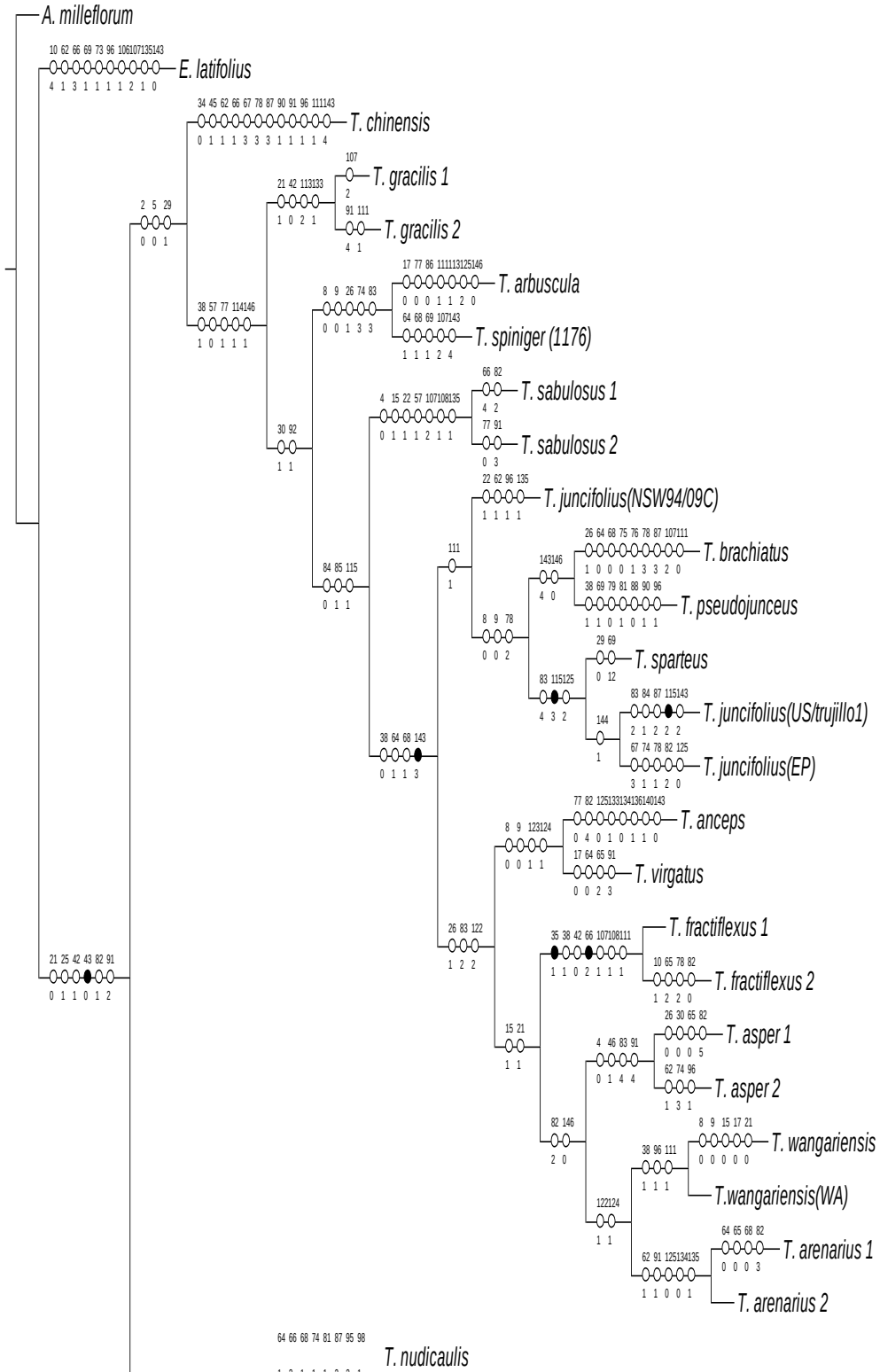


Fig. 2. Characters mapped to a randomly selected parsimonious tree; open circles indicate homoplasious characters, closed circles indicate synapomorphies. Numbers above the circles are the character numbers and the numbers below are states.

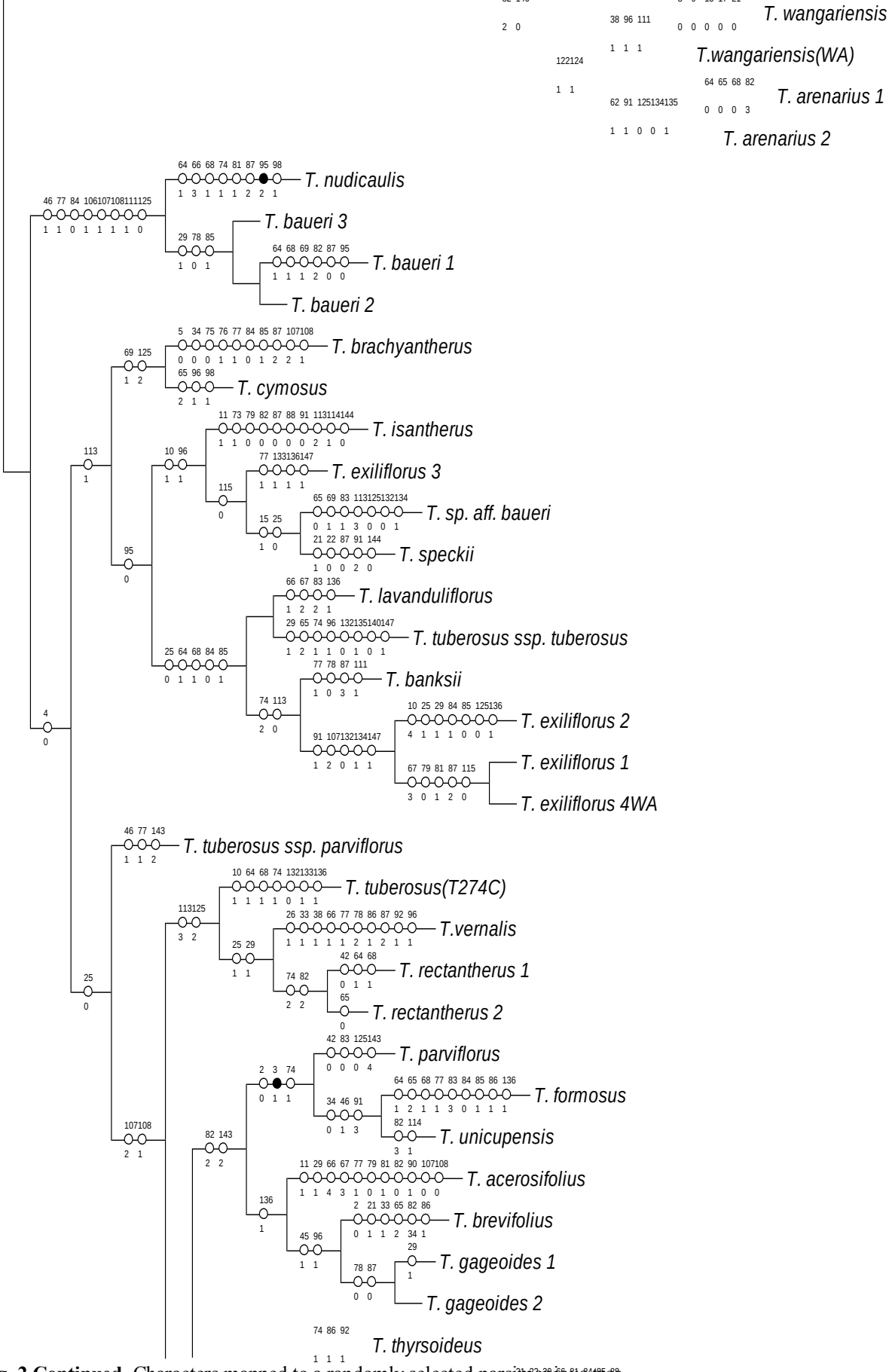
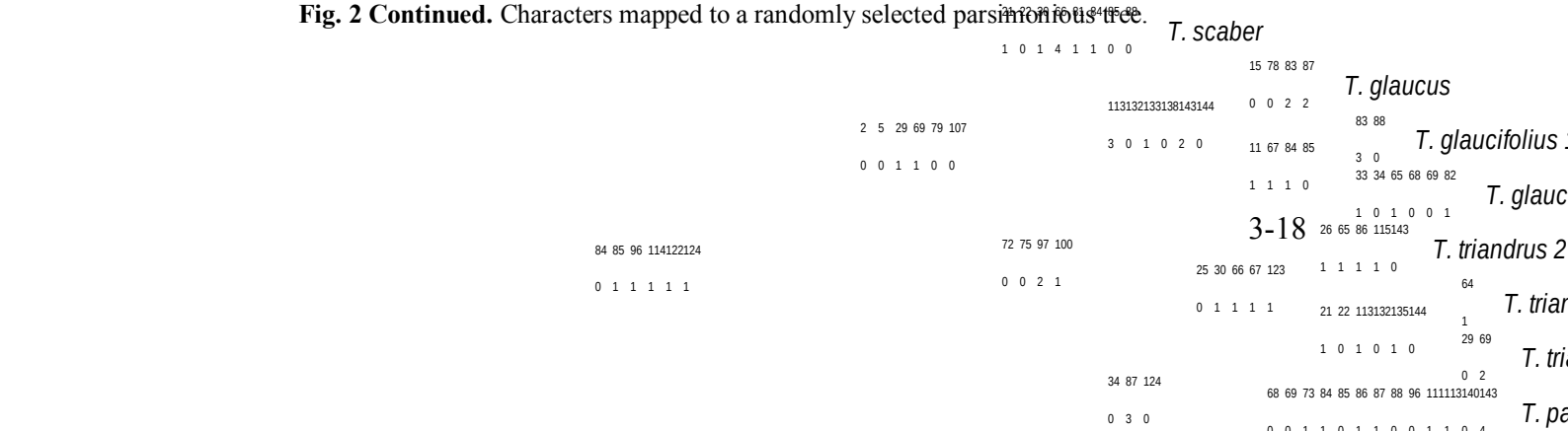


Fig. 2 Continued. Characters mapped to a randomly selected parsimonious tree.



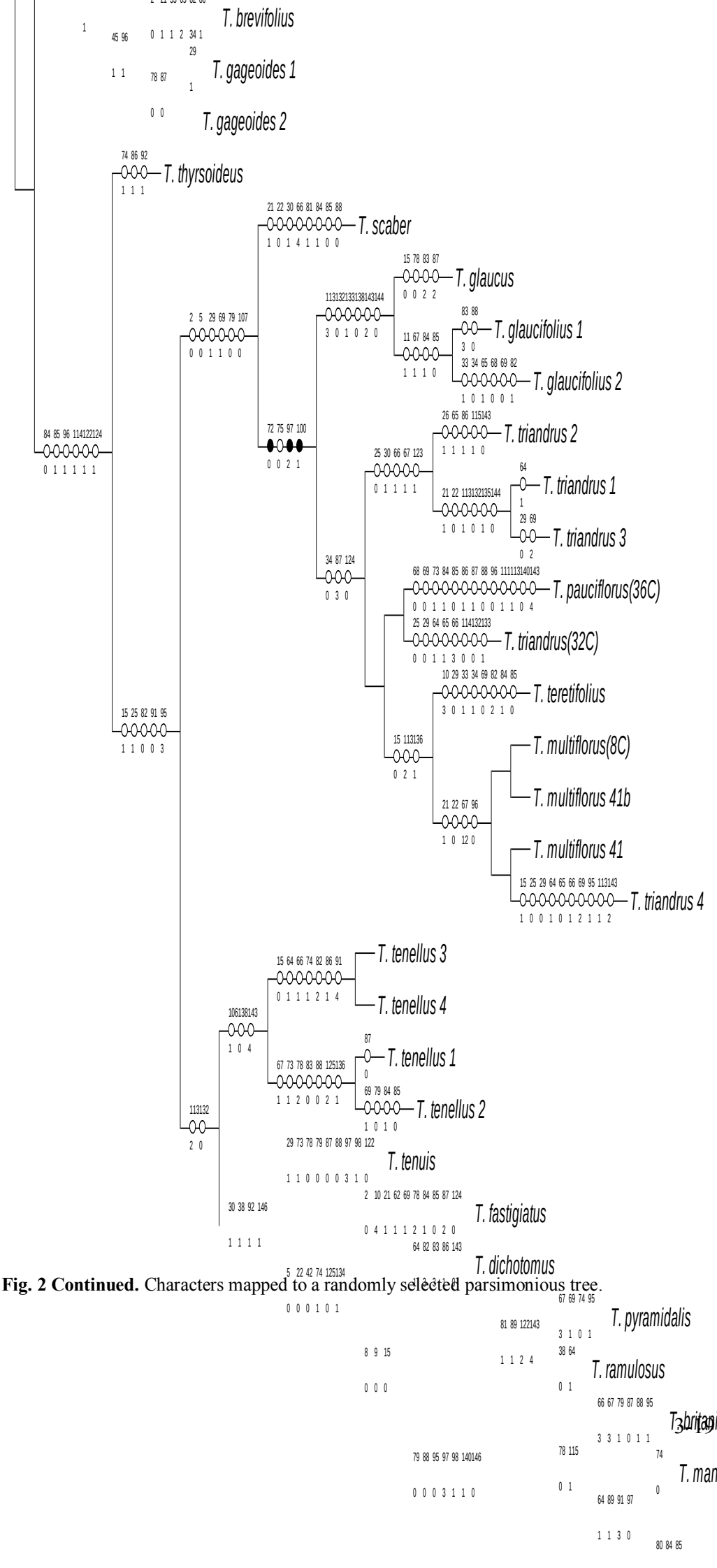


Fig. 2 Continued. Characters mapped to a randomly selected parsimonious tree.

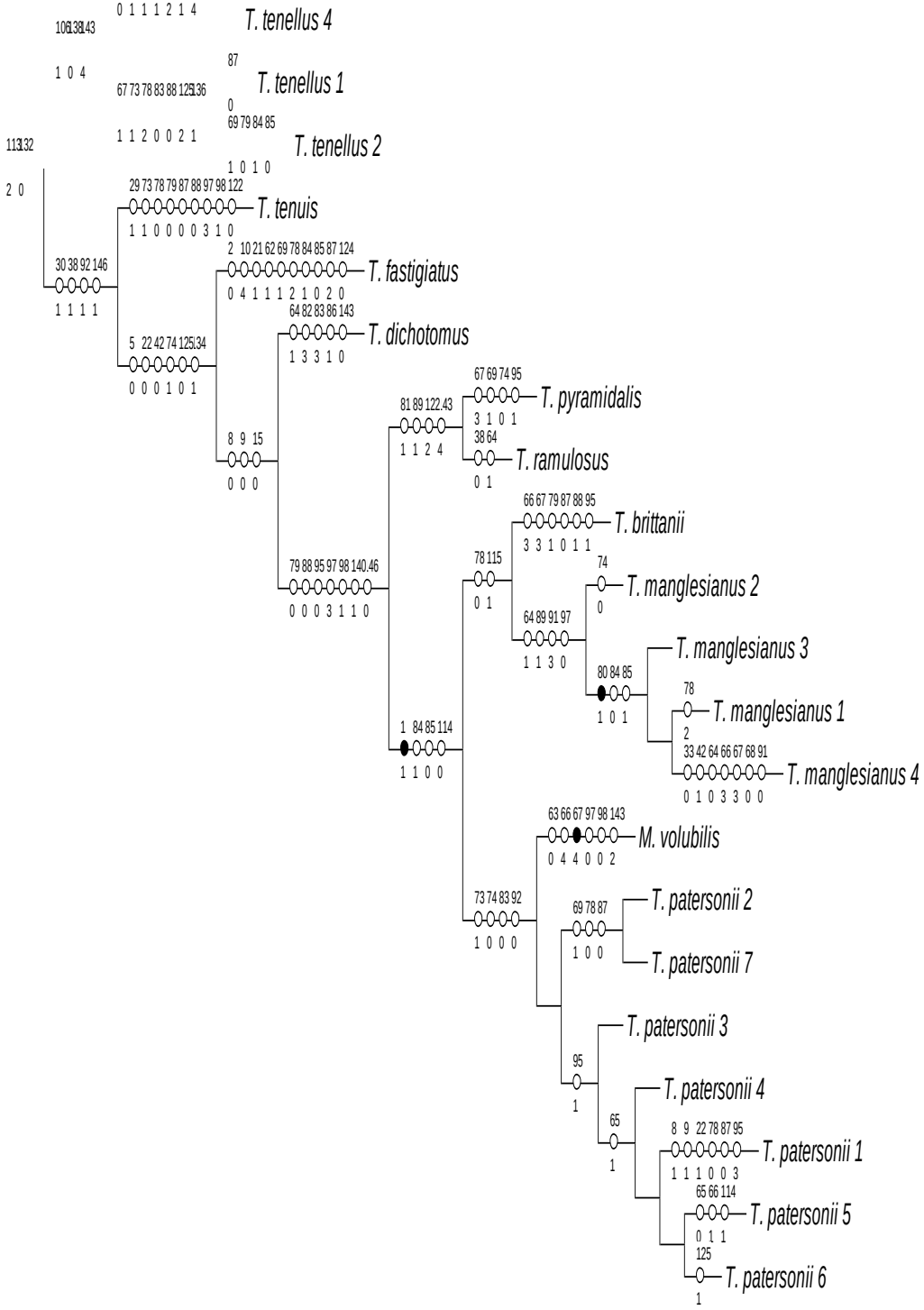
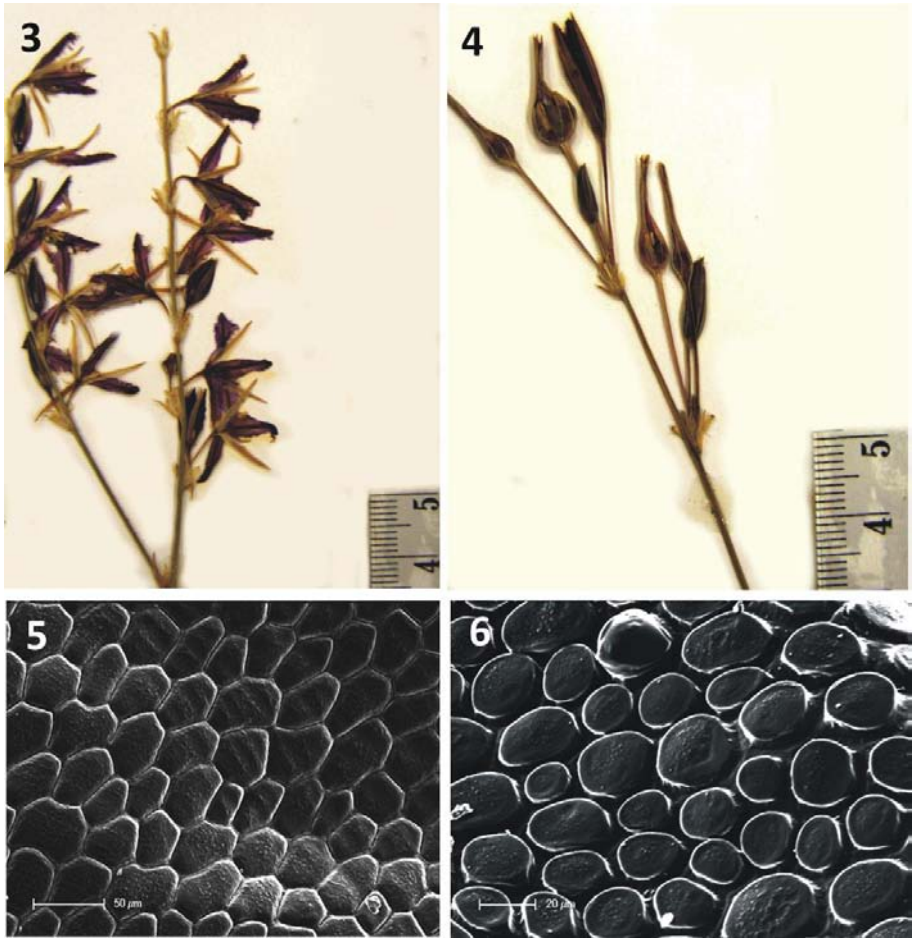


Fig. 2 Continued. Characters mapped to a randomly selected parsimonious tree.



Figs. 3–6. Aerial axes and seed epidermal cell comparisons of *T. baueri* and *T. nudicaulis*. 3. Aerial axis of *T. baueri* with sessile umbels along the scape. 4. Aerial axis of *T. nudicaulis* with sessile umbels along the scape. 5. Polygonal shaped seed epidermal cells in *T. baueri* with verrucose cell surface microsculpturing. 6. More or less circular shaped seed epidermal cells in *T. nudicaulis* with verrucose cell surface microsculpturing.

Clade 2ii was defined by a single homoplasious character: elliptical transverse stem sections (113/1). Proximal within this clade was the taxon pair of *T. cymosus* and *T. brachyantherus*. Brittan (1960, 1981) recognized similarities in the general habit of the inflorescence of *T. cymosus* and *T. brachyantherus*, however that the latter differs by possessing markedly twisted outer anthers in comparison with the non twisted anthers of *T. cymosus*. Furthermore, the leaves of *T. cymosus* are more marcescent than those of *T. brachyantherus* (Brittan 1981) and the phylogeny supports these two species as being closely related.

The characters, variable shaped transverse stem sections (113/3) and >1 epidermal cell h/w ratio (125/2) define Clade 2iii. The clade included *T. tuberosus* (TST 274), *T. vernalis* and *T. rectantherus*. *T. tuberosus* is non-monophyletic as one subspecies recovered in Clade 2ii and the other basal to Clade 2iii in an isolated position.

Clade 2iv represents all the 6 staminate species with simple scapes (*T. unicupensis*, *T. formosus*, *T. brevifolius* and *T. gageoides*) and this was further defined by outer anther pores 0.31–0.5 mm long (82/2) and half circular xylem shape (143/2). Sirisena *et al.* (2009) had assumed that *T. unicupensis* and *T. formosus* were closely related, and both formed a distal pair due to their possession of sessile umbels, in the former, clustered towards the apex (Figs 7–8). Furthermore, *T. parviflorus*, *T. unicupensis* and *T. formosus* formed a distinct clade sharing fleshy, non-tuberous roots (3/1) as a synapomorphy uniting them. Although paniculately branched, *T. acerosifolius* otherwise closely resembles *T. brevifolius* (Brittan 1981) and its placement immediately proximal to the terminal *T. brevifolius/T. gageoides* clade supports this.

Figs. 7–8. Aerial axes of *T. formosus* and *T. unicupensis*. 7. Sessile branches along the scape of *T. formosus*. 8. Sessile branches clustered towards the apex in *T. unicupensis*. (Scale bars: 7 = 3 cm; 8 = 10 mm)

Reduction of the androecium to three stamens (72/0) is a synapomorphy defining Clade 2v, along with elongated seeds (97/2) and long stalked arils (100/1) and a single homoplasious character absence of straight outer anthers (75/0). Possession of three stamens defines a distinct evolutionary lineage; however this should be further tested by including molecular data. The

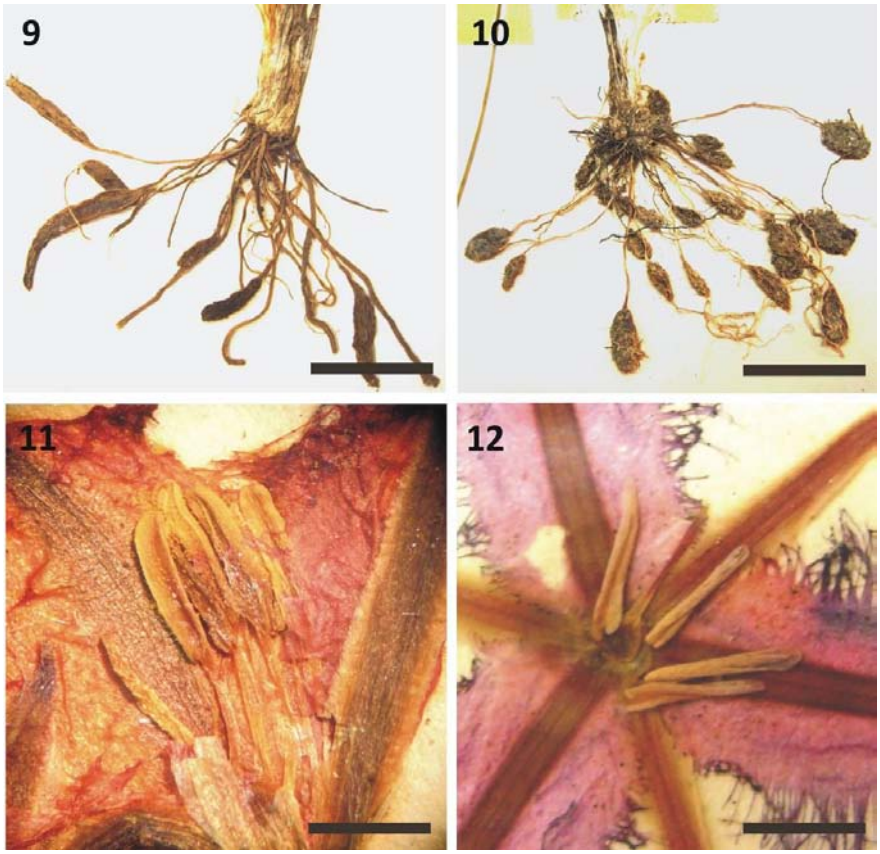
importance of tri-staminate flowers in this highly distinct evolutionary lineage was also recognised previously by Bentham (1878), Brittan (1962) and Thongpukdee (1989).

In contrast, Clade 2vi (the *T. tenellus* accessions) could be defined by slightly convex periclinal walls (106/1); absence of sclerenchyma below chlorenchyma (138/0) and presence of straight xylem (143/4). *T. tenellus* occurs in both South Australia and Western Australia and the two specimens were clustered as pairs representing each State. The distinguishing features between the Western Australian and South Australian specimens are listed below, indicating that there at least 2 different forms within *T. tenellus* that require to be circumscribed.

Table 4. Differences between *T. tenellus* from South Australia and Western Australia

Character	*T. tenellus* (SA)	*T. tenellus* (WA)
Tuber shape (Figs. 9, 10)	Cylindrical/bottle shaped	Elliptical
Leaf indumentum	Present	Absent
Petal length	< 10 mm	>10.1 mm
Anther dehiscence (Figs. 11, 12)	Slits	Pores
Inner and outer anther length	< 3 mm	3.1 mm–5 mm
Bilobed anther base	Absent	Present

All the annual climbing *Thysanotus* species and *M. volubilis* formed the terminal Clade 2vii united by the synapomorphy, twining inflorescence habit (1/1).



Figs. 9–12. Tubers and anthers of *T. tenellus*. 9. Elongate tubers of *T. tenellus* from SA (AD97235225). 10. Elliptical tubers of *T. tenellus* from WA (P0298192). 11. Anthers with simple bases dehiscing by slits in *T. tenellus* from SA (Specimen 1). 12. Anthers with bilobed bases dehiscing by pores in *T. tenellus* from WA (P0298192). (Scale bars: 9, 10 = 3 cm; 11 = 3 mm; 12 = 500 μm)

Discussion

Monophyly of Thysanotus

Thysanotus (once expanded to include *Murchisonia*) is monophyletic with strong support (94%) and the recognition of *Murchisonia* as a separate genus is not justified by our analysis. Therefore, returning this taxon to *Thysanotus*, where it was placed originally by Black (1943) should be considered.

Morphological character evolution of Thysanotus

The absence of pendent flowers (43/0) is the only synapomorphy for *Thysanotus* (*sens. lat.*). In many *Arthropodium* species and *Eustrephus*, the flowers are generally pendent (facing downwards), but this feature is not characteristic of *Thysanotus* flowers. Aril in seeds is also a derived condition

common to all *Thysanotus* taxa and *Murchisonia,* albeit one which also evolved in *Eustrephus*.

The habits of *Thysanotus* inflorescences are highly variable; however a simple distinction is whether they form erect or twining inflorescences. the phylogenetic significance of this character has not been studied previously, although Brittan (1970) attempted to categorise *Thysanotus* based on different morphotypes albeit without any phylogenetic significance. In the present study the erect habit found to be homoplasious in *Thysanotus*, while the twining habit is a derived condition restricted to distant Clade 2vii.

In areas with limited rainfall, the presence of underground fleshy tubers provides an enormous advantage for water storage and perenniating structures (2/1) and most *Thysanotus* are geophytes possessing tuberous roots. Presence of root tubers is homoplasious as well as plesiomorphic, shared with both outgroups, but this feature seems to have disappeared twice in *Thysanotus*. It is absent in Clade 1, present in Clade 2 and absent in Clade 2iv.

Leaf characters such as presence/absence of leaves during flower/fruit (8, 9), number of leaves (10), filiform leaves (11), leaf indumentum (15) and presence/absence of leaves during lifetime (17) are all homoplasious.

Well-developed rhizomes are restricted mainly to Clade 1 and occur in all species except *T. sabulosus* and *T. asper*. The feature is apparently plesiomorphic in *Thysanotus,* which also occurs in *Arthropodium* and *Eustrephus*. *Thysanotus chinensis, T. sabulosus* and *T. asper* leaves during flower and fruit whereas all other species are generally leafless at anthesis. *Thysanotus chinensis* possessesd a single terminal umbel on a simple scape, as opposed to other taxa in the same clade, which possess branched scapes. Possession of rhizomes and tuberous roots would appear to be ancestral conditions in *Thysanotus* while their absence is derived.

Development of indumentum on the aerial axes (26, 30) is highly homoplasious and absent from both outgroups. This condition has evolved multiple times within *Thysanotus* and its presence would seem to be the result of adaptations to reduce water loss.

Annual inflorescences (57) were also homoplasious and plesiomorphic as this character is shared with *Arthropodium* and *Eustrephus*. In contrast,

perennial inflorescences were characteristic of Clade 1. Prominent fimbriae along the full length of the margins of the inner tepals (62) are absent from *Arthropodium* and *Murchisonia* but present in *Eustrephus* and *Thysanotus* and therefore, homoplasious. Fimbriae may probably be an adaptation to facilitate pollination, but their exact function is unknown. Absence of fimbriae in *Murchisonia* is merely a reversal to the plesiomorphic condition that also occurs in *Arthropodium*.

Anther dehiscence in *Thysanotus* is by slits or pores (73:0/1). These are ambiguous in the tree as *Thysanotus* shares slit dehiscence with *Arthropodium* (although the *Arthropodium* species traditionally separated as *Dichopogon* Kunth have porate anther dehiscence), and porate dehiscence is shared with *Eustrephus*. Similar character distributions were also observed for other anther characteristics, but anteriorly extended outer anther pore lips (80/1, 89/1) were restricted to *T. manglesianus*.

Intra-specific taxa and new species in Thysanotus

Examination of the phylogeny showed that there were several species complexes where samples from different regions or morphotypes were separated, indicating that the taxonomy of these groups needs further study.

Thysanotus juncifolius samples from South Australia were distant from the eastern (type) form of *T. juncifolius* from NSW. Main differences were in the presence of sessile umbels, inner anther length and inner anther pore length. Therefore, a taxonomic separation of these taxa from different populations is required following a larger study.

There have been several collections of specimens from the eastern coast of WA that are very similar to the South Australian *T. wangariensis* and one of these WA specimens was included in this study for comparison. The accessions of *T. wangariensis* from South Australia and Western Australia were sisters in the analysis, indicating that they are very closely related, albeit with some differences. This suggests that they are probably subspecies and that *T. wangariensis* can no longer be considered endemic to South Australia. However, a further study is required for confirmation.

Four specimens of ambiguous *T. exiliflorus* from the very widespread, arid zone were included for analysis: specimen 1 from the NT/SA/WA border, specimens 2 and 4 from WA, and specimen 3 from SA. Specimens 1 and 4 were sister taxa above and rather different from specimen no 2, creating sister clade to *T. banksii*. Specimen 3 was instead paired with a *T.* sp. aff. *baueri*/*T. speckii* clade, distant from the other *T. exiliflorus* accessions. Although specimens 1 and 4 were classified as *T. exiliflorus*, they have more recently been reassigned provisionally to a new species (*T. inaequalis* ms; Macfarlane, pers. comm.). Our results also support the recognition of specimens 1 and 4 as a new species. Specimen 2 apparently represents another new species closely related to *T. inequalis*. When morphological characters of specimen 3 were compared with morphology descriptions of *T. exiliflorus* by Brittan (1981, 1987b), the specimen clearly represents the true *T. exiliflorus*, which is distinguished from all other *T. exiliflorus* specimens (specimens1, 2 and 4) by possessing broadly elliptical petals and purple anthers which are yellow distally. Specimens 1, 2 and 4 possessed oblong petals and purple anthers.

The specimen labelled *T.* sp. aff. *baueri* from the South Australian herbarium (AD) was distant from the other examined *T. baueri* specimens in the analysis and was instead related to *T. speckii* and *T. exiliflorus sens. str.* Features such as the absence of sessile umbels and presence of more than one branch at a node (Fig 13) clearly distinguish this taxon from *T. baueri* (Fig. 3). It differs from *T. speckii* in leaf length, width and indumentum and is also different from *T. exiliflorus* by having leaf indumentum, multi-branched nodes and 4–6 flowers per umbel cluster. *Thysanotus* sp. aff. *baueri* therefore probably represents a new species from the Lake Gairdner–Lake Torrens area of South Australia, but as only a single specimen is known, more samples and study are required.

Thysanotus thyrsoideus was in an isolated position below Clades 2v–2vii with no apparent close affinities. Bentham (1878) had claimed that *T. thyrsoideus* (from WA) was not easily separable from *T. tuberosus* (SE Aust); however, Brittan (1981) noted that in addition to their distributions, nutant fruiting pedicels and possession of usually two leaves in *T. thyrsoideus* clearly distinguished it from *T. tuberosus*, which has erect fruiting pedicels and more

than five leaves. The analysis results placed *T. thyrsoideus* distant from *T. tuberosus* (the latter is split between Clades 2ii–2iii). Although this study was not able to sample all of the described *T. tuberosus* morphotypes, the results indicate that the two subspecies, as presently understood, are not conspecific, and that at least one other *T. tuberosus* morph (Specimen 274) belongs to neither subspecies. The whole complex needs further sampling across both its geographic and morphological range, as well as clarification in relation to the other taxa with which the *T. tuberosus* forms were variously placed.

Mueller (1858) and Bentham (1878) considered the form of *T. tenellus* from the Flinders Ranges in SA to be a distinct, separate species (*T. exasperatus* F.Muell.; also *T. isantherus auct. non.* R.Br.: Benth.); however, Brittan (1981) considered it to be conspecific with *T. tenellus* from WA. As described earlier in Table 4, there are at least clearly definable two morphotypes of *T. tenellus* from SA and WA, based on tuber shape (Figs 9–10), leaf indumentum, anther dehiscence and anther bases (Figs 11-12) separating into SA and WA forms. Nevertheless, herbarium specimens from Flinders Ranges are incomplete and of poor quality, thus further collection and wider sampling are needed to determine if these differences are maintained over their respective ranges.

Further sampling is also required to delimit the nature of the different morphs of *T. triandrus* that were placed variously in a clade with *T. pauciflorus* and *T. multiflorus*. Although Brittan (1981) suggested that the variation in these species was a climatic response that affected scape/leaf length ratio, especially in *T. triandrus*, we observed that different ratios occur within the same population, suggesting that there are other factors at work which require more detailed investigation.

A leafless, climbing annual-inflorescence *Thysanotus* species from WA which resembles both *T. manglesianus* and *T. patersonii* has been recognised recently as new (*T. brittanii* ms: Macfarlane, per. comm.). In our analysis this taxon was sister to a highly variable *T. manglesianus* clade. *Thysanotus brittanii* can be distinguished by purple/yellow anthers, all of equal length and dehiscing by pores with a posteriorly extended lip. *T. manglesianus* is easily distinguished by its unequal anthers with anteriorly extended pore lips (41/1);

whereas, both differ from *T. patersonii* by having a long, conspicuous tail on the fruits, formed by the persistent remains of the perianth (Figs 14 and 15).

Fig. 13. Aerial axis of *T.* sp. aff. *baueri* with more than one branch from nodes and terminal umbels (pedicellate umbels). (Scale bar: 5 cm)

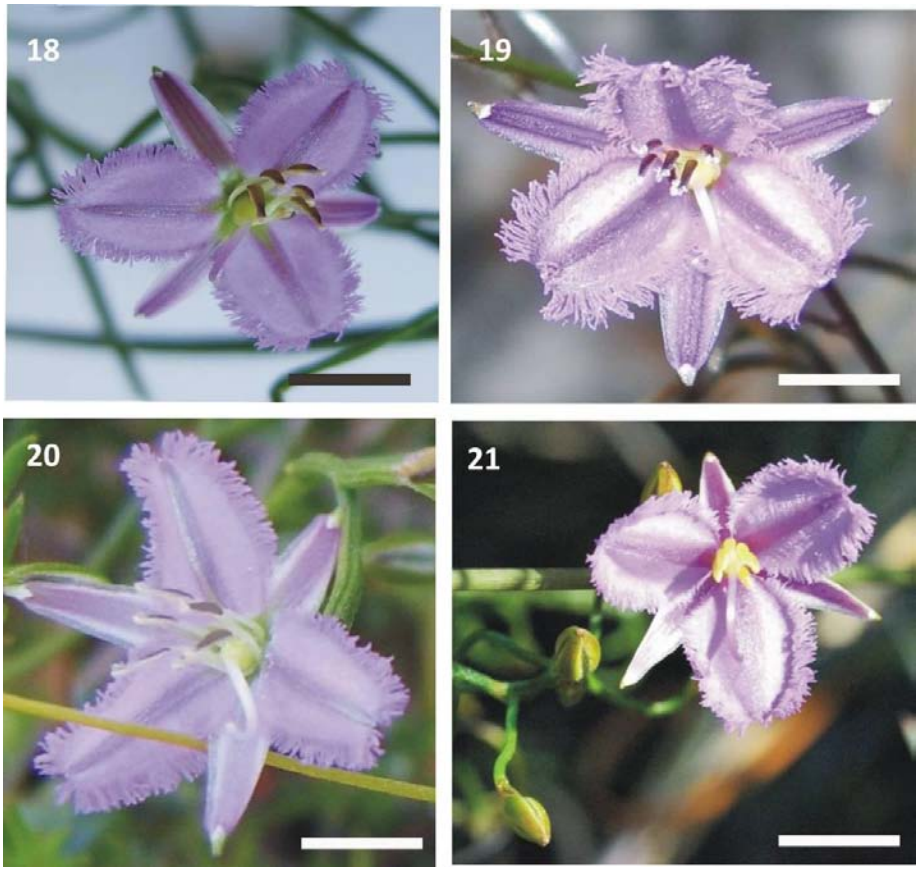
Murchisonia volubilis was sister to all of the sampled *T. patersonii* specimens, suggesting a close affinity to annual climbing *Thysanotus*, although inner tepals without fimbriae (62/0) and pale purple, almost white tepals (30/4) are autapomorphies defining *M. volubilis*. This indicates that *Murchisonia* is a *Thysanotus* without fimbriate petals and needs to be returned there. However,

as the name *T. volubilis* R.Br. already exists as a synonym of *T. patersonii,* the species name would then become *T. exfimbriatum nom. nov.*, based on *T. patersonii* var. *exfimbriatum* J.M.Black (1943) *nom. illeg*., which would be a legal epithet under Art. 58.1 of the Code (McNeill *et al.* 2007).

Thysanotus patersonii is the most widespread species in Australia, but is still disjunct between the east and the west (Keighery and Muir 2005). During our study we sampled *T. patersonii* from Western Australia and South Australia. Specimens collected from the Southern Lofty region of South Australia (numbers 5 and 6) were paired together, sister to *T. patersonii* (Specimen 1). Unlike most other examined *T. patersonii*, specimen 1 possessed numerous leaves at flowering and fruiting. The specimen was cultivated, although originally collected from Mount Lofty and this variation might have been being due in part to more favourable growth conditions. Nevertheless, similar, multi-leaved plants have also been seen in Onkaparinga Gorge Nat. Park, south of Adelaide and Mt Remarkable Nat. Park in the southern Flinders Ranges, SA, suggesting that there is a naturally leafy morphotype in South Australia.

The *T. patersonii* specimens from Western Australia (2 and 7) were also paired and formed a clade, separate from the SA accessions. All samples shared anthers dehiscing by slits, but the WA specimens had anthers 1.3 mm–2 mm long and globose capsules while in SA specimens the anthers were >2 mm long and the capsules were elliptical to oblong. Anther colour was also variable, with Specimens 2 and 7 yellow, Specimen 1 greenish yellow and the remainder purple to dark purple (Figs 16, 17, 18, 19, 20 and 21). In particular, the SA specimens from Newland Head Cons. Park (5) and Scott Creek Cons. Park (6) had very dark purple anthers. Accordingly, there are at least two forms *T. patersonii*, one each from SA and WA (Table 5). In SA there is at least leafless one form with dark purple anthers and elliptical capsules and a possible second leafy one with greenish yellow anthers. In WA there is at least one form with yellow anthers and globose capsules.

Figs. 14–17. Anther pores and fruit of *T. manglesianus* (specimen no 3) and fruits of *T. patersonii* collected from different localities possessing different shapes. 14. Front lip extended outer anther pores of *T. manglesianus*. 15. Globose fruit and long tail formed by the perianth in fruit of *T. manglesianus* from Western Australia. 16. Elliptic fruit of *T. patersonii* from Scott Mt. Lofty, SA (specimen no 1). 17. Globose fruit of *T. patersonii* from Western Australia (specimen 8). (Scale bars: 14=250 μm; 15= 4 mm; 16 = 2 mm; 17 = 1 mm)



Figs. 18–21. Flowers of *T. patersonii* with different anther colours. 18. *T. patersonii* from Mt. Lofty, SA with greenish yellow anthers (Specimen 1). 19. *T. patersonii* collected from Newland Head Conservation Park, SA with dark purple anthers (Specimen 6). 20. *T. patersonii* from Belair National Park, SA with pale purple anthers. 21. *T. patersonii* from Western Australia with yellow anthers (Specimen 8). (Scale bars: 18–20 = 8 mm; 21= 5 mm).

Table 5. Character similarities and/or differences observed in different forms of *T. patersonii*. Numbers in parentheses are specimens listed in Table 1

Character	SA (1)	SA (3, 4, 5, 6)	WA (2, 7)
Leaves present at anthesis	yes	no	no
Acuminate infl. bracts	Absent/present	Absent/present	Present
Fimbriae length	<0.90 mm	<0.90	2.1–3 mm
Anther colour	Greenish yellow	Purple/dark purple	Yellow
Anther length	2–2.8 mm	2–2.8 mm	1.30–2 mm
Fruit shape	Elliptical	Elliptical	Globose

Conclusions

Although the presence of erect inflorescence structures is homoplasious in *Thysanotus*, the specialised twining inflorescence habit is a synapomorphy for the distal Clade 2vii. Presence/absence of rhizomes, tubers, and leaves all are homoplasious characters in *Thysanotus* which have changed multiple times within the tree. Presence of rhizomes, tubers and leaves are plesiomorphic which are also shared with both outgroups and their absence is homoplasious which may have occurred as adaptations to environmental conditions.

Species Clade 1 represents an evolutionary line with all species rhizomatous, non tuberous with perennial inflorescences. Within Clade 1, there is a further specialised clade defined by the single synapomorphy, presence of square shaped xylem and homoplasies, rhizomatous, non tuberous, non leafy plants with perennial inflorescences.

Within the species Clade 2 there is a distinct derived lineage with three stamens and fibrous roots (Clade 2v). Also dichotomously branched, twining leafless species with tuberous roots and annual inflorescences (Clade 2vii) represent another evolutionary line in Clade 2. The remaining clades in the major Clade 2 include tuberous rooted leafy plants with erect annual inflorescences. The morphological character evolution in *Thysanotus* is relatively highly homoplasious and this could mean that these taxa have adapted to thrive in the very diverse Australian environments.

Species such as *Thysanotus juncifolius*, *T. exiliflorus, T. tuberosus, T. tenellus, T. patersonii* and *T. triandrus* require further study for clarification of ambiguity within those taxa. This requires further sampling and incorporation of molecular techniques such as AFLP/RFLPs.

Murchisonia is a *Thysanotus* without fimbriate petals and needs to be returned there.

Acknowledgments

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Phylogeny of *Thysanotus* R.Br. inferred from non coding chloroplast DNA sequences, nuclear sequences and morphological data

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Statement of contribution

Ms. Udani M. Sirisena carried out the field collections, experimental work, data analysis and preparation of the manuscript. Dr. John Conran collected specimens and helped in data analysis and manuscript preparation. Dr. Terry Macfarlane collected specimens and helped in manuscript preparation.

Udani M. Sirisena **(**January 2010)

John G. Conran **(**January 2010)

Terry D. Macfarlane **(**January 2010)

Phylogeny of *Thysanotus* R.Br. inferred from non coding chloroplast DNA sequences, nuclear sequences and morphological data

Abstract. *Thysanotus* is usually characterised by flowers borne in umbels and fringed inner tepals, however, phylogeny and character evolution within the genus is poorly understood and many taxonomic ambiguities require clarification. Thirty-eight species and variants (62 specimens) were included in a phylogenetic study using molecular and morphological data. *Murchisonia* was also included, due to the controversy over its generic placement. The cp DNA (*trn*L intron and *trn*L–F intergenic spacer) and nuclear ITS2 gene regions were amplified and the results compared and combined with a morphological analysis using vegetative, reproductive and anatomical features.

The results show that the two *Murchisonia* species were embedded separately within *Thysanotus,* indicating that loss of fringed inner tepals has occurred twice and that *Murchisonia* is artificial. Similarly the distinction between taxa with three and six fertile stamens in *Thysanotus* was not supported phylogenetically. Instead, there were four main lineages within the genus, each representing life history adaptations. The basal lineage appears to be perennial-leaved, rhizomatous and fibrous-rooted, with two derived lineages: leafless fibrous-rooted rhizomatous perennial inflorescences; and leafy tuberous-rooted geophytes with annual inflorescences. Within the latter, there is a further specialised crown lineage possessing leafless, annual, twining inflorescences.

Introduction

Thysanotus is a genus of 49 species, chiefly in Australia, but one species (*T. chinensis* Benth.) extends to China, Hong Kong, Thailand, Vietnam, the Malay Peninsula, the Philippines, Lesser Sunda Is., Celebes, Aru Is. and 2 species to New Guinea (Brittan 1987b). The genus is usually characterised by flowers borne in umbels and fringed inner tepals. A number of character states are shared by many species of *Thysanotus* e.g. glabrous aerial axes and leaves,

straight anthers, anthers dehiscing by pores, straight style and the sessile aril (Brittan 1987b). *Thysanotus* belongs to the order Asparagales according to Thorne (1992), APG II (2003) and APG III (2009). The genus was previously placed in Liliaceae (Cronquist 1981) and Anthericaceae (Marchant *et al.* 1987), but was subsequently allocated into a new family Lomandraceae (=Laxmanniaceae) (Chase *et al.* 1996; Thorne and Reveal 2007) and most recently into a significantly expanded Asparagaceae, subfam. Lomandroideae (Mabberley 2008; APG III 2009).

Since the pioneering work carried out by Brittan (1960, 1962, 1970, 1971b; 1971a; 1972, 1978, 1981, 1987b), no detailed systematic studies on the genus exist. Phylogeny of the genus is poorly understood and few studies including *Thysanotus* have been conducted; primarily to understand relationships at a higher level i.e. monocot-wide or family level relationships (Thongpukdee 1989; Chase *et al.* 1995; Chase *et al.* 1996; Fay *et al.* 2000).

Molecular studies including *Thysanotus* are scarce (Chase *et al.* 1995; Fay *et al.* 2000). Relationships within the order Asparagales were understood by continued molecular studies but family limits were rearranged and more monogeneric and small families were recognised by this process (APG II 2003). The relationships patterns of Asparagales are now relatively clear (Fay *et al.* 2000) therefore; more comprehensive studies can be carried out within the new family limits. When higher level relationships are recovered, the need arises for lower level (generic and species level) studies employing rigorous phylogenetic character analyses (Zomlefer *et al.* 2001). Extensive molecular level studies are unknown for *Thysanotus* and many taxonomic ambiguities related to this genus can be clarified by the combination of both molecular and morphological data sets (Chase and Reveal 2009; Chase *et al.* 2009). In the past, two other generic names/series have been raised by the differences in number of stamens (Bentham 1878). Therefore, recognition of any generic names/series within a phylogenetic framework is important.

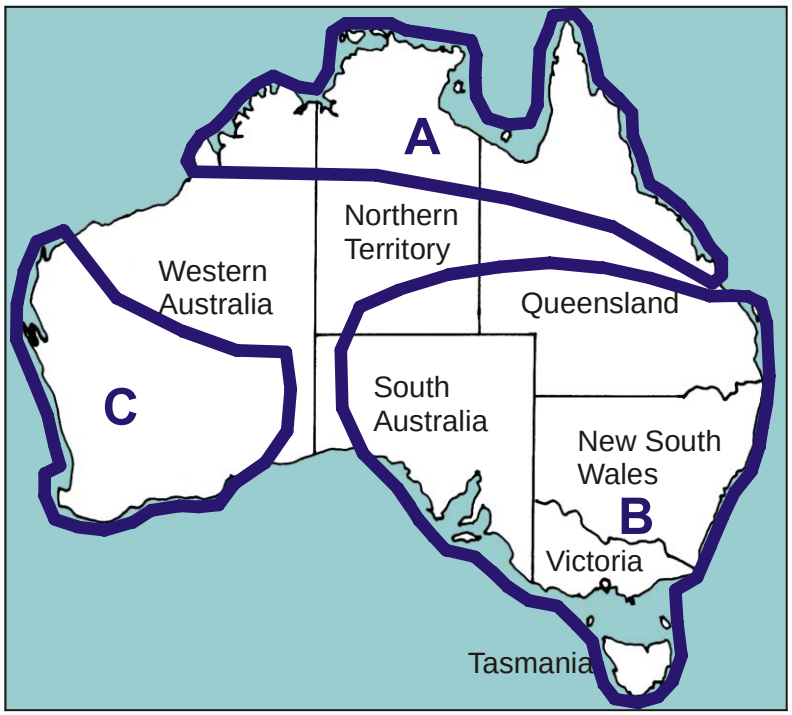


Fig. 1. The distribution zones of *Thysanotus* in Australia. (a) a tropical zone running across the continent, in which *T. banksii* and *T. chinensis* occur, (b) a zone from south-east Queensland to western South Australia, including Tasmania and (c) south-west Western Australia (Brittan 1981).

Three areas of *Thysanotus* distribution can be delimited within Australia: (a) a tropical zone running across the continent, in which *T. banksii* R.Br. and *T. chinensis* Benth. occur, (b) a zone from temperate Queensland to western South Australia, including Tasmania and (c) south west of Western Australia (Brittan 1981) (See map in Fig. 1) . In these three zones, *Thysanotus* generally occurs in: open sclerophyllous heaths, salt-bush plains, coastal sand heaths, desert sands of Eremaean, and dry eucalyptus forests (Brittan 1981). The highest diversity of species is concentrated to south west of WA indicated by zone C with about 40 species.

Existing knowledge on *Thysanotus* suggests that its species prefer different ecological conditions; therefore they mostly grow on different habitats. Some species are recorded from very diverse habitats. Many species disjunct in the east and the west are reported to have variable habitats. Commonly known disjunct species; *T. patersonii* R.Br. grows in a variety of

habitats in extra tropical Australia. Personally observed habitats in South Australia varied from *Eucalyptus* woodlands with nutrient richer soil to open edges of *Banksia* woodlands with sandy soils. Although it grows in many habitats, apparently not growing in wet near coastal forest in Queensland (Brittan 1987b). *T. tenellus* Endl. also has a disjunct distribution between the east and the west. In Western Australia this species was observed in swampy habitats with relatively low shrubby vegetation while in South Australia this was observed from drier Mallee habitats. Bentham (1878) regarded *T. tenellus* from Flinders ranges from South Australia as a distinct species however Brittan (1981) considered this to be conspecific with *T. tenellus*.

Thysanotus triandrus (Labill.) R.Br. is distributed within WA where it has colonised a large number of habitats with sandy soils which include the coastal Balcatta sands with associated *Banksia* low forest and sand plain vegetation which is found in a north westerly direction from Esperence Brittan (1981). In the south west corner of WA it is found in darker, humus rich sands. Brittan proposed *T. triandrus* to be considered a polymorphic species due to the high variability of specimens until proper taxonomic work is carried out on the species.

Taxonomic differentiation of these polymorphic/disjunct species into intraspecific categories or new species is difficult solely using traditional techniques such as morphology and anatomy. Therefore, DNA sequence data can be utilised to provide phylogenetic insights to solve these problems such as elucidating intraspecific categories or recognising proper placements for certain taxa in a phylogeny which is otherwise difficult due to lack of or conflicting morphological characters (Crawford *et al.* 2001).

Chloroplast DNA (cpDNA) regions are widely used for phylogenetic studies at all taxonomic levels (Drabkova *et al.* 2004). The cpDNA evolution is conservative and therefore the changes in structure and gene content may provide significant phylogenetic information (Vijverberg *et al.* 1999). The *trn*L–F intergenic spacer and *trn*L intron have been used from intra- and interspecific levels to the subfamilial and tribal levels (Mes *et al.* 2000) and the most frequently used non-coding cpDNA region is the *trn*L–F intergenic spacer (Richardson *et al.* 2000; Chassot *et al.* 2001; Feliner *et al.* 2002). To determine

generic and species relationships, the ITS nrDNA regions have often been used (Crawford *et al.* 2001; Pepper and Norwood 2001; Zomlefer *et al.* 2001). ITS regions are often best suited for comparing species and closely related genera as they are rapidly evolving compared to the highly conserved coding regions (18S, 26S rDNA) (Soltis and Soltis 1998).

Accordingly, we used non coding cpDNA (*trn*L intron and *trn*L–F intergenic spacer), ITS2 sequence data and combined morphological and molecular data to:

(1) Present a comprehensive and more resolved phylogeny for the first time in the taxonomic history of the genus

(2) Evaluate resulting inter-species and intra species relationships of *Thysanotus*

(3) Recognise any sections within the genus

Materials and methods

Taxon sampling

Thirty-eight *Thysanotus* species and known variant forms (64 specimens for molecular analysis) were included in the study (Table 1). Live specimens were used where possible, but where live specimens were unavailable, herbarium specimens were utilised.

Outgroups

Representative species from the genera *Eustrephus* R.Br. (*E. latifolius* R.Br. ex. Ker.Gawl.) and *Arthropodium* R.Br. (*A. milleflorum* (DC.) J.F.Macbr.) were selected as the outgroups based on the gene tree for monocot classification by Chase *et al.* (1995).

Murchisonia volubilis Brittan was also included as an outgroup taxon as it was described originally as *T. patersonii var. exfimbriatum* J.M.Black *nom illeg.* (Black 1943) and is thought to be closely related to *Thysanotus* (Brittan 1987a). Both share many common vegetative features; however flowers of *Murchisonia* flowers lack fringed inner perianth margins. The second

Murchisonia species (*M. fragrans* Brittan) was also included as its inner tepals are apically fringed (Brittan 1987a), making it even more *Thysanotus*-like.

Morphological data

Morphological characters were scored from live and herbarium specimens and taxonomic descriptions. A total of 148 characters were coded into discrete states (Tables 2 & 3). Some characters were coded as binary variables while most as multistate due to extended variation.

Table 1. List of specimens used in this study with their voucher information.

Specimen	Collector/source	Herb.	Accession no
A. milleflorum R.Br.	John G. Conran	ADU	Kuranga Nursery VIC
E. latifolius R.Br. ex. Ker.Gawl.	John G. Conran	ADBG	ADBG88058 7
M. fragrans Brittan	T. D. Macfarlane	PERTH	4044
M. volubilis Brittan	John G. Conran	ADU	1186
T. arbuscula Baker	Udani M. Sirisena & T. D. Macfarlane	PERTH	20
T. arenarius Brittan	T. D. Macfarlane	PERTH	1605
T. asper Lindl.	T. D. Macfarlane	PERTH	1613
T. banksii R.Br.	John G. Conran	ADU	800
T. banksii R.Br.	John G. Conran	ADU	815
T. baueri R.Br.	F. J. Badman	AD	99106157
T. brevifolius Brittan	Udani M. Sirisena & T. D. Macfarlane	PERTH	38
T. britanii H.R. White & T.D. Macfarlane	A. Chant	PERTH	6417728
T. chinensis Benth.	W.R. Barker	AD	9792103
T. cymosus Brittan	Udani M. Sirisena & T. D. Macfarlane	PERTH	28
T. dichotomus (Labill.) R.Br.	John G. Conran	AD	1126
T. dichotomus (Labill.) R.Br	John G. Conran	AD	2108
T. dichotomus (Labill.) R.Br	T. D. Macfarlane	PERTH	3995
T. exiliflorus F.Muell. *NT/WA form*	H. P. Vonow	AD	199674
T. exiliflorus F.Muell. *SA form*	John G. Conran	ADU	2178
T. exiliflorus F.Muell. *SA form*	T. S. Te	AD	233
T. fastigiatus Brittan	John G. Conran	ADU	1432
T. formosus Brittan	Udani M. Sirisena & T. D. Macfarlane	PERTH	7
T. fractiflexus Brittan	D. J. Duvall	AD	1480
T. gageoides Diels	Udani M. Sirisena & T. D. Macfarlane	PERTH	35
T. glaucus Endl.	B.J. Keighery & N. Gibson	PERTH	4463501
T. gracilis R.Br.	T. D. Macfarlane	PERTH	3905
T. isantherus Benth.	Udani M. Sirisena & T. D. Macfarlane	PERTH	37
T. juncifolius (Salisb.)Willis & Court *(NSW form)*	Dalby et. al.	PERTH	94/09
T. juncifolius (Salisb.)Willis & Court *(SA form)*	B. Saunders	ADU	*s.n.*
T. juncifolius (Salisb.)Willis & Court *(SA form)*	Udani M. Sirisena & C. Trujillo	ADU	1
T. manglesianus Kunth.	John G. Conran	ADU	1180
T. manglesianus Kunth.	John G. Conran	ADU	2210

T. multiflorus R.Br.	Udani M. Sirisena	PERTH	41
T. multiflorus R.Br.	Udani M. Sirisena & T. D. Macfarlane	PERTH	8
T. patersonii R.Br. *(WA form)*	John G. Conran	ADU	2212
T. patersonii R.Br. *(WA form)*	John G. Conran	ADU	2255
T. patersonii R.Br. *(SA form)*	John G. Conran	ADU	2119
T. patersonii R.Br. *(SA form)*	Udani M. Sirisena	ADU	1
T. patersonii R.Br. *(SA form)*	Udani M. Sirisena	ADU	3
T. patersonii R.Br. *(SA form)*	Udani M. Sirisena	ADU	4
T. pauciflorus R.Br.	Udani M. Sirisena & T. D. Macfarlane	PERTH	36
T. pseudojunceus Brittan	T. D. Macfarlane	PERTH	3994
T. pseudojunceus Brittan	Udani M. Sirisena & T. D. Macfarlane	PERTH	5
T. rectantherus Brittan	Udani M. Sirisena & T. D. Macfarlane	PERTH	22
T. sp. aff. *baueri*	F.J. Badman	AD	99324113
T. sparteus R.Br.	John G. Conran	ADU	2085
T. sparteus R.Br.	T. D. Macfarlane	PERTH	3969
T. speckii Brittan	T. D. Macfarlane	PERTH	1904
T. tenellus Endl. *SA form*	Udani M. Sirisena	ADU	18
T. tenellus Endl. *WA form*	Udani M. Sirisena & T. D. Macfarlane	PERTH	12
T. tenellus Endl. *WA form*	Udani M. Sirisena & T. D. Macfarlane	PERTH	44
T. tenuifolius sp. nov.	Udani M. Sirisena & T. D. Macfarlane	PERTH	11
T. tenuis Lindl.	Udani M. Sirisena & T. D. Macfarlane	PERTH	26
T. thyrsoideus Baker	John G. Conran	ADU	2271A
T. triandrus (Labill.) R.Br.	John G. Conran	ADU	1430
T. triandrus(Labill.) R.Br.	Udani M. Sirisena	ADU	32
T. triandrus (Labill.) R.Br.	T. D. Macfarlane	PERTH	3997
T. triandrus (Labill.) R.Br.	Udani M. Sirisena & T. D. Macfarlane	PERTH	6
T. tuberosus ssp. parviflorus (Benth.) Brittan	John G. Conran	ADU	324
T. tuberosus R.Br. *ssp. tuberosus*	John G. Conran	ADU	JGC- Home garden
T. tuberosus R.Br.	T. S. Te	AD	274
T. unicupensis Sirisena, T.Macfarlane & Conran	Udani M. Sirisena & T. D. Macfarlane	PERTH	13
T. virgatus Brittan	Dalby et. al.	PERTH	94/08
T. volubilis sp.nov.	T. D. Macfarlane	PERTH	8001146
T. wangariensis Brittan	T. S. Te	PERTH	238
T. wangariensis Brittan	A. S. George	PERTH	2980568

Table 2. List of morphological and anatomical characters with their character states

1. *Habit of inflorescence: erect = 0, twining = 2
2. *Tuberous roots: absent = 0, present = 1
3. Fleshy roots: absent = 0, present = 1
4. *Rhizomes: absent = 0, present = 1
5. Stalked, distant tubers: absent = 0, present = 0
6. Sessile tubers: absent = 0, present = 0
7. Tuber shape: NA = 0, cylindrical/bottle shaped = 1, elliptical = 2, oblong = 3, irregular = 4
8. *Leaves at flowering time: absent = 0, present = 1
9. Leaves at fruiting: absent = 0, present = 1
10. No of leaves: < 5 = 0, 6–10 = 1, 11–15 = 2, 16–20 = 3, >20 = 4
11. Filiform leaves: absent = 0, present = 1
12. Leaf blade length: <10cm = 0, 10.1–20cm = 1, 20.1–30cm = 2, >30.1cm = 3

13. Leaf blade width: < 1mm = 0, 1.1mm–2mm = 1, 2.1–3mm = 2, >3.1 = 3
14. Leaf surface ridges: absent = 0, present = 1
15. Leaf surface indumentum: absent = 0, present = 1
16. Persistent leaves: absent = 0, present = 1
17. *Leaves during plant life: absent = 0, present = 1
18. Basal wings on leaves: absent = 0, present = 1
19. Leaf sheath length: <1cm = 0, 1–3cm = 1, 3.1–5cm = 2, >5.1cm = 3
20. Leaf sheath width: <1 = 0, 1.1–3mm = 1, 3.1–5mm = 2, >5.1mm = 3
21. Flat leaves: absent = 0, present = 1
22. Terete leaves : absent = 0, present = 1
23. Length of aerial axes: 1–10cm = 0, 10.1–20cm = 1, 20.1–30cm = 2, 30.1–40cm = 3, 40.1–50cm = 4, >50.1 = 5
24. Width of aerial axes: < 1mm = 0, 1.1–2mm = 1, 2.1–3mm = 2, 3.1–4mm = 3, 4.1–5mm = 4, >5.1mm = 5
25. *Ridges on aerial axes (Upper part): absent = 0, present = 1,
26. *Aerial axes indumentums (Upper part): absent = 0, present = 1
27. Hairy indumentum (Upper part): absent = 0, present = 1
28. Warty indumentum (Upper part): absent = 0, present = 1
29. *Ridges on aerial axes (Lower part): absent = 0, present = 1,
30. *Aerial axes indumentum(Lower part): absent = 0, present = 1
31. Hairy indumentum (Lower part): absent = 0, present = 1
32. Warty indumentum (Lower part): absent = 0, present = 1
33. *Bracts along the stem/scape: absent = 0, present = 1
34. *Branches on aerial axes: absent = 0, present = 1
35. *Zigzag branches: absent: 0, present = 1
36. Nodes without branches: absent = 0, present = 1
37. More than one branch from nodes: absent = 0, present = 1
38. *Dichotomous branching pattern: absent = 0, present = 1
39. Branches restricted to upper 2cm of stem: absent = 0, present = 1
40. Branching starts from middle or above: absent = 0, present = 1
41. Branches from base of stem: absent = 0, present = 1
42. *Umbels: absent = 0, present = 1
43. *Pendent/downward facing flowers: absent = 0, present = 1
44. No of flowers per umbel/cluster: 1–3 = 0, 4–6 = 1, 7–9 = 2, 10–12 = 3, >12 = 4
45. *Single terminal umbels: absent = 0, present = 1
46. *Sessile umbels: absent = 0, present = 1
47. Pedicellate umbels: absent = 0, present = 1
48. Lanceolate inflorescence bracts: absent = 0, present = 1
49. Ovate inflorescence bracts: absent = 0, present = 1
50. Elliptical inflorescence bracts: absent = 0, present = 1
51. Deltoid inflorescence bracts: absent = 0, present = 1
52. Oblong inflorescence bracts: absent = 0, present = 1
53. Acuminate tip in inflorescence bract: absent = 0, present = 1
54. Inflorescence bract length: <1mm = 0, 1.1–3mm = 1, 3.1mm–5mm = 2, >5.1mm = 3
55. Inflorescence bract width: <1mm = 0, 1.1–2mm = 1, >2.1mm = 2
56. Transparency of inflorescence bract: non transparent = 0, <50% transparent = 1, >50% transparent = 2, 50% transparent = 3
57. *Annual inflorescence: absent = 0, present = 1
58. Articulations of flowering pedicels: absent = 0, present = 1
59. Basal articulation of flowering pedicels: absent = 0, present = 1
60. Middle articulation of flowering pedicels: absent = 0, present = 1
61. Flowering pedicels articulated at all positions: absent = 0, present = 1
62. *Fimbriae in tepals: absent = 0, present = 1
63. Habit of flowering pedicels: erect = 0, nutant = 1
64. Petal length: <10mm = 0, 10.1–20mm = 1, >20.1 = 2
65. Petal width: 1–3mm = 0, 3.1–5mm = 1, >5.1mm = 2
66. Petal shape: elliptical = 0, broadly elliptical = 1, obovate = 2, oblong = 3, ovate = 4

67. Petal colour: purple = 0, dark purple = 1, bluish purple = 2, pink purple = 3, pale purple(almost white) = 4
68. Sepal length: <10mm = 0, 10.1–20mm = 1, >20.1mm = 2
69. Sepal width: 1–2mm = 0, 2.1–3mm = 1, 3.1–4mm = 2, 4.1–5mm = 3, >5.1mm = 4
70. Sepal shape: linear = 0, oblong = 1, elliptical = 2, lanceolate = 3, ovate = 4
71. Fimbriae length:<0.9 = 0, 1–2mm = 1, 2.1–3mm = 2, 3.1–4mm = 3, 4.1–5mm = 4, >5.1mm = 5,
72. *No of stamens: 3 = 0, 6 = 1
73. *Anther dehiscence: terminal pore = 0, longitudinal slits = 1
74. Outer anther length: <3mm = 0, 3.1–5mm = 1, 5.1–7 = 2, 7.1–9 = 3, >9.1 = 4
75. Straight outer anthers: absent = 0, present = 1
76. Curved outer anthers: absent = 0, present = 1
77. *Twisted outer anthers: absent = 0, present = 1
78. Outer anthers colour: all yellow = 0, all purple = 1, yellow above purple below = 2, purple above yellow below = 3
79. *Posteriorly extended outer anther pore lips: absent = 0, present = 1
80. *Anteriorly extended outer anther pore lips: absent = 0, present = 1
81. *Even outer anther pores: absent = 0, present = 1
82. *Outer anther pore length: <0.1mm = 0, 0.11–0.3mm = 1, 0.31–0.5mm = 2, 0.51–0.7 = 3, 0.71–0.9mm = 4
83. Inner anther length: <3mm = 0, 3.1–5mm = 1, 5.1–7 = 2, 7.1–9 = 3, >9.1 = 4
84. Straight inner anthers: absent = 0, present = 1
85. Curved inner anthers: absent = 0, present = 1
86. *Twisted inner anthers: absent = 0, present = 1
87. Inner anthers colour: all yellow = 0, all purple = 1, yellow distally, purple proximally = 2, purple distally yellow proximally = 3
88. *Posteriorly extended inner anther pore lips: absent = 0, present = 1
89. *Anteriorly extended inner anther pore lips: absent = 0, present = 1
90. *Even inner anther pores: absent = 0, present = 1
91. *Inner anther pore length: < 0.1mm = 0, 0.11–0.2mm = 1, 0.21–0.3mm = 2, 0.31–0.4mm = 3, >0.4mm = 4
92. *Tail formed by perianth in fruit: absent = 0, present = 1
93. Fruit length:1–3mm = 0, 3.1–5mm = 1, >5mm = 2
94. Fruit width: 1–3mm = 0, 3.1–5mm = 1, >5mm = 2
95. Fruit shape: globose = 0, elliptical = 1, ovate = 2, oblong = 3
96. Habit of fruiting pedicels: erect = 0, nutant = 1
97. *Seed shape: orbicular/globose = 0, elliptical/ovoid = 1, elongated = 2, angular = 3
98. *Median ridge: absent = 0, present = 1
99. *Aril/arillate appendage: absent = 0, present = 1
100. *Aril stalk with aril positioning: sessile with aril positioned close = 0, long stalk with aril positioned distant = 1, aril absent = 2
101. Aril shape: elongated = 0, flat = 1, aril absent = 2
102. Shiny seed coat in naked eye: absent = 0, present = 1
103. Seed surface texture: smooth = 0, rough = 1
104. Seed length: ≤ 2mm = 0, > 2mm = 1
105. *Outer epidermal cell shape: polygonal = 0, rounded = 1, irregular = 2
106. *Outer periclinal wall shape: strongly convex = 0, slightly convex = 1, flat = 2
107. Convex periclinal wall surface shape: flat = 0, rounded = 1, uneven = 2
108. Cell surface microsculpturing: absent = 0, present = 1
109. Verrucose microsculpturing: absent = 0, present = 1
110. Striated microsculpturing: absent = 0, present = 1
111. *Anticlinal cell wall boundary: raised = 0, channelled = 1
112. Shallowly channelled anticlinal boundaries: absent = 0, present = 1
113. *Shape of transverse section: terete = 0, elliptical = 1, polygonal = 2, other = 3
114. Cuticle surface: even = 0, ferrulate = 1
115. *Cuticle thickness: 0–10 µm = 0, 10.1–20 µm = 1, 20.1–30 µm = 2, >30 µm = 3
116. Ridges on the surface: absent = 0, present = 1
117. Furrows on the surface: absent = 0, present = 1

118. Projections on surface: absent = 0, present = 1
119. Hairy projections: absent = 0, present = 1
120. Projections on ridges: absent = 0, present = 1
121. Projections distributed outside ridges: absent = 0, present = 1
122. Length of projections: NA = 0, ≤ 200 µm = 1, >200 µm = 2
123. Projections of highly variable lengths: absent = 0, present = 1
124. Tuberculate hairs: absent = 0, present = 1
125. *Epidermal cells h/w ratio: <1 = 0, 1 = 1, >1 = 2
126. Rectangular epidermal cells: absent = 0, present = 1
127. Square epidermal cells: absent = 0, present = 1
128. Columnar epidermal cells: absent = 0, present = 1
129. Irregular shaped epidermal cells: absent = 0, present = 1
130. Chloroplasts in epidermal cells: absent = 0, present = 1
131. No of chlorenchyma layers: <3 = 0, >4 = 1
132. Elliptical chlorenchyma: absent = 0, present = 1
133. Globose chlorenchyma: absent = 0, present = 1
134. *Elongated chlorenchyma: absent = 0, present = 1
135. Irregular shaped chlorenchyma: absent = 0, present = 1
136. *Raphide canals in chlorenchyma: absent = 0, present = 1
137. Parenchyma/collenchyma layer below chlorenchyma: absent = 0, present = 1
138. Sclerenchyma below chlorenchyma: absent = 0, present = 1
139. Vascular bundles adjacent to Chlorenchyma: absent = 0, present = 1
140. *Raphide canals around sclerenchyma: absent = 0, present = 1
141. No of central vascular bundles: 1–10 = 0, 11–20 = 1, >21 = 2
142. No of central vascular bundle rings:1 = 0, 2 = 1, >2 = 2
143. *Shape of xylem: irregular = 0, v–shaped = 1, half circular = 2, half square shaped = 3, straight = 4
144. Average no of vessels in a bundle: <4 = 0, 5–10 = 1, >10 = 2
145. Parenchymatous pith: absent = 0, present = 1
146. *Lignified pith: absent = 0, present = 1
147. *Raphide canals in pith: absent = 0, present = 1
148. Diameter of the transverse section: <1mm = 0, 1.1mm–2mm = 1, 2.1 = 2

DNA extraction

Total DNA was extracted from fresh, silica gel dried and herbarium material (leaves and stems) following the standard protocol for DNeasy® Plant mini kit (Qiagen: Germantown, Maryland).

PCR and sequencing

PCR reactions were carried out after optimising the premix using Failsafe PCR Premix Selection Kit® (Epicentre Biotechnologies: Madison, Wisconsin) following the standard protocol given by the manufacturers. Failsafe enzyme mix and Premixes „E" and „G" provided by the manufacturers were used in the PCR reactions.

PCR reactions were carried out using 25ul reactions (Table 4). The universal primers used in White *et al.* (1990) and Taberlet *et al.* (1991) were used in all the reactions (Table 5).

Table 4. Reaction volumes used in PCR reactions

cp DNA: Reagents	Quantities (µl)	ITS2: Reagents	Quantities (µl)
PCR water	0.25	PCR water	0
10% DMSO	0	10% DMSO	3
Premix E	12.5	Premix G	12.5
Template	10	Template	10
Enzyme	0.25	Enzyme	0.5
Primers(Forward and Reverse)	2	Primers(Forward and Reverse)	2
Total volume	25	Total volume	28

Table 5. Forward and reverse primers used (White *et al.* 1990; Taberlet *et al.* 1991)

Primer	Sequence 5‟–3‟
*trn*L forward (C)	CGAAATCGGTAGACGCTACG
*trn*L reverse (D)	GGGGATAGAGGGACTTGAAC
*trn*L–F spacer forward (E)	GGTTCAAGTCCCTCTATCCC
*trn*L–F spacer reverse (F)	ATTTGAACTGGTGACACGAG
ITS2 forward primer	GCATCGATGAAGAACGCAGC
ITS2 reverse primer	TCCTCCGCTTATTGATATGC

Amplification profile

The gene regions were amplified separately using the relevant primers. The amplification profile for cp DNA regions was: 35 cycles with 94^{o}C for 1 min to denature the DNA, 60^{o}C to 1 min to anneal the primers, and 72^{o}C for 2 min for primer extension. At the end of 35 cycles, temperature was held at 20^{o}C for 1 min.

The amplification profile for ITS2 nr DNA region was: 35 cycles with 94^{o}C for 1 min to denature the DNA, 52^{o}C to 2 min to anneal the primers and 72^{o}C for 2 min for primer extension. At the end of 35 cycles, the temperature was held at 72^{o}C for 10 min to allow all the new strands to finish polymerisation.

PCR purification

The PCR products were cleaned with QIAquick® PCR purification kit (Qiagen: Germantown, Maryland), following the manufacturers‟ protocol.

Sequencing

For the *trn*L intron *and trn*L–F intergenic spacer sequencing, a more or less a standard sequencing protocol was applied using a total volume of 20 µl, which contained 3 µl Big dye terminator ver. 3.1, 5 µl of 3 pmol primer, 10 µl of water with 2 µl of template concentration adjusted depending on the quality of PCR product were used. Thermocycling included 25 cycles of 96^{o}C for 30 secs, 50^{o}C for 15 secs, and 60^{o}C for 4 mins.

ITS2 sequencing following Bogler and Simpson (1996): Before beginning the sequencing reaction, the primers were annealed to the DNA using the „snap chill‟ technique which consisted of boiling the sequencing reaction buffer (including 10% DMSO), primer (3 pmol) and double stranded DNA template for 5 minutes to separate the DNA strands. The tubes were then plunged into wet ice to anneal the primers. Then the standard sequencing protocol was used as above.

Phylogenetic analyses

DNA sequences were aligned using CLUSTAL X2 (Larkin *et al.* 2007) and edited in MacClade ver. 4.0 (Maddison and Maddison 2000). The molecular matrix and the combined matrix were analysed using PAUP* ver. 4.0b10 (Swofford 2002) Both data matrices were analysed using the heuristic search option with tree-bisection-reconnection (TBR) branch swapping and the MULTREES option in effect. 100 random stepwise addition replicates were performed. All characters were given equal weight and considered unordered. Further analyses were performed after applying successive weighting of all characters according to the retention index. After constancy was reached strict consensus and 50% majority rule consensus trees were calculated. Bootstrap analyses were carried out using 1000 bootstrap replicates, and 10 random stepwise addition replicates.

The characters were mapped using WinClada ver. 1.00.08 (Nixon 2002). Certain characters were not mapped due to high intra-specific variation and variability. Mapped characters are indicated in asterisks in table 2.

Results

Molecular data analysis

In the study taxa, *trn*L length varied from 538–554 and the aligned length was 584 bps, of which 72 characters were parsimony informative. *trn*L–F spacer region length varied from 259–340 bps with an aligned length of 376bps and 75 parsimony informative characters. ITS2 region varied from 241–263 bps and the aligned length was 300 bps with 131 parsimony informative characters. The combined molecular matrix thus resulted in 1,260 positions and 278 parsimony informative characters. Number of OTUs for the molecular data set was 66 including the four outgroup taxa.

The heuristic search (after removing uninformative characters) initially yielded 150,278 equally parsimonious trees of length 1,028 steps, CI = 0.406, RI = 0.666 and RC = 0.270. Successive weighting based on Rescaled Consistency Index (RC) till constancy was reached yielded 12 trees of 208 steps, CI = 0.637, RI = 0.856 and RC = 0.545. The strict consensus of these 12 trees is shown in Fig. 2.

Our results strongly support the monophyly of *Thysanotus* with 100% BS support, but with the two species of *Murchisonia* embedded separately within *Thysanotus*. The cladogram divided into three major clades (1–3) with 65%, 99% and 52% BS support respectively.

Clade 1 included all the 3-staminate species as well as the 6-staminate *T. chinenesis* and *T. unicupensis* and consists of species with a tufted habit, perennial leaves, fibrous to fleshy, non-tuberous roots.

The Clade 2 represents rhizomatous species with more or less leafless, perennial, branched inflorescences, with *T. pseudojunceus* sister to the remainder of the clade. The WA *Thysanotus dichotomus* and *T. fastigiatus* were closely related as were the SA species *T. fractiflexus* and *T. wangariensis*.

Clade 3 represents all of the geophytic species with annual leaves and tuberous roots. It is divided into a proximal grade including a number of small clades and isolated taxa (*T. banksii* and *T. baueri*) and 2 clades (Clades 3a and 3b) above the much smaller grade in "3". The annual climbers are just a specialised crown lineage inside a larger clade with non-climbers. Clade 3a consisted of *Thysanotus tuberosus* (both sub species), *T. rectantherus, T. tenuifolius, T. tenellus* (12, 44), *T. isantherus* and *T. thyrsoideus* and Clade 3b consisted of *T. speckii, T. tuberosus, T. exiliflorus* (233, 2178), *T.* sp. aff. *baueri*, *T. tenellus* (18) and all the species with climbing, annual inflorescences which formed a specialised crown lineage with 98% BS support. *M. volubilis* was basal to all other annual climbing *Thysanotus* species in the clade and closely related to *T. brittanii*.

Combined data analysis

The combined data matrix included 57 OTUs with 1,408 positions (148 morphological, seed micromorphological and anatomical characters and 1,260 molecular positions), 405 characters were parsimony informative. Heuristic search initially yielded 25 equally parsimonious trees with 1,731 steps, CI = 0.345, RI = 0.590 and RC = 204. After successive weighting based on the RC till constancy is reached, only one MPT was obtained with 293 steps, CI = 0.627, RI = 0.820 and RC = 0.514, is given in Fig. 3.

The monophyly of *Thysanotus* including *Murchisonia* is strongly supported with 100% BS support. The tree topology of the combined analysis was very similar to that of the molecular analysis alone and most of the major clades were recovered unchanged in the combined analysis.

The first clade in the combined analysis included the 3-staminate species and two 6-staminate species *T. unicupensis* and *T. chinensis* (84% BS support). The second large clade and its inclusive species were unchanged in the combined analysis although the relationships between species were slightly different from those in the molecular analysis. The clade received a stronger support (91%) than in the molecular tree (68%).

Clade 3 recovered with the similar obvious subdivisions in the molecular analysis with few differences in species relationships, Clades 3a and 3b were

formed above the proximal grade. The Clade 3a only included all the *T. tenellus* specimens, *T. isantherus* and *T. thyrsoideus*. All the climbers with annual inflorescences were again sister to *M. volubilis* forming the terminal the Clade 3b instead of a crown lineage that was formed in the molecular analysis. The clade received 96% BS support in the combined analysis. Remainder of the taxa in Clade 3 formed the proximal Grade 3.

Character mapping

When morphological characters were plotted on to the molecular tree to evaluate the morphological traits with respect to phylogenetic signal, a single synapomorphy to support the monophyly of *Thysanotus* could be recognised (Fig. 4). The only synapomorphy was the absence of pendent flowers (43/0) and homoplasies were presence of ridges on upper part of aerial axes (25/0), presence of umbels (42/1) and back lip extended inner anther pores (88/1). Molecular tree was chosen for character mapping mainly because trees from both molecular and combined analyses had similar tree topologies and combining morphological data only slightly affected the resolution of the *Thysanotus* phylogeny. Also molecular tree included higher number of variable specimens of certain species than in the combined tree.

Clade 1 could be characterised by the absence of aerial branches (34/0) (Figs. 5–6) and all of the 3–staminate species were in this group. It also included *T. unicupensis* which is a new species from WA (Sirisena *et al.* 2009) and *T. chinensis*, both of which are 6–staminate. The close relationship between *T. multiflorus* and *T. triandrus* which was previously recognised by Brittan (1981, 1987) was reflected in the phylogeny by forming a clade together, however, *T. multiflorus* was basal to *T. triandrus*. The 3-staminate *T. pauciflorus* was basal and closely related to *T. triandrus* than to *T. multiflorus*.

The possession of branched, perennial inflorescences characterised Clade 2 (57/0) and the species composition was unchanged between the molecular and combined analyses. *Thysanotus juncifolius* (NSW) was closely related to *T. arenarius* and *T. asper* in both analyses, whereas *T. juncifolius* (SA) accessions were distant from the NSW specimen in both analyses and more closely related to the South Australian endemic *T. fractiflexus* in the molecular analysis, or

sister to a *T. fractiflexus, T. virgatus, T. wangariensis* and *T. volubilis* crown lineage in the combined tree. It is therefore clear that *T. juncifolius* from SA and NSW are both genetically and morphologically different and represent different, unrelated species (see Chapter 8).

Within Clade 2 different accessions of several „species" were fragmented across the clade, and *T. dichotomus* (for example) is known to have several morphs which have not been investigated properly (Macfarlane, pers. comm.). In the molecular analysis, the specimen no 2108 was sister *T. fastigiatus* and the specimen no 1126 was sister to *T. gracilis*. These genetic differences and phylogenetic relationships of *T. dichotomus* might probably represent some of these putative morphs. *T. fractiflexus* is endemic to KI and could be defined by presence of zigzag branches (35/1, Fig. 7).

Clade 3 could be defined by the homoplasy; outer anther pore length 0.31–0.50 mm (82/2). All the clade 3 species characterised by presence of tuberous roots, absence of rhizomes and presence of leaves till/during flower and fruit which generally united them together. The third large clade could be further divided into a small proximal grade including number of isolated taxa (see molecular analysis), however only the crown lineage of Clade 3b could be characterised by a synapomorphy. The crown lineage of Clade 3b could be characterised by synapomorphy; twining inflorescence habit (1/1) (Figs.4 & 8), and homoplasies; absence of leaves during flowering time (8/0), presence of ridges on aerial axes lower part (29/1), presence of indumentum on aerial axis lower part (30/1), presence of bracts along the stem/scape (33/1), presence of dichotomous branching pattern (38/1), absence of many flowered umbels (42/0, flowers are mostly solitary) and elongated chlorenchyma in stem TS (134/1). Presence of front lip extended outer and inner anther pores (80/1, 89/1) characterise *T. manglesianus*. *T. tenuis* also nested among the annual climbers although it is not a climbing species. *T. patersonii* (WA)*, T. manglesianus* and *T. tenuis* were basal to *T. patersonii* from SA forming a polytomy.

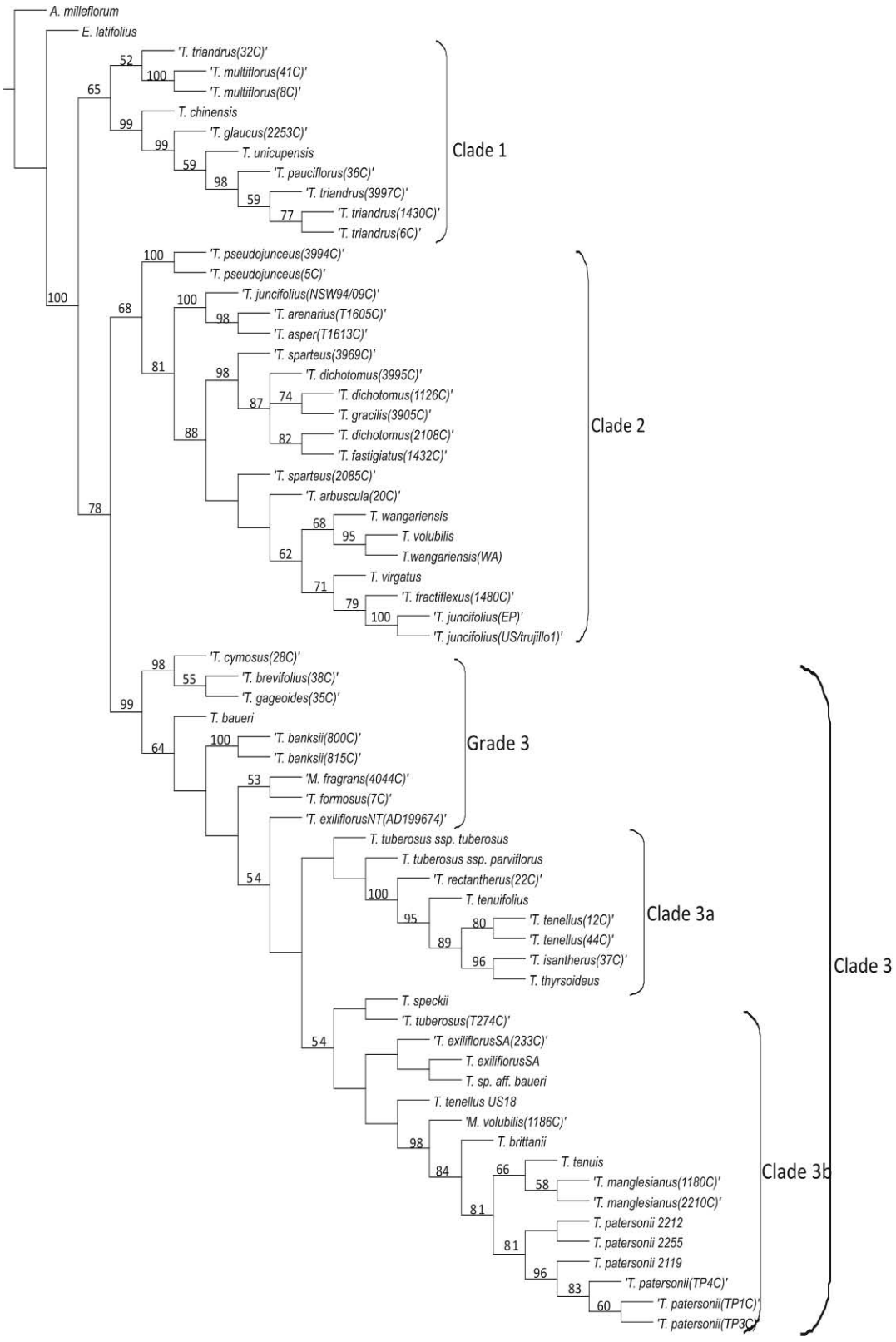


Fig. 2. Strict consensus tree of the 12 most parsimonious trees resulted from the analysis of the molecular data set (278 parsimony informative characters). 208 steps, CI = 0.637, RI = 0.856 and RC = 0.545. Bootstrap values are indicated above the branches.

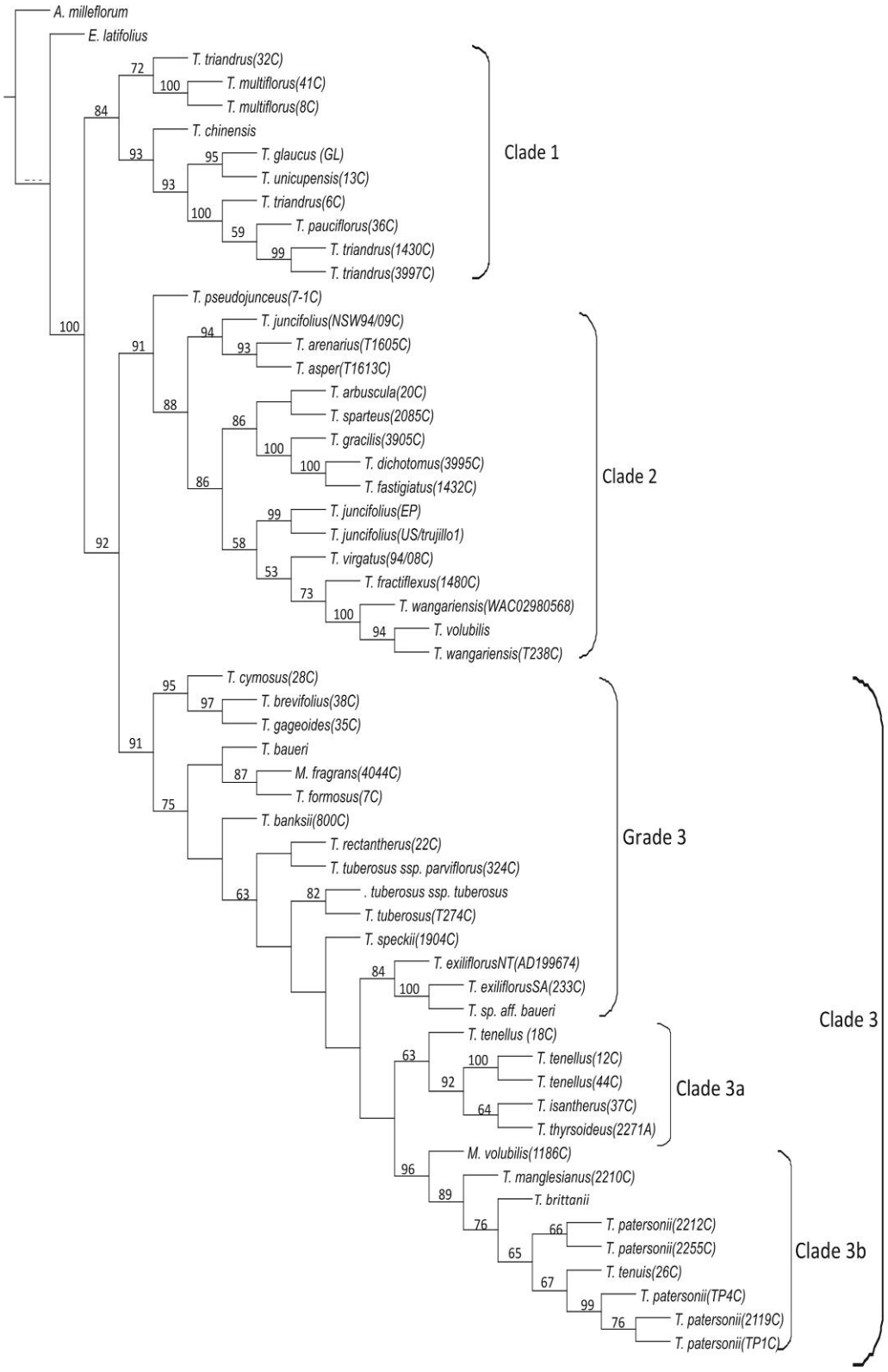


Fig. 3. Single most parsimonious tree resulting from the analysis of the combined data set (400 parsimony informative characters). 293 steps, CI = 0.627, RI = 0.820 and RC = 0.514. Bootstrap values are indicated above the branches.

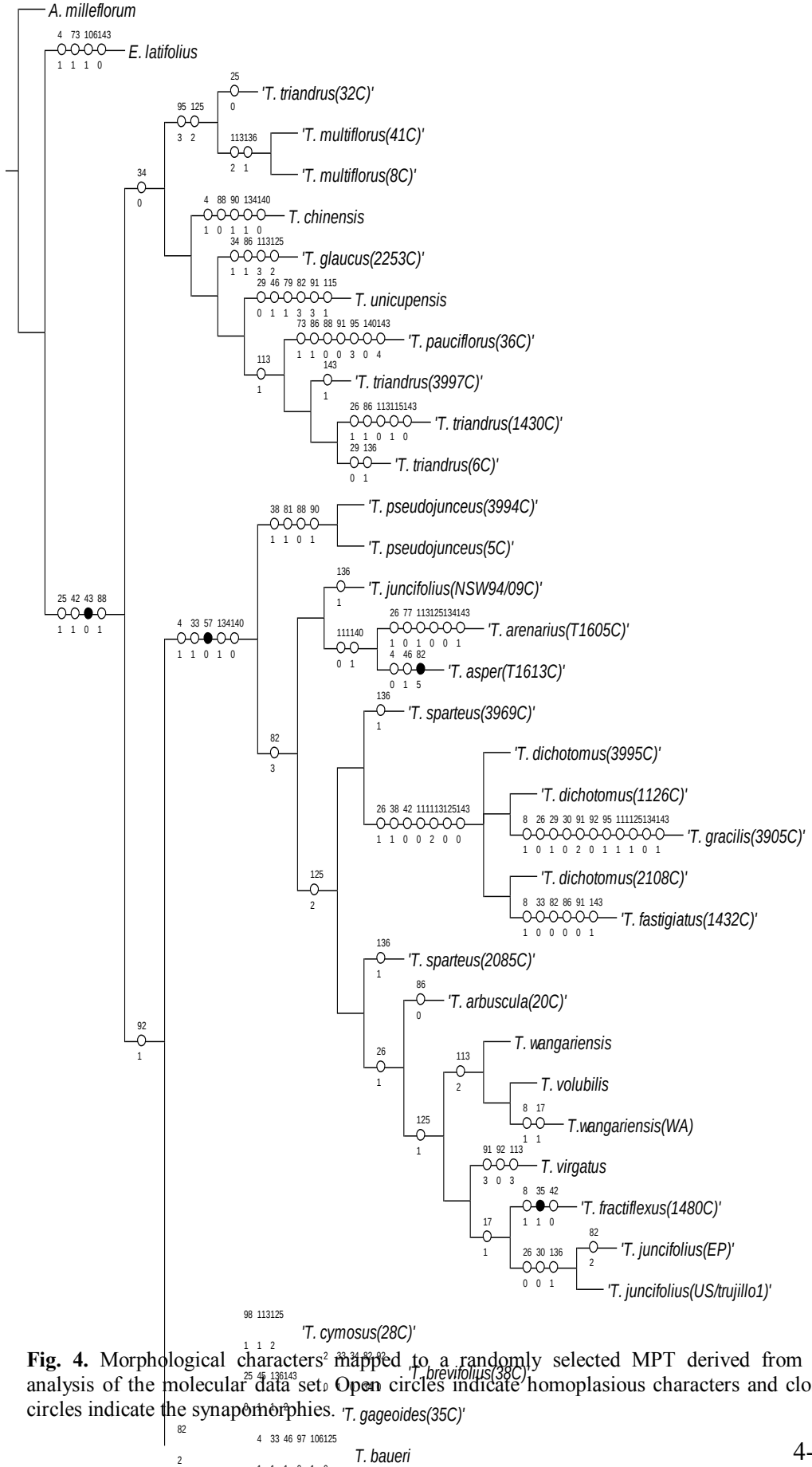


Fig. 4. Morphological characters mapped to a randomly selected MPT derived from the analysis of the molecular data set. Open circles indicate homoplasious characters and closed circles indicate the synapomorphies.

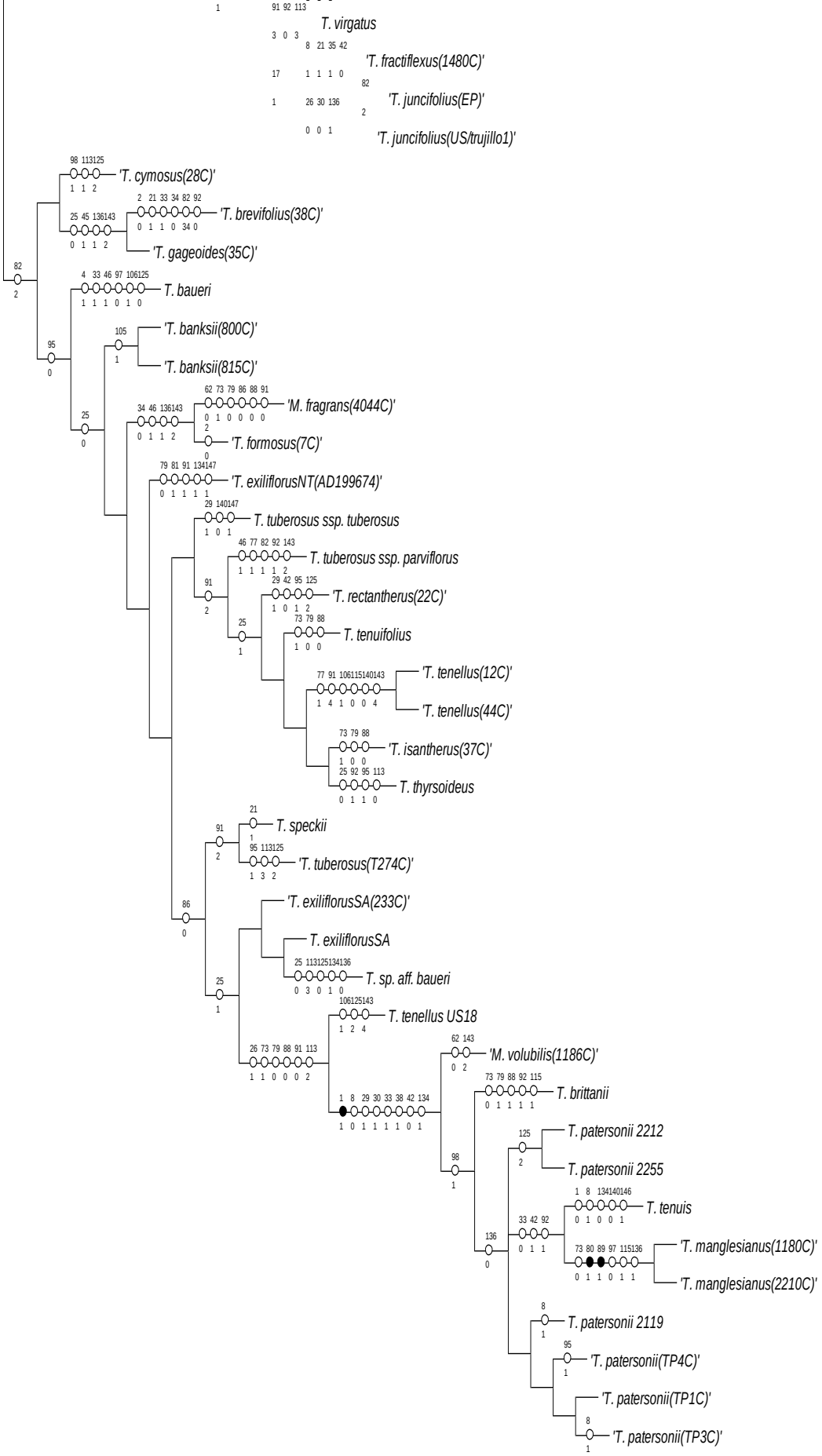
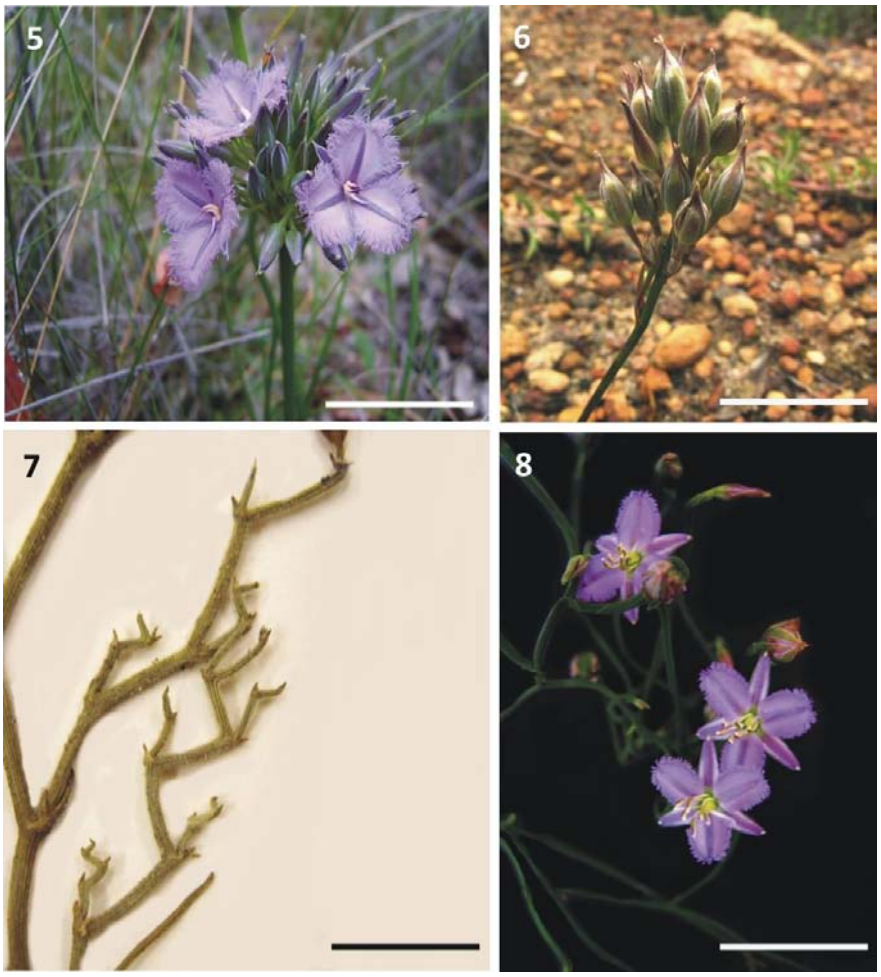


Fig. 4. Morphological characters mapped to a randomly selected MPT derived from the analysis of the molecular data set continued.



Figs. 5–8. Inflorescences, zig-zag branches and climbing habit of *Thysanotus*. 5. Single terminal umbellate inflorescence of *T. multiflorus* (41) from WA. 6. Terminal cluster of umbels in *T. unicupensis* from WA. 7. Peculiar zigzag shaped branches of *T. fractiflexus* endemic to Kangaroo Is. (SA). 8. Twining inflorescence habit of *T. patersonii* (Scale bars: 5, 6 = 2 cm; 7 = 1 mm; 8 = 1 cm)

Murchisonia was clearly polyphyletic, with the two species nested separately within *Thysanotus* in both analyses. *M. fragrans* was sister to *T. formosus* while *M. volubilis* was proximal to the annual climbing *Thysanotus* species crown lineage.

Presence of arillate seeds were common to all clades (Figs. 9–10) and shared with outgroup taxon *Eustrephus*, therefore not a synapomorphic condition for *Thysanotus*.

Discussion

Phylogenetic relationships and character evolution in Thysanotus

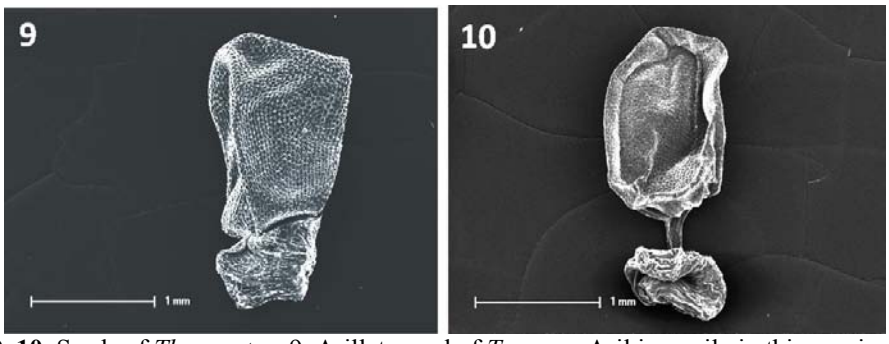
Based on both our molecular and combined morphological data sets it is confirmed that *Thysanotus* including *Murchisonia* is monophyletic which is presented for the first time in the taxonomic history of *Thysanotus* and *Murchisonia*. Congruence between molecular and combined data to a larger extent illustrates the stability of this resulting phylogeny. Monophyly is supported by 100% BS value in both analyses. Discussion will be based on characters when mapped on to the molecular tree that showed character state evolutionary changes in relation to major clades and the characters which have been useful in discussing relationships in the morphological phylogeny in Chapter 3.

After mapping the morphological characters onto the molecular tree, the only reliable morphological synapomorphy was the absence of pendent flowers (43/0). The most easily recognisable inflorescence habits of *Thysanotus* were whether erect or twining/climbing. When this character was mapped, erect inflorescence habit seems to be homoplasious condition in *Thysanotus* restricted to major Clades 1, 2, Grade 3, Clade 3a and basally isolated taxa of Clade 3b while the twining habit is the derived condition restricted to the crown lineage in Clade 3b. Erect inflorescence habit is shared with outgroups, *Arthropodium* and *Eustrephus* and therefore plesiomorphic. This is also congruent with the morphological phylogeny (Chapter 3).

The roots of Laxmanniaceae vary from fleshy or tuberous to fibrous or in some cases wiry roots and stilt roots are present (Pate *et al.* 1984). In *Thysanotus* there are tuberous, fleshy and/or fibrous roots. Tuberous rooted species formed Clade 3 in the molecular analysis, while the basal clades 1 and 2 possessed non tuberous fleshy/fibrous roots. *Thysanotus* shares tuberous roots with both out group taxa and therefore the condition in homoplasious and a reversal. Absence of tuberous roots is a derived condition in *Thysanotus*.

The character state „ridges on the upper part of the aerial axis“ is homoplasious (25/1). This has originated independently in *Thysanotus* and absent from both outgroup taxa. The condition had disappeared and reappeared

several times in the tree. Similar pattern was observed in indumentum on aerial axis (26, 30). Both characters showed a similar evolutionary pattern in the morphological phylogeny (Chapter 3) and can be considered as adaptations to reduce water loss in dry environmental conditions.



Figs. 9–10. Seeds of *Thysanotus*. 9. Arillate seed of *T. asper*. Aril is sessile in this species. 10. Long stalked aril of *T. triandrus* which is common to all 3-staminate species.

Umbels are characteristic for *Thysanotus* and *Murchisonia* (42) and absent from *Arthropodium* and *Eustrephus* (Conran 1998). The character has therefore originated independently in *Thysanotus* and *Murchisonia*, however then reduced to solitary flowers in certain *Thysanotus* species (*T. patersonii, T. dichotomus*) and *M. volubilis*, therefore homoplasious (42/1). Another phylogenetically defining floral feature for *Thysanotus* is the absence of pendent flowered inflorescences (Figs. 11–13). Both outgroup taxa have pendent flowered inflorescences but absent from *Thysanotus* resulting a synapomorphy for *Thysanotus* (43/0).

Presence of annual inflorescences (57) is plesiomorphic condition in *Thysanotus* shared with outgroups and homoplasious. Absence of annual inflorescences (57/0) is a derived condition restricted to molecularly defined Clade 2 which is a synapomorphy for the clade (Clade 2 species possess perennial inflorescences). Therefore the character is phylogenetically significant in understanding *Thysanotus* relationships.

Murchisonia is considered very closely related to *Thysanotus* but differs mainly by the absence of fringed inner tepals (62/0) and *Eustrephus* shares fringed tepals with *Thysanotus* (Conran 1998). There are also some

Arthropodium species with finely fimbriate inner tepals (Conran 1998), however, *A. milleflorum* included in the analysis clearly lacks the fimbriate inner tepals. Therefore the presence of fimbriate inner tepals is homoplasious in *Thysanotus* and is a retained condition from *Eustrephus*. This character supports the phylogenetic affinity between *Eustrephus* and *Thysanotus*.

Figs. 11–13. Inflorescences of *A. milleflorum*, *E. latifolius* and *T. juncifolius*. 11. Pendent flowers of *Arthropodium*. 12. Pendent flowers of *Eustrephus*. 13. Erect flowers of *Thysanotus*. (Scale bars: 11 = 10 mm; 12, 13 = 2 cm)

In the morphological analysis (Chapter 3), presence of three stamens (72/0) clearly formed a synapomorphy representing a distinct evolutionary lineage once recognised by Bentham (1878) and Thongpukdee (1989), however, the molecular results are contradictory to this. Presence of three stamens is not a synapomorphy in the molecular analysis and does not represent a distinct

evolutionary lineage. 3–staminate species clade also includes two 6–staminate species in the molecular analysis. 3–staminate *T. glaucus* nested between 6–staminate *T. chinensis* and *T. unicupensis* showing that loss of outer whorl of stamens occurred three times in the phylogeny.

Thysanotus has both slit and porous dehiscence of anthers (73/0, 73/1) and these features are homoplasious. Slit dehiscence has been retained from *Arthropodium* and porous dehiscence from *Eustrephus* and homoplasious in *Thysanotus* however; front lip extended outer and inner anther pores (80/1; 89/1) form synapomorphies to define *T. manglesianus*. Seed features and their systematic utility are largely unknown for *Thysanotus* and other Laxmanniaceae (Rudall and Cutler 1995; Rudall and Chase 1996; Conran 1998) but present results indicate a high reliability of certain seed characters in understanding phylogeny of *Thysanotus* and related taxa. Presence of arillate seeds (99/1) is a derived condition uniting all *Thysanotus* and *Murchisonia* species and *Eustrephus*.

The significance of anatomical characters in explaining phylogenetic affinities of *Thysanotus*, *Arthropodium* and *Eustrephus* is dealt separately (Chapter 6) for a more detailed understanding.

Previously *T. wangariensis* was considered endemic to SA (Brittan 1978) however; there have been several collections of similar specimens from the south eastern coast of WA. We included one WA specimen in the molecular analysis which revealed that WA and SA specimens are closely related to each other supporting results of the morphology analysis in Chapter 3. Therefore there is greater chance that *T. wangariensis* from WA and SA represents the same species that makes *T. wangariensis* no longer endemic to SA. *T. volubilis* which was previously placed in *T. dichotomus* and *T. patersonii* (Brittan 1981) recovered closely related to *T. wangariensis*.

The close evolutionary relationship of *T. gageoides, T. brevifolius* and *T. cymosus* could perhaps be due to their similar distribution and environmental conditions as they are restricted to south west of WA (Fig. 14). *Thysanotus gageoides* and *T. brevifolius* both share single terminal umbels on the scape with former possessing only two leaves while the latter possessing many leaves. Both possess equal to subequal anthers dehiscing by pores. *Thysanotus*

cymosus possess a branched scape (cymosely branched) which can be considered a convergence which it shares with other distant taxa in the cladogram. Furthermore, *T. cymosus* shares characters such as few leaves (1–2) with basal *T. gageoides* and equal to sub equal anthers dehiscing by pores with both *T. gageoides* and *T. brevifolius* which could probably be considered as retained conditions.

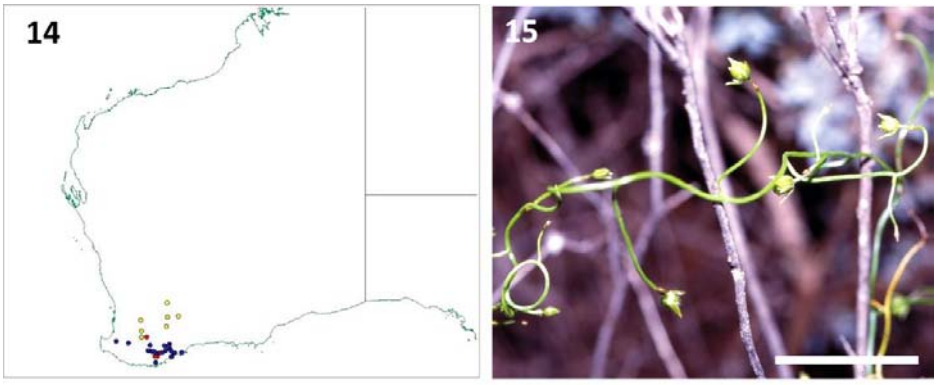
T. exiliflorus specimen from NT /WA border was basal to and distant from *T. exiliflorus* specimens from South Australia. *T. exiliflorus* specimens similar to the NT/WA border specimen (AD199674) have been recently allocated into a new species called *T. ineaqualis* (Macfarlane, pers. comm.). This species is known to be distributed in northern parts of SA and WA and southern NT. Our molecular results clearly support the recognition of it as a new species. Both SA and NT/WA specimens shared a unique feature of having raphide canals and raphides in pith in their stem anatomy. Neither the other *Thysanotus* species nor the out groups possessed this feature. *T.* sp. aff. *baueri* was closely related to *T. exiliflorus* from SA than to *T. baueri* in both analyses, therefore it can be considered a close relative of *T. exiliflorus* from SA. However, this specimen lacked the raphide canals in pith unique to *T. exiliflorus* and possessed many branches from nodes unlike *T. exiliflorus* which possessed 1–2 branches from nodes.

The two subspecies of (*T. tuberosus ssp. tuberosus* and *T. tuberosus ssp. parviflorus*) were closely related to each other in both analyses. Surprisingly, the single *T. tuberosus* specimen from eastern South Australia (specimen no 274) was distant and basal to *T. speckii* without forming a clade with the two subspecies of *T. tuberosus*. Some of the distinguishing features of *T. tuberosus* morphs are as follows. Epidermal cells of the stem TS in *T. tuberosus* (274) were columnar with >1 h/w ratio while the two subspecies possessed square and irregular epidermal cells. *T. tuberosus* (274) had raphide canals in chlorenchyma while these were absent from the chlorenchyma of the other two. Therefore, it may represent a closely related different species/subspecies of *T. tuberosus* however, more sampling from suspected and known localities are required to reach a conclusion.

Our analyses also reveal a distinction between *T. tenellus* from SA and WA. Molecular data indicate a relatively larger distance between *T. tenellus* from SA and WA. The morphological differences between SA and WA forms were previously listed in Chapter 3. Separation of *T. tenellus* from SA and WA in to proper categories should be possible after further sampling and analyses.

The distribution of western and southern annual climbing species in the phylogeny clearly shows a geographical pattern as western species (*T. brittanii, T. patersonii* (WA)*, T. tenuis and T. manglesianus*) were basal to southern ones.

Brittan (1962) and Keighery and Muir (2005) stated the still disjunct species *T. patersonii*. Molecular results also indicate that Western specimens are basal to South Australian specimens and that they are distant, however larger molecular and phenetic studies are required for further clarification. Morphological and anatomical differences between the Western and the South Australian *T. patersonii* were illustrated previously in Chapter 3.



Figs. 14–15. Distribution map of *T. gageoides, T. brevifolius* and *T. cymosus* in WA and the climbing habit of *M. volubilis*. 14. *T. gageoides*–blue, *T. cymosus*–yellow and *T. brevifolius*–red, with similar distributions. 15. Habit of *M. volubilis* is similar to annual climbing *Thysanotus* species. (Scale bar = 5 cm).

Murchisonia and Thysanotus

Thysanotus and *Murchisonia* are closely related by possessing umbellate inflorescences Brittan (1987a; 1987b) and similar seed features (Sirisena 2010). Although *M. volubilis* lack fringed inner tepals and articulation of flowering pedicels, vegetatively resembles an annual climbing *Thysanotus*

species (Fig. 15). Therefore, Black (1943) considered *M. volubilis* to be *T. patersonii* var. *exfimbriatum*, a variety of *T. patersonii*. The close affinity between *Thysanotus* and *M. volubilis* indicated by our molecular and combined results may lead to reconsider this varietal treatment in future Australian flora treatments.

Vegetative features such as presence of tubers, presence of terminal umbellate inflorescence, leaves during flower and fruit and articulation of flowering pedicels also relate *M. fragrans* to *Thysanotus*. Its treatment as a non *Thysanotus* (Brittan 1987a) has always been controversial without a proper systematic study. Our molecular and combined results strongly support the recognition of *M. fragrans* as a *Thysanotus* relative.

Absence of fimbriae in tepals has occurred in *Murchisonia* twice in the phylogeny and the two species were embedded separately in the tree indicating that *Murchisonia* is an artificial assemblage.

New sections for Thysanotus

Taxonomic history of *Thysanotus* does not provide any sections for the genus although Bedford *et al.* (1986) separated other larger genera such as *Lomandra* in Laxmanniaceae into sections. The molecular data and the combined data yielded highly resolved strict consensus trees and enabled us to recognise three sections for the genus based on the three main lineages recognised in the molecular tree (Fig. 16).

Three main lineages within the genus, each representing life history adaptations could be recognised. The basal Clade 1 consists of perennial-leaved, non-rhizomatous and fibrous-rooted plants with annual inflorescences. Of the two distal clades, Clade 2 consists of leafless, fibrous-rooted, rhizomatous plants with perennial inflorescences; and Clade 3 consists of leafy, tuberous-rooted geophytes with annual inflorescences. Within the latter, there is further specialised crown lineage possessing leafless, annual, twining inflorescences.

Section 1- This section includes *T. triandrus, T. multiflorus, T. pauciflorus, T. chinensis* and *T. unicupensis* which form Clade 1 of the molecular tree. The

species are characterised by absence of tubers, presence of fibrous and fleshy roots and presence of simple inflorescences with single terminal umbels or terminal cluster of umbels.

Section 2 – T. juncifolius (SA and NSW forms)*, T. arenarius, T. asper, T. sparteus, T. dichotomus, T. fastigiatus, T. gracilis, T. arbuscula, T. virgatus, T. wangariensis* (from SA and WA), *T. pseudojunceus* and *T. fractiflexus* are included in this section. These species form Clade 2 in the cladogram. Characteristic features are, absence of tubers, presence of rhizomes, absence of leaves during flower and fruit (sometimes leaves present), absence of annual inflorescences (presence of perennial inflorescences) and dichotomous to monopodial branching inflorescences.

Section 3 – This section includes all the species of Clade 3.
Species possess tuberous roots and annual, erect/twining, branched/unbranched inflorescences. Rhizomes are absent from most species. Leaves are produced annually, which last till anthesis except in the annual-climbing species in the crown lineage, the leaves wither very early before anthesis.

Comparison of these three sections is given in Table 6. High level of homoplasy is observed in all sections and this could probably be due to the vast range of habitats that *Thysanotus* occurs in and adapting to these habitat conditions.

Table 6. Comparison of characters of major sections

Character	Section 1	Section 2	Section 3
Habit of inflorescence	Erect	Erect	Erect /twining
Roots	Fibrous	Fibrous	Tuberous
Rhizomes	Absent	Present	Absent
Branched inflorescence axis	Absent	Present	Present/absent
Dichotomous branches	Absent	Present/ absent	Present / absent
Perennial leaves	Present	Absent	Absent
Tuberculate hairs	Absent	Present /absent	Present /absent
Lignified pith	Absent	Present/absent	Absent

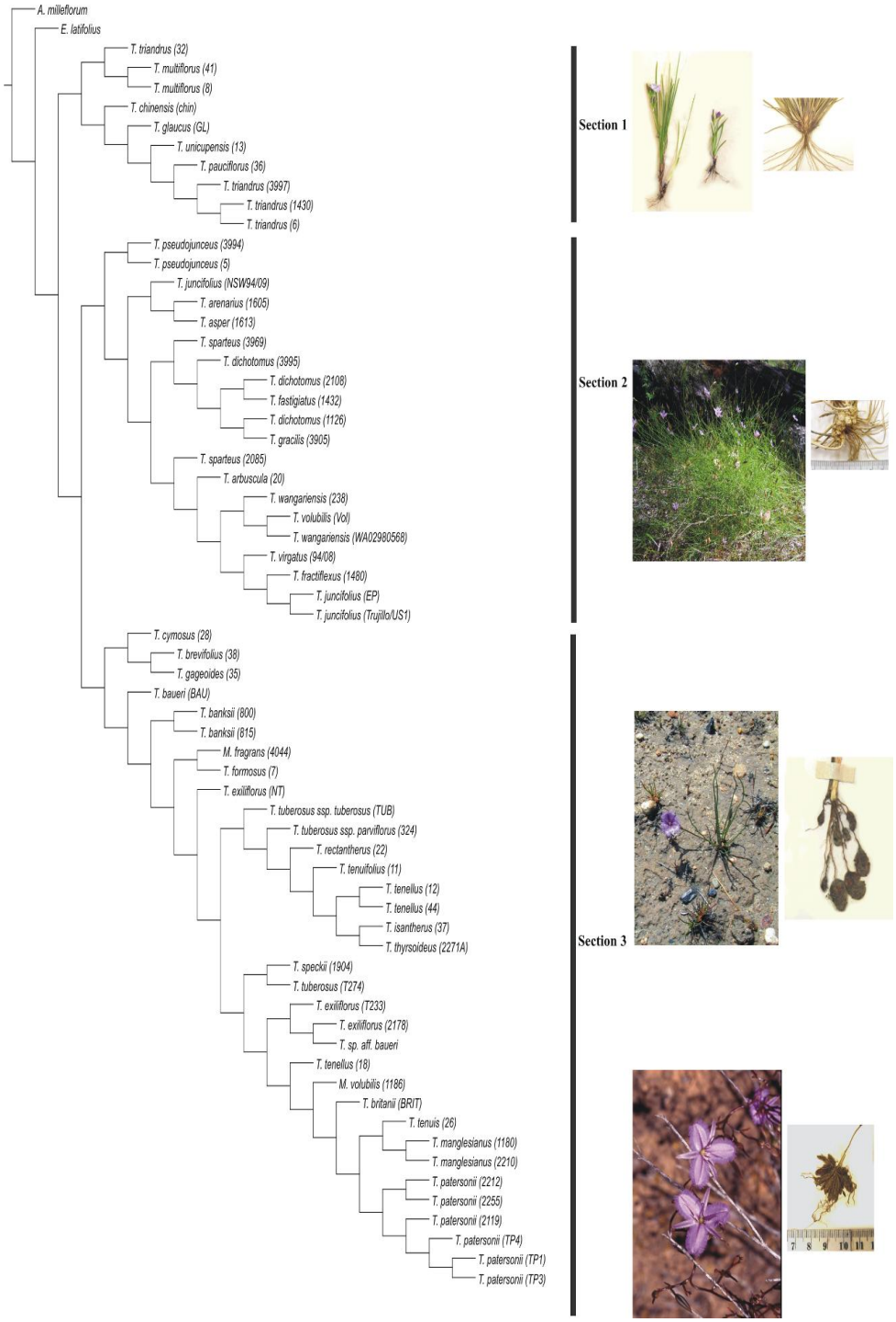


Fig. 16. Strict consensus derived from the molecular analysis with major sections of *Thysanotus*. Sections correspond to the sections described in the text.

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