A Study of Molecular and Morphological Variation Within and Between *Tetratheca* (Tremandraceae) from Windarling and Die Hardy Ranges; Coolgardie District, WA.

By

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Report Prepared for Portman Iron Ore Pty. Ltd.

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Summary & Recommendations:

In contrast to the large amount of sequence variation seen between *Tetratheca paynterae* Alford and both *T. aphylla* F. Muell. and *T. harperi* F. Muell. in the nuclear ribosomal DNA Internal Transcribed Spacer (ITS) region (Butcher *et al.* 2001), there are no unambiguous differences in ITS base pair composition evident between *T. paynterae* and *Tetratheca* collections made from the Die Hardy Range. However, unambiguous sequence variation exists in the non-coding *trn*L-*trn*F region of chloroplast DNA and, although this is small, it is sufficient enough to differentiate these two closely related taxa, both from one another as well as from other species of *Tetratheca* within the same geographical area.

As well as variation at the molecular level, *T. paynterae* and *Tetratheca* (Die Hardy Range) display a large number of consistent and statistically significant morphological differences which readily identify these as distinct taxa, despite their similarity. The best characters for the discrimination of these taxa include the pubescence of the ovary, the shape of the receptacle, the pubescence of the upper and lower leaf surfaces, the pubescence of the calyx and peduncle, and the colour and fusion of the anthers. It is therefore proposed that *Tetratheca* (Die Hardy Range) be recognised as a new species. Additional taxonomic findings of this report, based on both molecular and morphological variation, are that plants previously identified as *T. aphylla* collected from just south of Eneabba should be recognised as a new species, whilst collections from near Newdegate should be recognised as a subspecies of *T. aphylla*.

Cladistic analyses of the separate and combined ITS and *trn*L-*trn*F sequence data sets illustrate a well supported sister taxon relationship between *T. paynterae* and *Tetratheca* (Die Hardy Range). Analyses also show that these two species are highly divergent in both nuclear and chloroplast sequences from other members of the *T. aphylla* group and this finding is consistent with morphological differences in phylogenetically significant features such as ovule number across the group. Based on *trn*L-*trn*F sequences, *T. paynterae* and *Tetratheca* (Die Hardy Range) can be seen to be more closely related to *T. rupicola*, a New South Wales endemic with which they share

the possession of two ovules per locule, than to the other *Tetratheca* included in this study from the same geographical area, all of which possess a single ovule per locule. This would indicate that *T. paynterae* and *Tetratheca* (Die Hardy Range) belong to a genetic lineage that diverged from that of *T. aphylla* (*et al.*) in the distant past and that the superficial similarity of these 'leafless' species is due to convergence in response to environmental pressures.

Introduction & Background:

This report follows on from previous work (Butcher *et al.* 2001) funded by Portman Iron Ore to investigate the nuclear ribosomal DNA (nrDNA) Internal Transcribed Spacer (ITS) sequence variation amongst Declared Rare *Tetratheca* growing at Bungalbin Hill (*T. aphylla* F. Muell.), Mt Jackson and Muddarning Hill (*T. harperi* F. Muell.) and 'Windarling Range' (*T. paynterae* Alford). The findings of this earlier report supported morphological evidence that these three species were distinct despite the superficial similarity of *T. aphylla* and *T. paynterae*. Background information on the taxonomic issues in the genus and this 'species group' (*sensu* Thompson 1976), as well as a discussion of the morphological characters useful for the differentiation of *T. aphylla* and *T. paynterae* can be found in Butcher *et al.*(2001) as well as in Alford (1995) and will not be repeated in detail here.

Continued survey of the banded ironstone hills north of Koolyanobbing in late 2001, associated with Portman's Expansion Project, located a new population of *Tetratheca* in the Die Hardy Range. Plants collected from this location as voucher specimens had a close morphological affinity to *T. paynterae,* as represented by the type population at Windarling, but differed most noticeably in vestiture of the calyx, peduncles and ovary, as well as the degree of fusion of the anther filaments and the shape of the receptacle (see Butcher 2001 for a full discussion). Morphological assessment of these specimens by R. Butcher (Department of Plant Biology, The University of Western Australia) and M. Duretto (Royal Botanic Gardens, Melbourne) identified that the plants from the Die Hardy Range represented a new, undescribed taxon of *Tetratheca*, but based on the limited flowering material available for study at that time, it was unclear whether these morphological differences were consistent and whether *Tetratheca* (Die Hardy Range) should be recognised as a new species or as a subspecies of *T. paynterae sensu stricto*.

The morphological characters shared between *T. paynterae* and *Tetratheca* (Die Hardy Range) are both taxonomically and evolutionarily significant (Thompson 1976, Alford 1995) and include the possession of two ovules per locule (total of four ovules per flower), broadly rounded stem tubercules, short anther filaments relative to the

anther body, the size and shape of the calyx segments, a yellow spot at the base of the petals and a distinctive, musky floral scent (Butcher 2001). As the Die Hardy Range is only *c.* 10 km NE of 'Windarling Range', the close geographical proximity of these taxa and the shared possession of these characters suggests a sister taxon relationship exists between *T. paynterae* and *Tetratheca* (Die Hardy Range).

In addition to the newly discovered *Tetratheca* (Die Hardy Range), two collections with very close morphological affinity to *T. aphylla* have also been made in recent years; the first from the Eneabba area and the second from near Newdegate. Like *T. aphylla*, both of these taxa have one ovule per locule, a leafless appearance and dense, acute tubercules on their stems, but they are ecologically distinct from *T. aphylla* and have been collected from upland heath communities in grey-white sands over laterite. Although both taxa have been identified as *T. aphylla* in the past, floral morphology clearly indicates that plants from near Eneabba belong to a new, undescribed species, but the taxonomic distinctness of the Newdegate material from *T. aphylla* is still uncertain. From the small number of herbarium specimens examined to date it would appear that *T. aphylla*, as represented from the Helena and Aurora Range, and *T.* aff. *aphylla* (Newdegate) may be the same species despite their disjunct distribution, as their floral morphology is nearly identical. However, there are slight differences evident in the curvature of the anthers and the length and thickness of the anther filaments that suggest that plants from the Newdegate area might warrant formal recognition at the rank of subspecies. As these plants clearly belong in the *T. aphylla* group and their taxonomic status has not been determined, individuals from both the Eneabba and Newdegate populations have been included in this sequencing study. The sequence data obtained from these taxa will allow their relationship to *T. aphylla* and other taxa within this species group to be investigated as well as provide a comparative base for the assessment of patterns of morphological and sequence variation between *T. paynterae* and *Tetratheca* (Die Hardy Range).

Molecular tools for species discrimination:

As outlined in Butcher *et al.* (2001), molecular genetic evidence at both population and species level has proved to be an extremely useful and powerful tool for identifying conservation units and determining taxon boundaries (Coates & Sokolowski 1992, Byrne 1999, Byrne *et al.* 1999, Coates & Hamley 1999, Coates 2000), with DNA sequencing studies now commonplace for the investigation of organismal relationships at the generic and specific ranks. For the investigation of species-level relationships, the most frequently sequenced regions of the plant genome are ITS (see Baldwin *et al.* 1995 for a review, Bena *et al.* 1998a, 1998b) and *trn*L-*trn*F (see Sang *et al.* 1997 for a review, Bayer *et al.* 2000), although research is ongoing into the utility of the nrDNA External Transcribed Spacer (ETS) (Baldwin & Markos 1998, Bena *et al.* 1998a, 1998b, Clevinger & Panero 2000, Linder *et al.* 2000) as well as the non-coding chloroplast DNA (cpDNA) spacer *psb*A-*trn*H (Sang *et al.* 1997) for low level phylogenetic reconstruction, with these both these regions purported to evolve more rapidly than ITS and *trn*L-*trn*F, respectively.

The ITS region is part of a tandemly repeated, multicopy, nuclear gene family coding for ribosomal RNA (ribonucleic acids) and is comprised of the 5.8S gene and two flanking spacer regions, ITS-1 (between the 18S and 5.8S genes) and ITS-2 (between the 5.8S and 26S genes) (Figure 1 A, B). Whilst the genes are highly conserved across a wide range of plants and fungi, the spacer regions are not subject to the same evolutionary constraints and evolve rapidly, making them extremely useful for low level systematic studies. However, due to the high number of repeated copies in the genome, ITS is subject to a degree of intraindividual and intraspecific polymorphism (Takabayashi *et al.* 1998) which can make the interpretation of sequence data and resultant taxon relationships difficult. This was evident in sequence data obtained for *T. paynterae*, *T. aphylla* and *T. harperi* in 2001, where double peaks, representing the presence of two different nucleotides at a particular base position in different copies of ITS, were frequently seen in chromatograms (Butcher *et al.* 2001).

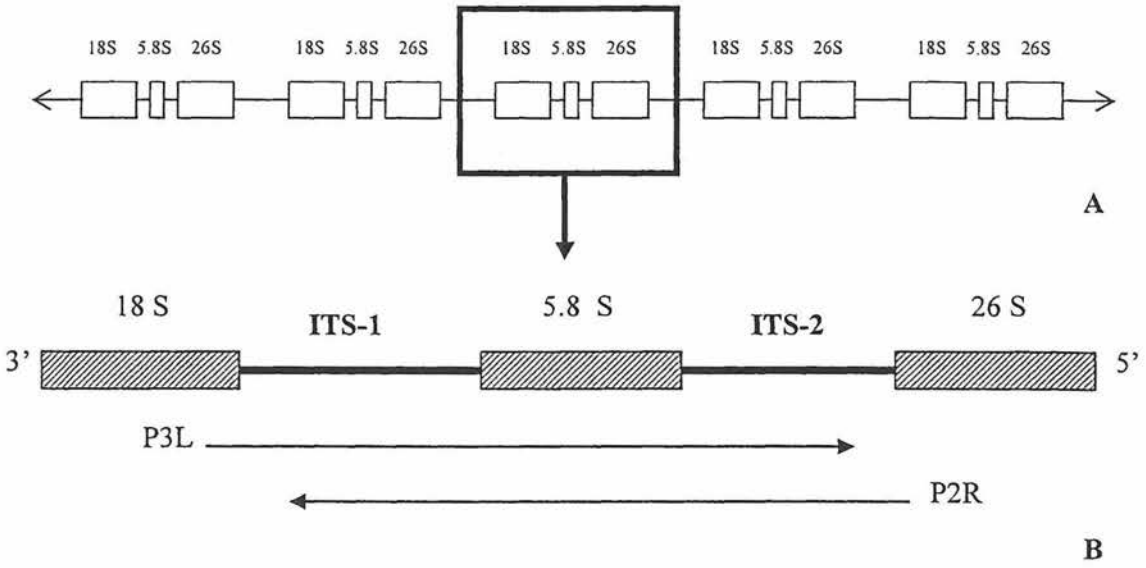

Figure 1: Diagrammatic representation of nuclear ribosomal DNA illustrating the tandem repeat structure of the multi-copy RNA gene family (A) and the positions of ITS-1 and ITS-2 flanking the 5.8 S gene (B). The annealing positions and directionality of the primers P3L and P2R used in the amplification and sequencing of the ITS region are illustrated.

In contrast to the nuclear genome, the chloroplast genome is not subject to recombination and is inherited, in angiosperms, as a single copy through the maternal line. As a consequence, cpDNA evolves slowly and coding sequences are generally not particularly informative of relationships at the specific and intraspecific level, but non-coding regions have been shown to display a high frequency of mutation in some taxa (Palmer *et al.* 1988). Variation in genes and spacers is usually manifest as length polymorphisms due to insertion/deletion events, which may include long repeats and inverted repeats, as well as single and multiple base nucleotide substitutions. The phylogenetically informative (Sang *et al.* 1997, Bayer *et al.* 2000) *trn*L-*trn*F region is comprised of the *trn*L intron (between the 5' and 3' *trn*L exons) and the intergenic spacer between *trn*L and *trn*F (Figure 2). This region has proved to be a useful and convenient tool in molecular genetic studies as several hundred base pairs of non-coding sequence are interspersed between conserved genes, allowing universal primers anchored in the genes to be designed and the non-coding regions to be sequenced (Taberlet *et al.* 1991).

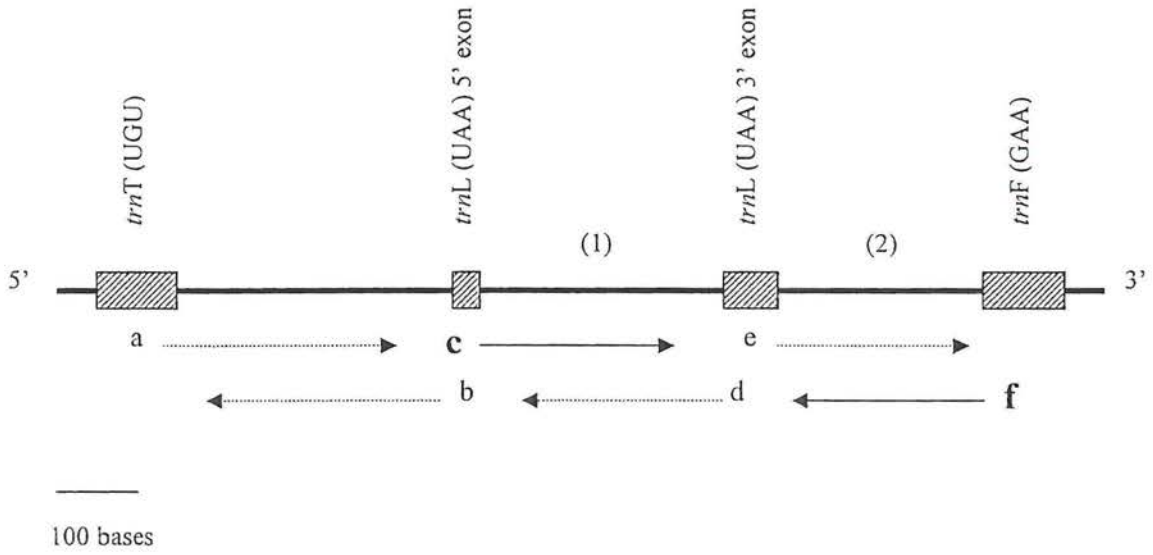

Figure 2: Composition of the *trn*T-*trn*F region of chloroplast DNA indicating the coding (shaded boxes) and non-coding regions commonly used in phylogenetic studies. This study utilised the *trn*L intron (1) and the intergenic spacer between the *trn*L 3' exon and the *trn*F gene (2). Primers *trn*-c and *trn*-f (emboldened) were used in this study and their annealing positions and directionality are indicated.

As the nuclear and chloroplast genomes display different modes of inheritance it is highly recommended (Doyle 1992) that both plastid (chloroplast and mitochondrial) and nuclear sequence data are obtained and compared for any study group, and that data are combined for further analyses where congruence is observable in tree topologies. In this manner, data from different sources can inform on, and lead to improved resolution of, relationships between taxa. As sequence data obtained from the ITS region was found to provide a large number of nucleotide characters for the discrimination of *T. paynterae*, *T. aphylla* and *T. harperi* (Butcher *et al.* 2001), this region is being examined again in this study, in conjunction with the *trn*L intron and the *trn*L-*trn*F intergenic spacer (collectively called the *trn*L-*trn*F region), to assess the level of distinctness of *Tetratheca* (Die Hardy Range) from *T. paynterae*, and the relationships of the *T.* aff. *aphylla* collections (Eneabba and Newdegate) to other taxa within the *T. aphylla* group.

Multivariate morphometrics for distinguishing taxa within species complexes:

Discriminant analyses are used in systematic studies such as this, primarily as a means of differentiating between similar groups of organisms (e.g. Lamont *et al.* 1987, Mackay & Morrison 1989, Hart & Henwood 1996, Krauss 1996, Elliot *et al.* 2002) and are powerful tools for the simple separation of taxa and the determination of statistically significant differences between *a priori* determined groups (e.g. individuals of *Tetratheca* growing at 'Windarling Range' *versus* individuals growing at the Die Hardy Range). Through discriminant analysis, variables, such as vegetative and floral measurement characters, are identified which have the power to accurately discriminate between groups. These are then used to compute a canonical variate that represents the differences between the groups and this in turn can be used as an axis for the graphical representation of total variation between the groups. Canonical variates analysis (CVA) is widely used due to its ability to maximise the variation between groups relative to the variation within groups and is very robust to departures from homogeneity in data, with multivariate normality required only when statistical testing is being performed (Blackith & Reyment 1971, Krauss 1996).

Study sites:

The species of *Tetratheca* examined in this study exhibit highly restricted distributions, and of the taxa occurring north of Koolyanobbing, each is known from only a single, small range (Figure 3; Alford 1995, Brown *et al.* 1998). Of the three Declared Rare taxa sequenced in 2001, *T. harperi* is found only on Mount Jackson and Muddarning Hill, *c.* 65 km NNW Bullfinch, where this species is locally abundant but restricted to very shallow soils and rock crevices in cliff faces and rocky outcrops. In this habitat *T. harperi* occupies the same ecological niche as the Priority Listed species *Jacksonia jackson* Chappill. *Tetratheca aphylla sensu stricto* has been collected from throughout the Helena and Aurora Ranges and is locally common over an area of *c.* 12 km, growing in shallow, well drained, gravelley soils on moderately exposed, steep, stony slopes as well as at the base of hills. Comparatively, *T. paynterae* is restricted to

the 'Windarling Range' (an unnamed range *c.* 7 km N of Windarling Peak) where the majority of plants (*c.* 2000) grow in rock crevices on the north facing, exposed slopes of the 'W3 Deposit'. Survey carried out in late 2001 and early 2002 located additional plants of *T. paynterae* on the western end of the 'W5 Deposit', such that three small subpopulations of *c.* 30 plants each can be found on this low ridge to the south of 'W3'. However, the recorded sighting of two plants of *T. paynterae* on 'W4' could not be confirmed in follow up surveys and may represent a misidentification.

At the time material was collected for molecular analysis, two populations of *Tetratheca* (Die Hardy Range) had been identified from the Die Hardy Range, *c.* 10 km NNE of the 'Windarling Range'. Recent survey of the Die Hardy Range by Ecologia consultants estimated that each of these populations comprises *c.* 3000 individuals, and also located a third, smaller, population of *c.* 800 plants (Figure 4). Like *T. paynterae* and *T. harperi*, plants of *Tetratheca* (Die Hardy Range) are restricted to exposed cliff faces and ironstone breakaways which, in this Range occur primarily on the eastern and south-western faces. Survey of the Yokradine Hills, which run more-or-less parallel with the Die Hardy Range (Figure 4), did not reveal the presence of any *Tetratheca* species although small areas of rocky outcrop occur. Similarly, no *Tetratheca* species were located on or around Mount Geraldine, which lies *c.* 4 km SE of the Die Hardy Range (Figure 4).

Of the other taxa examined in this study, *Tetratheca* aff. *aphylla* (Newdegate) has been collected from between 15-20 km E of Newdegate along the Newdegate-Lake King Road and from along Creek Road, *c.* 20 km SE of Newdegate and at both of these sites occurs in grey-white clayey sands in remnant heath and low shrubland near hill crests in undulating landscape (Figure 5). Comparatively, *Tetratheca* (Eneabba) has been identified as occurring just south of Eneabba to the west of the Brand Highway and in the South Eneabba Nature Reserve and appears locally restricted to this region of the northern sandplain. This taxon grows in gravelly sand in upland areas on lateritic ridges and small breakaways in kwongan heath communities (Figure 6).

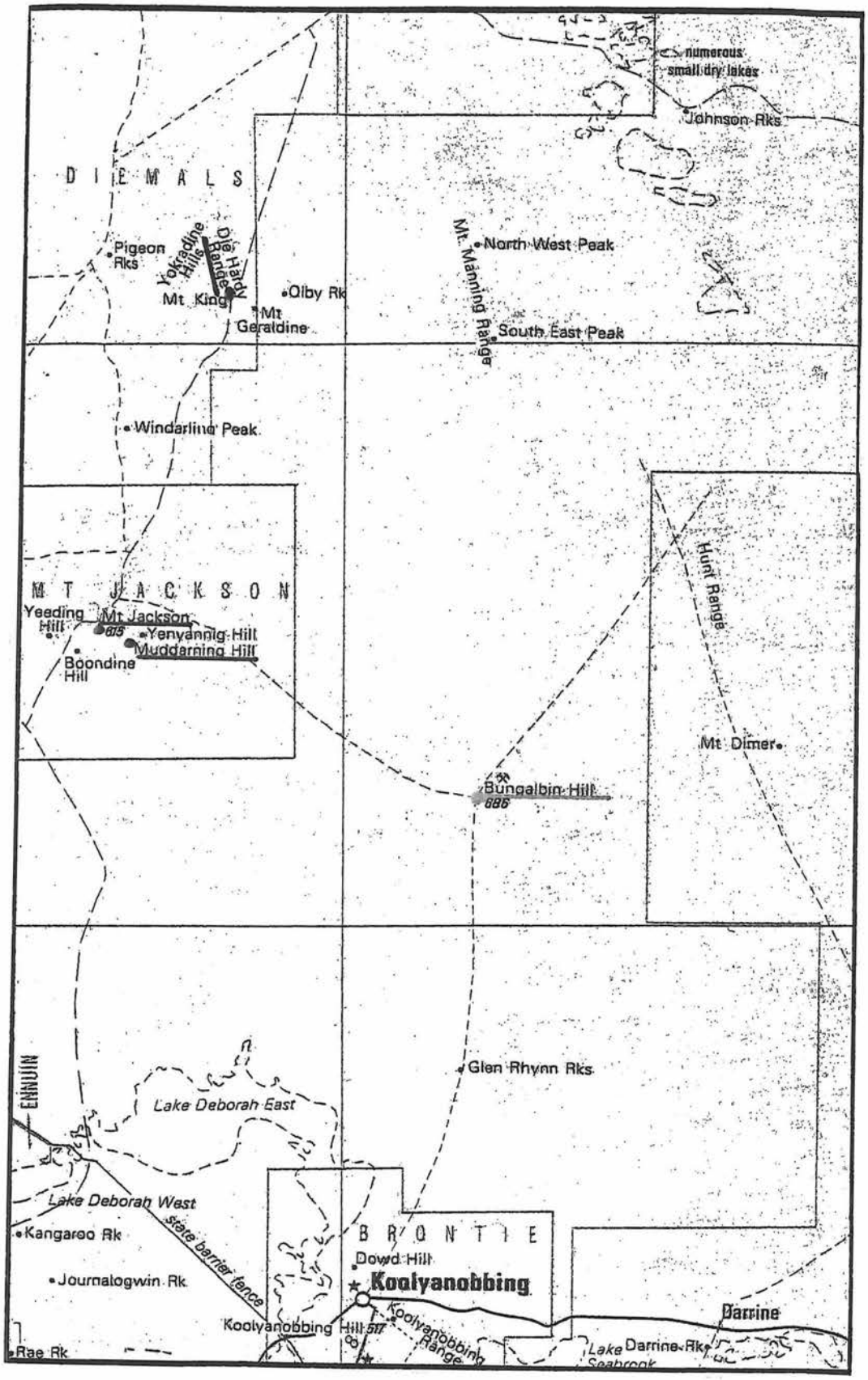

Figure 3: Map showing the location of the hills north of Koolyanobbing upon which the various species of *Tetratheca* grow. Their distributions are as follows and are presented in a south to north order; *T. aphylla*- Bungalbin Hill, Helena and Aurora Range; *T. harperi*- Mt Jackson and Muddarning Hill; *T. paynterae*- 'Windarling Range', *c.* 7 km N of Windarling Peak and *Tetratheca* (Die Hardy Range)- Die Hardy Range. Survey in June 2002 determined that *Tetratheca* was not found on the Yokradine Hills or on Mt Geraldine.

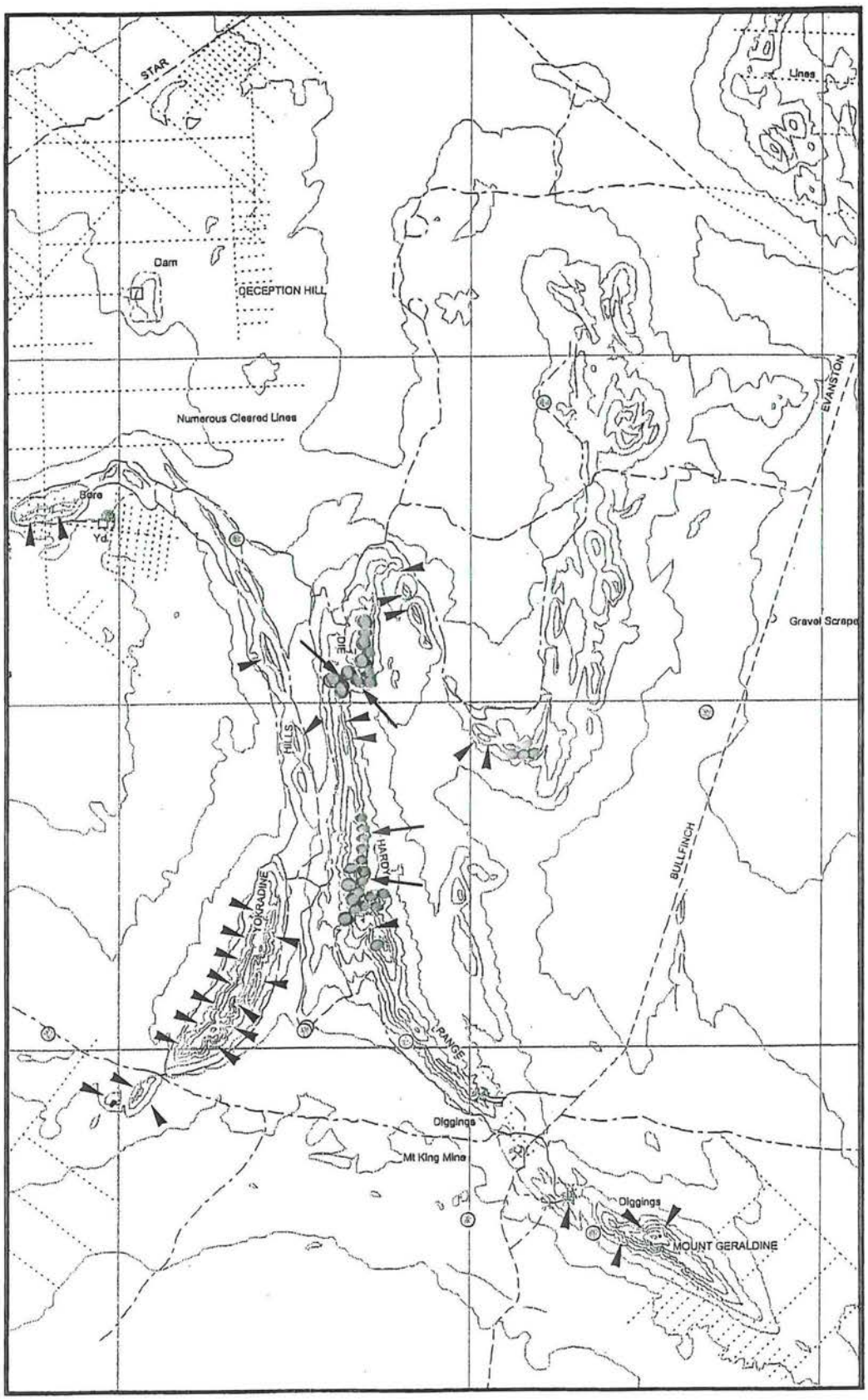

Figure 4: Distribution *Tetratheca* (Die Hardy Range). Three populations have been identified with material for the molecular study being taken from Populations 1 and 2, with the specific collection sites indicated on the map by ⟶. Plants for morphometric analysis were collected from across all three populations and are indicated by aquamarine dots. The close proximity of the Yokradine Hills and Mt Geraldine, where *Tetratheca* is not present, can be discerned from this map. Areas surveyed without success indicated by ►.

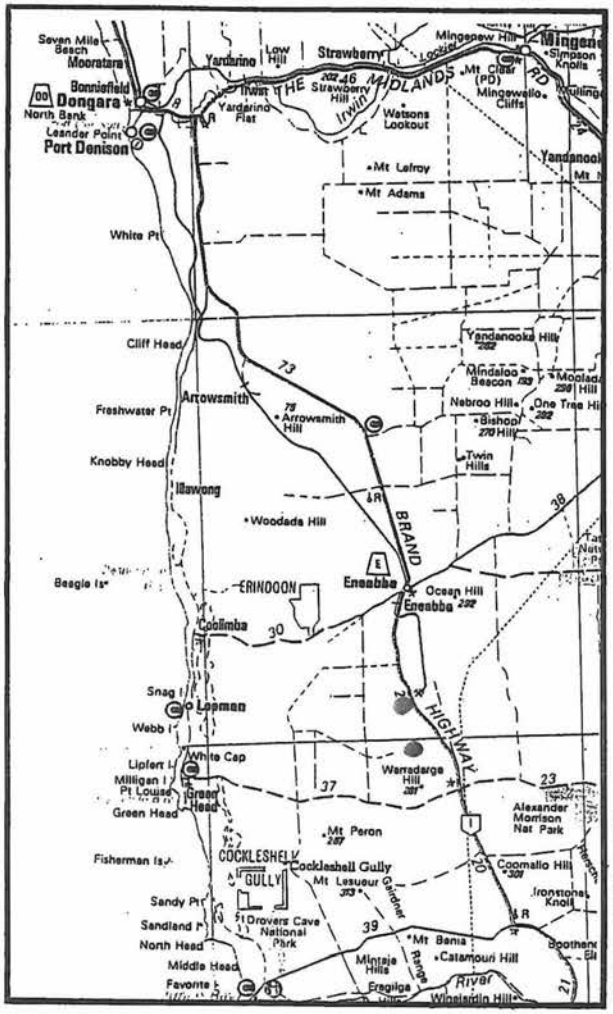

Figure 5: Map showing the distribution of *Tetratheca* (Eneabba) collections to the south-west of Eneabba represented by red dots. It is probable that this taxon occurs at other suitable sites in this immediate area.

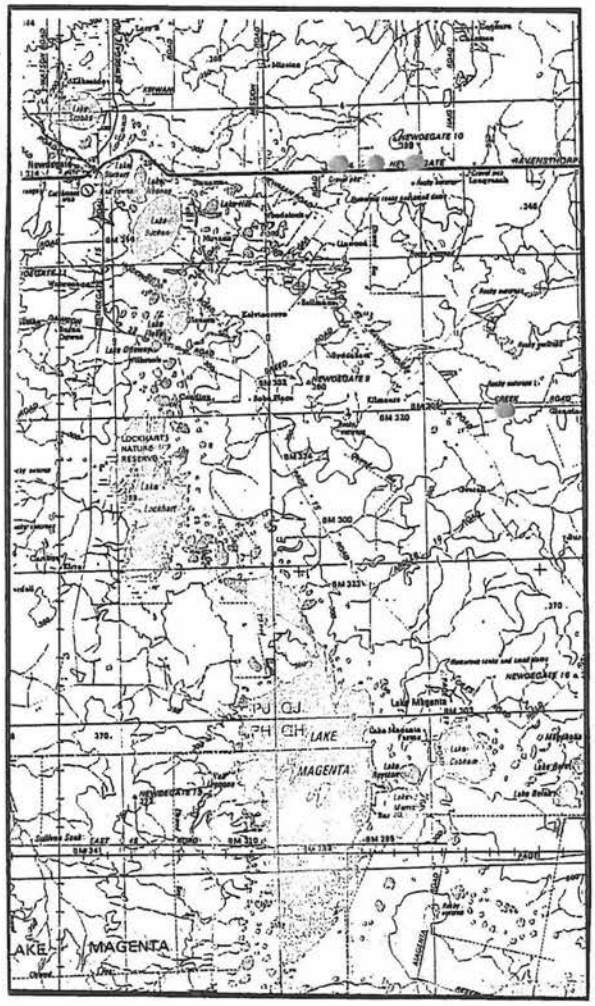

Figure 6: Map showing the distribution of *Tetratheca* aff. *aphylla* (Newdegate) collections to the east and south-east of Newdegate represented by aquamarine dots. It is probable that this taxon occurs at other suitable sites in this immediate area.

Study Aims:

- To examine whether ITS and *trn*L-*trn*F sequence variation exists between individuals of *T. paynterae* and *Tetratheca* (Die Hardy Range), in order to determine whether *Tetratheca* (Die Hardy Range) represents a distinct taxon.
- To compare the degree of sequence divergence between these two populations with ITS and *trn*L-*trn*F sequence data from *T. aphylla* and *T. harperi* as well as *T.* aff. *aphylla* (Newdegate) and *Tetratheca* (Eneabba) to assess the evolutionary patterns in the group and the possible taxonomic ranking that *Tetratheca* (Die Hardy Range) should be given.
- To compare vegetative and floral features of *T. paynterae* and *Tetratheca* (Die Hardy Range) by means of a multivariate morphometric study to determine whether these taxa can be unambiguously differentiated by their morphology.

As this report comprised two phases of study: the first involving the molecular level investigation of sequence variation between taxa in the *T. aphylla* group, and the second involving the morphological investigation of variation within and between *T. paynterae* and *Tetratheca* (Die Hardy Range), the remainder of this report will be divided into two sections (Molecular and Morphology), treating the methods and results of each of these study phases separately and then bringing the conclusions of each into a General Discussion.

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Methods:

Material sampling:

Young buds, leaves and stem material were collected for DNA extraction from 15 individuals of *Tetratheca* (Die Hardy Range) from across the extents of the two large populations known to exist at the time (Figure 4). As conditions had been drier in the Newdegate area, there were few buds and green leaves present on plants of *T.* aff. *aphylla* (Newdegate) and mainly stem material was collected for 15 individuals across both known locations for this taxon. Comparatively, buds were abundant on plants of *Tetratheca* (Eneabba) and these were collected in addition to stem and leaf material for 18 individuals across the two known populations. Although plant material from a large number of individuals was taken, the actual number sequenced per taxon was much lower due to difficulties in obtaining enough material for DNA extraction from some plants, as well as low DNA levels and problems with PCR amplification and sequencing. Similar problems were encountered in the sequencing of *T. aphylla*, *T. harperi* and *T. paynterae* (Butcher *et al.* 2001).

DNA extraction, amplification and sequencing:

The methods for the extraction of DNA from *Tetratheca* (Die Hardy Range), *Tetratheca* (Eneabba) and *T.* aff. *aphylla* (Newdegate), as well the amplification and sequencing of the ITS and *trn*L-*trn*F regions are as outlined in Butcher *et al.* (2001) and are included here as Appendix 1. Variations from these methods include the following:

- In this molecular section *Tetratheca* (Die Hardy Range) has been given the short-hand notation of TDH, representing its locality, *Tetratheca* (Eneabba) has been designated as TAE, representing its affinity with *T. aphylla* and its locality, and *T.* aff. *aphylla* (Newdegate) has been called TAN for the same reasons. Other species

are represented as for Butcher *et al.* (2001), i.e. *T. aphylla* (Bungalbin Hill) as TAB, *T. paynterae* ('Windarling Range') as TW and *T. harperi* as TH.

- DNA extractions were made from mature leaves when present, the bases of deciduous leaves and bracts, and young buds collected and stored in liquid nitrogen in the field, as well as from stem scrapings stored at -80°C prior to extraction. Leaf base and bud material was very scarce per plant for TDH with DNA yields from these tissues very low in some individuals. DNA extracted from stem scrapings was more degraded than that obtained from buds or leaf/bract material.
- Extractions from TAN and TAE plants using the Qiagen DNeasy® Plant Mini Prep Kit were made utilising a 20% higher volume of Buffer AP1 and Buffer AP2 than in the manufacturer's instructions (J. Bradford pers com.) with apparently good results, but amplification difficulties in these two taxa suggested that additional compounds in the fresh buds were interfering with PCR reactions.
- Two primer pairs, ITS4 & ITSLeu1 (Mast 1998 after White *et al.* 1990) and P3L & P2R (P. Weston, pers. comm.) were found to successfully amplify and sequence the ITS region in *Tetratheca*, with the best sequence results for TW and TAB being obtained using the Weston primers (Butcher *et al.* 2001). Consequently, the primers P3L and P2R have been used exclusively to amplify and sequence TDH, TAE and TAN individuals in this study. Their annealing positions are indicated in Figure 1.
- Purification of the PCR amplified ITS and *trn*L-*trn*F regions in TDH, TAN and TAE individuals was carried out using a Qiagen QIAQuick® PCR Purification Kit according to the manufacturers specifications and the final elution volume comprised 30 µl of supplied EB Buffer (10 mM Tris.Cl, pH 8.5). Purified DNA was not precipitated and resuspended as for TAB, TW and TH (Butcher *et al.* 2001).
- Sequence chromatograms were checked and manually corrected where polymorphisms were observed using SeqEd v 1.0.3 (Kececioglu and Myers 1992), and pair-wise, multiple sequence alignments for all data sets obtained for the six taxa were performed using ClustalW (Thompson *et al.* 1994) according to the default settings. ITS and *trn*L-*trn*F sequence data for all the *Tetratheca* taxa in these studies will be lodged with GenBank.

Phylogenetic analysis:

Following their correction and alignment, ITS and *trn*L-*trn*F sequence characters were entered into a data matrix in MacClade (v 3.05, Maddison & Maddison 1992) and analysed using the phylogenetic software PAUP* (v 4.0b4a, Swofford 2000). Previously published *trn*L intron and *trnL-trn*F intergenic spacer sequences for *Tetratheca rupicola* J. Thompson (Bradford & Barnes 2001), a Sydney region endemic, were downloaded from GenBank and included in some analyses to provide further comparison for sequence divergence in these non-coding chloroplast regions. No additional ITS sequences were available. Where ITS and *trn*L-*trn*F data sets were combined, two individuals of each taxon which had been sequenced for both spacers were included, with data for two different individuals of TDH (TDH 5 & TDH 10) needing to be combined to provide a complete data set for a second TDH terminal taxa.

Both parsimony and maximum likelihood analyses were performed for each of the data sets as well as for the data sets combined. Parsimony analyses of the data were performed using the following parameters: all characters were unweighted and unordered, transitions (i.e. purine to purine or pyrimidine to pyrimidine) were regarded as twice as likely as transversions (i.e. purine to pyrimidine and *vice versa*), variable sites (where two or more different bases were evident at the same position in the chromatogram), were coded as polymorphisms, gaps (representing indels) were coded as fifth bases and trees were unrooted. Maximum likelihood analyses employed the following parameters: empirical base frequencies were used, among site rate variation was treated as equal and a molecular clock model was not enforced, gaps were treated as missing data and all trees were unrooted.

Heuristic searching was employed with initial trees generated by simple, step-wise addition sequences prior to 1000 random addition sequence replicates employing tree bisection-reconnection branch swapping being performed. Bootstrap values, providing an estimate of branch support, were calculated from 10 000 bootstrap replicates of 10 random addition sequences with trees rooted using TH. Although this did not represent an ideal situation, this taxon had not been hypothesised as being part of a sister-species association that required further investigation (e.g. TW & TDH; TAB

& TAN + TAE), and so its position as the root was not felt to compromise tree topology. In *trn*L-*trn*F analyses, trees were rooted using both TH and *T. rupicola* to investigate the placement of each species and the resultant topologies.

Results:

ITS:

Aligned ITS sequences for all the samples utilised in the study showing the positions of the spacers ITS-1 and ITS-2 and the 5.8 S gene are provided in Appendix 2 and the base positions at which robust variations occur are highlighted in yellow. It can be seen that within species variation is negligible and occurs almost exclusively as single base change events i.e. one individual will possess a different base to all other individuals at a particular site; either a substitution, an inserted or deleted base, or an actual polymorphism with more than one base being represented at that site. This variability was generally correlated with samples for which the quality of the DNA extracted was poor or the quantity was low.

It is evident from the ITS sequence data that TAB, TW, TH and TAE are four extremely distinct species, as clearly indicated by morphology (Thompson 1976, Alford 1995, Brown *et al.* 1998), with 65 unambiguous nucleotide sites found to be informative for species discrimination (Table 1). Of these variable sites, the majority (44) can be found in the ITS 1 region, which possesses an area of *c.* 50 difficult to align bases just before the start of the 5.8 S gene. The greater variability of the ITS-1 region is consistent with findings for other taxa in a wide range of families (e.g. Baldwin 1992, Baldwin *et al.* 1995, Mast 1998). The ITS 2 region yields 21 informative sites, and there is a significant single base substitution in the highly conserved 5.8 S gene (at bp 400) which unifies TW and TDH (T) and differentiates these from all other taxa (G).

Table 1: A summary of variable bases in the ITS region for *Tetratheca* sequenced in this study. Both unique and shared single bp substitutions and insertion/deletion events have been shown.

Base position	Region	TW	TDH	TAE	TH	TAB	TAN
49	ITS 1	C	C	C	T	T	T
60	ITS 1	T	T	T	A	T	T
70	ITS 1	G	G	C	C	C	C
71	ITS 1	A	A	A	G	G	G
77	ITS 1	A	A	A	G	T	T
103	ITS 1	T	T	T	G	T	T
104	ITS 1	G	G	G	T	G	G
177	ITS 1	-	-	-	G	-	-
206	ITS 1	T	T	C	T	C	C
213	ITS 1	T	T	T	C	C	C
214	ITS 1	G	G	A	A	A	A
217	ITS 1	C	C	C	T	C	C
222	ITS 1	C	C	C	T	C	C
225	ITS 1	A	A	A/G	T	A	A
227	ITS 1	A	A	A	T	A	A
228	ITS 1	C	C	T	T	T	T
232	ITS 1	A	A	A/C	-	T	T
233	ITS 1	-	-	-	A	A	A
234	ITS 1	-	-	-	T	T	T
235	ITS 1	-	-	G	G	G	G
239	ITS 1	A	A	G	A	G	G
241	ITS 1	A	A	-	A	A	A
242	ITS 1	T	T	-	T	T	T
243	ITS 1	G	G	-	A	A	A
244	ITS 1	T	T	-	T	T	T
245	ITS 1	-	-	-	-	T	T
246	ITS 1	-	-	-	-	T	T
247	ITS 1	-	-	-	-	T	T
248	ITS 1	-	-	-	-	T	T
249	ITS 1	-	-	-	-	A	A
250	ITS 1	-	-	-	-	T	T
251	ITS 1	-	-	T	-	T	T
252	ITS 1	-	-	A		A	A
253	ITS 1	A	A	G	G	G	G
254	ITS 1	T	T	C	C	C	C
255	ITS 1	G	G	T	T	T	T
256	ITS 1	T	T	C	C	C	C
258	ITS 1	T	T	A	A	A	A
260	ITS 1	T	T	G	G	G	G
262	ITS 1	T	T	A	G	G	G
264	ITS 1	T	T	G	G	G	G
265	ITS 1	T	T	C	C	C	C
266	ITS 1	T	T/A	C	C	C	C
267	ITS 1	G	G	A/T	A	A	A
400	**5.8 S**	T	T	G	G	G	G
435	ITS 2	A	A	A	T	A	A
449	ITS 2	A	A	A	C	A	A
455	ITS 2	G	G	G	T	G	G
469	ITS 2	C	C	G	G	G	G
471	ITS 2	C	C	-	-	-	-
472	ITS 2	G	G	-	-	-	-
473	ITS 2	C/T	C/T	-	-	-	-
483	ITS 2	G	G	A	G	G	G
496	ITS 2	C	C	T	C	C	C
509	ITS 2	A	A	C/T	C	C	C
510	ITS 2	G	G	C/T	G	T	T
515	ITS 2	C	C	T	T	T	T
520	ITS 2	C	C	A/G	C	G	G
538	ITS 2	T	T	A	C	T	T
565	ITS 2	C	C	C	C	G	G
566	ITS 2	G	G	T	T	T	T
597	ITS 2	A	A	A	G	A	A
620	ITS 2	T	T	T	C	T	T
622	ITS 2	C/A	C	T	T	T	T
636	ITS 2	T	T	T	G	T	T
637	ITS 2	C	C	C	T	C	C

By comparison, ITS sequences of TW and TDH were invariable across the 669 nucleotides examined (Table 1), confirming the close evolutionary relationship between plants from the Windarling and Die Hardy Ranges. Pair-wise distances between TW and TDH are very low and range from 0 to 0.00306 (mean character differences), with total character differences ranging from 0 to 2. Comparatively, pair-wise distances between individuals of TW and TAB are high and range from 0.05837 to 0.07099 (mean character differences), with total character differences ranging from 38 to 46. At some base positions polymorphisms are evident in TDH that are not shared with TW and *vice versa* (i.e. at bp 266 TDH shows a T/A whilst TW clearly shows a single T peak), but as these are ambiguous and not phylogenetically informative, they have not been used to assess the relationship between these two taxa. This stance was also taken in the previous study (Butcher *et al.* 2001) where an extremely high number of polymorphic base sites were observed in TAB, TH and TW ITS sequences, and the issue is discussed at greater length in that report. As observed in TW and TDH, the ITS sequences of TAN plants are not divergent from those of TAB and there are no bases different between these two taxa. Pair-wise distances between TAB and TAN are comparable to those between TW and TDH also, and mean character differences range from 0.00303 to 0.00760; with total character differences ranging from 2 to 5.

Parsimony analysis of ITS data for these *Tetratheca* yielded over 10 000 shortest trees (713 steps) and the 50% bootstrap consensus tree is presented as a phylogram in Figure 7, with branch lengths and bootstrap values indicated on the tree. Trees have not been rooted, and four distinct, well supported clades are evident; the first containing the TH individuals (100% bootstrap support), the second containing TW and TDH (100%), the third first comprising the TAE individuals (90%), and the last containing TAB and TAN (100%). The lower bootstrap value and long internal branches in the TAE clade are the result of poor DNA quality and/or sequence reads for the TAE samples and a high number of missing values (N) in the data matrix. Despite this, the distinctiveness of TAE, as well as TH, is clear. Comparatively, the four remaining taxa form two very well supported clades, but the relationships between TW and TDH individuals, and between TAB and TAN individuals is not resolved using ITS data. The sister taxon relationship evident between TAB 3.1 and TAN 12 is dubious as TAB 3.1 sequences contain a large number of polymorphisms and bootstrap support for this relation is low (53%).

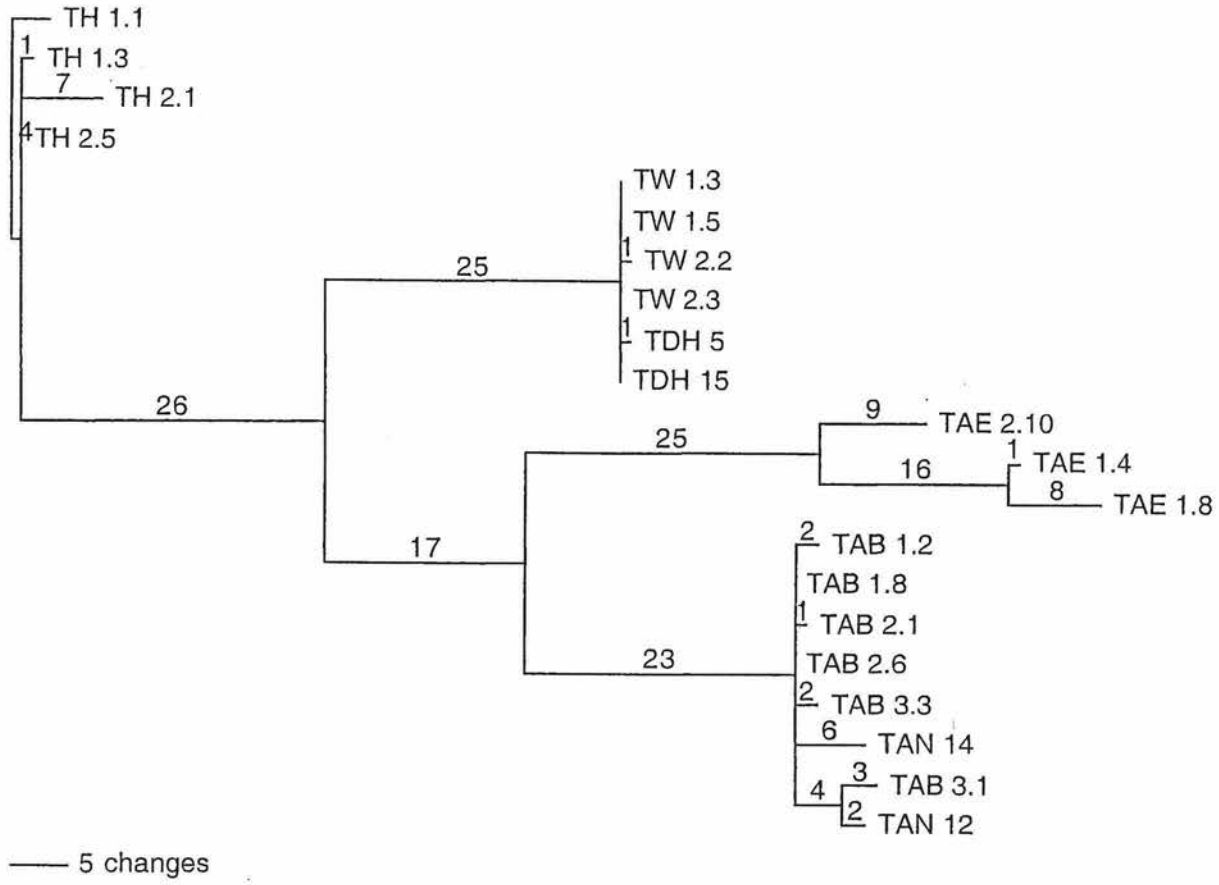

Figure 7: Bootstrap consensus of over 10 000 shortest trees generated for *Tetratheca* ITS data presented as a phylogram. Trees were obtained through parsimony analysis and were unrooted. Branch lengths, indicating sequence variation, are presented above the lines and bootstrap values below the lines.

Fourteen trees of equal length were generated by maximum likelihood analysis and the strict consensus tree is topologically congruent with that generated by the parsimony analysis and is not presented here. The four major clades outlined above are again evident with long branch lengths indicating their distinctiveness, but there is a small amount of additional resolution amongst terminal taxa of the TAN/TAB clade with TAN 14 indicated as sister to the remaining samples. The sister taxon relationship between TAB 3.1 and TAN 12 is again indicated. The relationship between TW and TDH individuals is completely unresolved in all 14 trees.

*trn*L-*trn*F:

Aligned *trn*L-*trn*F sequences (including that of the eastern states species *T. rupicola*; Appendix 3) indicate a total of 168 variable sites including three large indel regions (Table 2) characterising TAE (bp 223-309) and TW/TDH (bp 336-359 and bp 776-816) and 31 single base substitutions and indels (Table 3). As seen in the ITS data set, the *trn*L-*trn*F sequences of TW and TDH are very similar and are highly diverged from the other species. For example, nine substitutions and a two bp indel at position 238-239 differentiate TW from TAB (Table 3) but TH can only be differentiated from TAB at five sites (three indels and two substitutions) in the *trn*L-*trn*F data, despite being highly divergent in both morphology and ITS sequences. A comparison of the *trn*L-*trn*F sequences of TW and TDH individuals shows that these two taxa can be distinguished at three base positions, including a two bp indel at positions 238-239 where TW has a string of 10 As and TDH has 12, and a transversion substitution at position 793 within a shared indel region, where TW has a G and TDH has a T. The *trn*L-*trn*F sequences of TAB and TAN vary at only one base position, with TAN having a unique transversion substitution at position 860 (A compared with G in all other species). TAE can be seen to differ from the other species primarily in it's possession of an 86 bp indel, which is not shared with any of the species sampled here, as well as one unique transversion substitution at position 2 (Table 3). Across all variable sites TAE generally shares the same sequence as TAB/TAN and TH rather than TW/TDH, indicating a closer relationship between these taxa.

Table 2: Positions of large insertion/deletion events in the *trn*L-*trn*F sequences for the *Tetratheca* taxa sequenced in this study. The positions indicated are those represented by gaps (-) in the actual data matrix (see Appendix 3). Taxon abbreviations are as indicated in the text.

	Positions of Large Indels		
Taxon	**1**	**2**	**3**
T. rupicola	-	336-353 (17 bp)	776-816 (40 bp)
TW	-	-	-
TDH	-	-	-
TAE	223-309 (86 bp)	335-359 (24 bp)	776-816 (40 bp)
TH	-	336-354 (18 bp)	776-816 (40 bp)
TAB	-	336-354 (18 bp)	776-816 (40 bp)
TAN	-	336-354 (18 bp)	776-816 (40 bp)

Table 3: A summary of variable bases in the *trn*L-*trn*F region for the *Tetratheca* taxa sequenced in this study. Both unique and shared single base position substitutions and insertion/deletion events have been shown. Taxon abbreviations are as indicated in the text.

	Base Composition per Taxon						
Base Position	*T. rupicola*	TW	TDH	TAE	TH	TAB	TAN
2	A	A	A	G	A	A	A
47	T	A	A	T	T	T	T
75	C	T	T	T	T	T	T
101	G	A	A	A	A	A	A
176	G	A	A	G	G	G	G
217	C	C	C	T	T	T	T
230	A	A	A	-	T	T	T
238	A	-	-	-	A	A	A
239	G	-	-	-	G	G	G
320	A	T	T	T	T	T	T
327	T	A	A	A	A	A	A
336	-	A	-	-	-	-	-
337	-	A	-	-	-	-	-
359	A	A	A	-	-	A	A
360	T	A	-/A	T	T	T	T
393	C	A	A	A	A	A	A
422	C	T	T	C	C	C	C
426	-	T	T	T	T	T	T
435	C	-	-	-	-	-	-
553	N	A	A	A	T	A	A
593	N	T	T	C	C	C	C
608	-	C	C	C	C	C	C
636	G	A	A	G	G	G	G
641	T	A	A	T	T	T	T
712	G	T	T	T	T	T	T
737	A	A	A	A	C	A	A
746	-	-	-	-	T	-	-
765	G	T	T	G	G	G	G
793	-	G	T	-	-	-	-
860	G	G	G	G	G	G	A
871	-	A	A	A	A	A	A

Parsimony analysis of *trn*L-*trn*F sequences yielded four shortest trees of 194 steps and the relationships between the taxa are congruent with, but better resolved than, those obtained for the ITS data. In each of the four most parsimonious trees, the four major clades outlined in the ITS results are evident, with TAE and TW/TDH being highly divergent from the remaining taxa, but with TH demonstrating a closer relationship to the clade comprising TAB/TAN. The bootstrap tree (Figure 8) illustrates branch lengths of 89 and 72 for TAE and TW/TDH respectively, compared a branch length of only three steps between the TH clade and TAB/TAN. Bootstrap values for all relationships are presented on the tree and show high support for the major clades, even when branches are very short. Better resolution within the major clades shows a clear sister taxon relationship between TW and TDH (Figure 8), although bootstrap supports for these branches are relatively low due to one sample in each taxon having a single base position polymorphism. Due to their possession of a shared substitution at

bp 860 (Table 3), the two TAN individuals can be seen to be resolved as sisters nested within the polychotomous TAB clade.

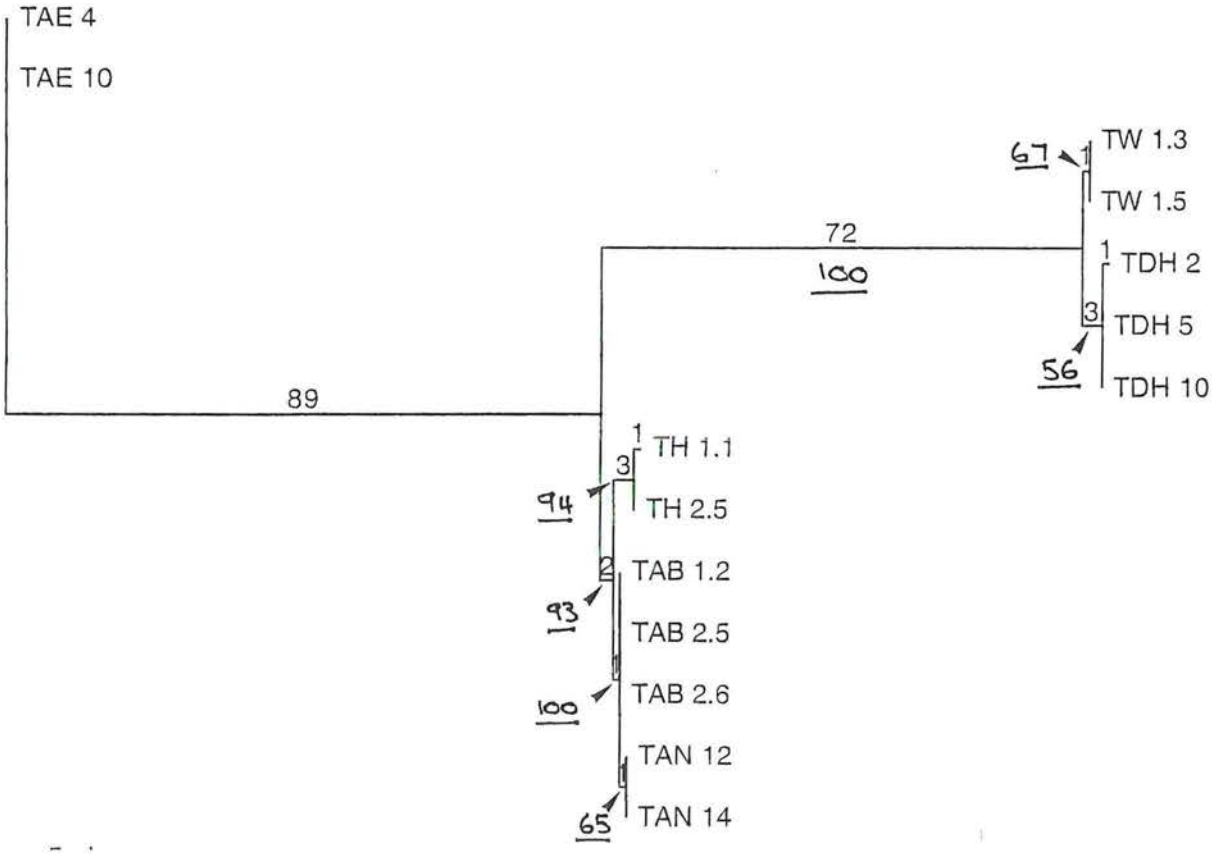

Figure 8: The single parsimony bootstrap tree generated for *Tetratheca trn*L-*trn*F data presented as a phylogram. The tree was rooted at TAE as its distinctness had been shown in prior parsimony analysis. Branch lengths, indicating sequence variation, are presented above the lines and bootstrap values below the lines.

Maximum likelihood analysis of the same data set yielded one tree with a score of 1272.69. In the unrooted network (Figure 9), the TW/TDH clade is highly diverged from the remaining samples, and TAE can be seen to be closely allied to TAB/TAN. As maximum likelihood does not allow coding of gaps as a separate character, the large indel (Table 2) which characterises TAE in its *trn*L-*trn*F sequences is not a component of branch length. Within the TW/TDH clade, the three TDH individuals form a discrete clade nested within TW and the relationship between TAB and TAN individuals is also fully resolved with the two TAN samples separated from the unresolved TAB samples by a short branch.

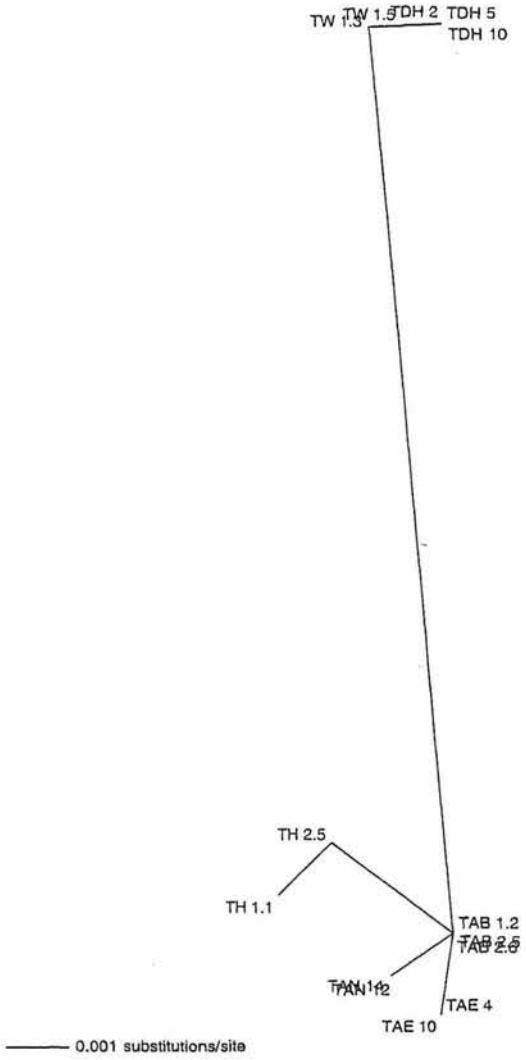

Figure 9: The single maximum likelihood tree generated for *Tetratheca trn*L-*trn*F data presented as an unrooted network. The distinctness of the TW/TDH clade from the other taxa is clear, and lower level distinction is evident between TW and TDH, and between TAN and TAB.

The addition of *trn*L-*trn*F data from the morphologically distinctive eastern states species *Tetratheca rupicola* does not change the topological relationships between the study species, but does throw an interesting slant on the phylogenetic relationships of these leafless south-west WA taxa. In both the parsimony and maximum likelihood analyses, *T. rupicola* can be seen to be the closest relative to the TW/TDH clade (Figure 10), being more similar to these taxa in its chloroplast sequences than these are to TH, TAB and TAN, taxa to which they are closer both geographically and phenotypically.

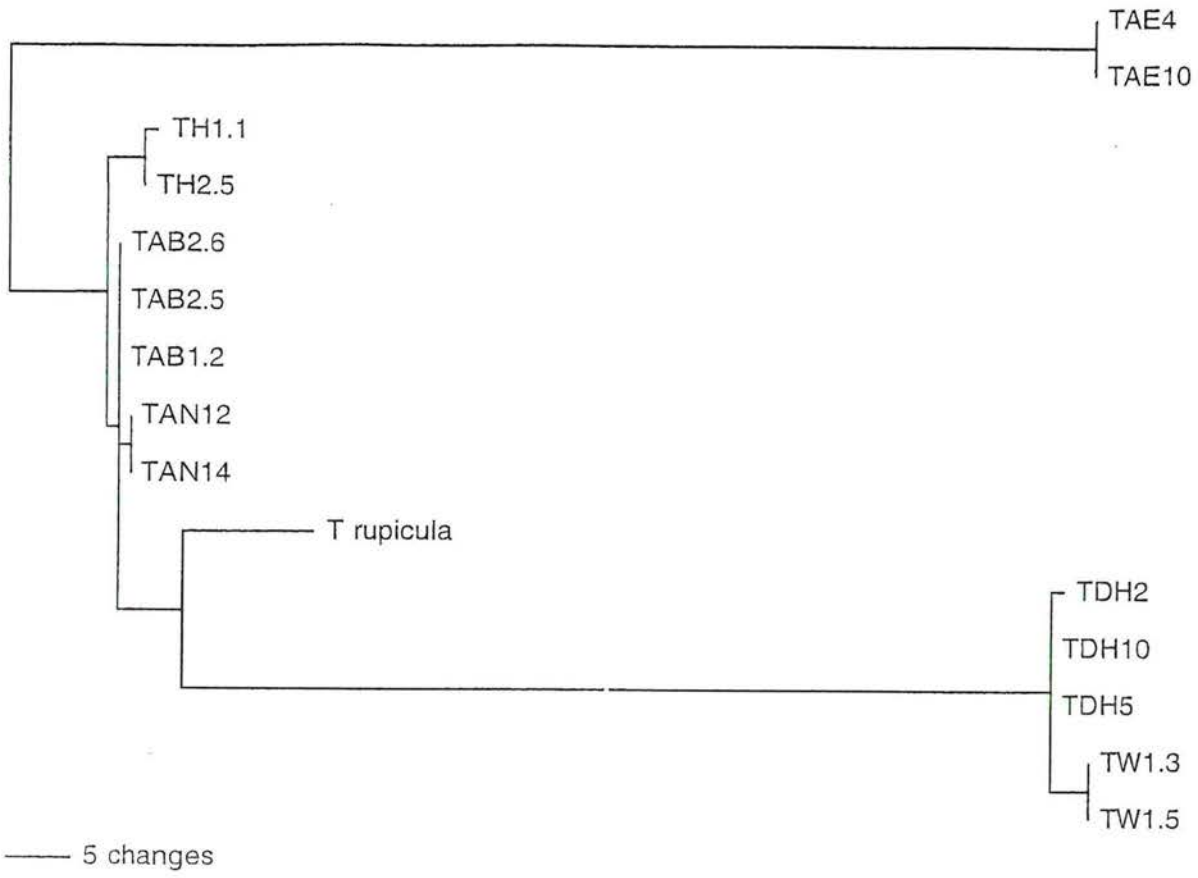

Figure 10: Strict consensus tree of *trn*L-*trn*F sequence data generated through parsimony analysis of the *T.* aphylla group *Tetratheca* taxa in addition to the eastern-states species *T. rupicola*. The hylogenetic relationships amongst these taxa is presented as a phylogram.

Combined ITS – *trn*L-*trn*F:

As the topologies of the ITS and *trn*L-*trn*F trees were congruent it was possible to combine the two data sets for additional cladistic analysis. Parsimony analysis yielded six shortest trees of 617 steps and the strict consensus of these demonstrates that the two TDH samples are distinct from the TW individuals, being separated within this polytomy by a short branch. Similarly, the TAN samples are resolved as sisters within the TAB clade (Figure 11). The single tree derived from maximum likelihood analysis of this combined data set is topologically congruent with those generated by parsimony analysis (Figure 12), and it can be seen that the combined use of nrDNA and cpDNA data sets affords greater resolution of relationships than either data set alone, with the more recent evolutionary divergence of TAN & TAB and TW and TDH reflected in branch lengths.

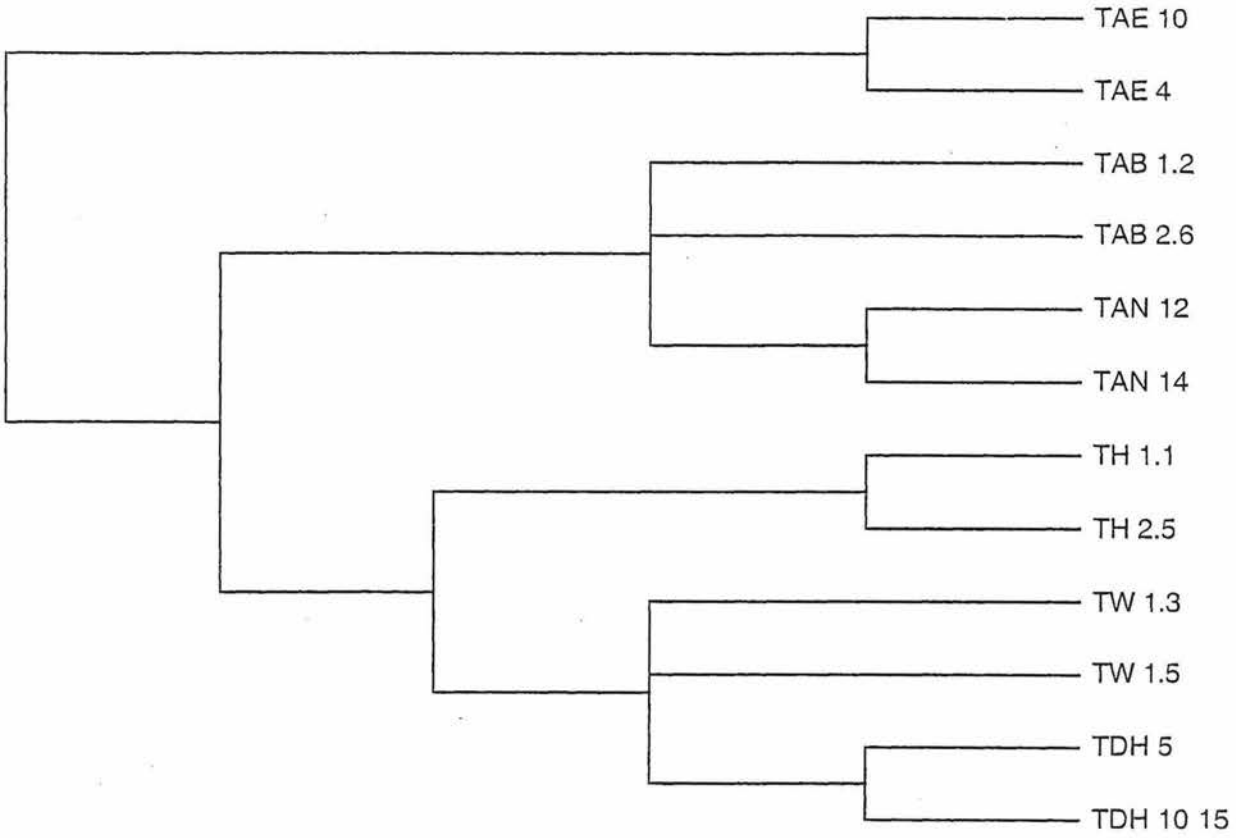

Figure 11: Strict consensus tree of six shortest trees generated for *Tetratheca* combined ITS and *trn*L-*trn*F data through parsimony analysis. Tree is presented as a cladogram.

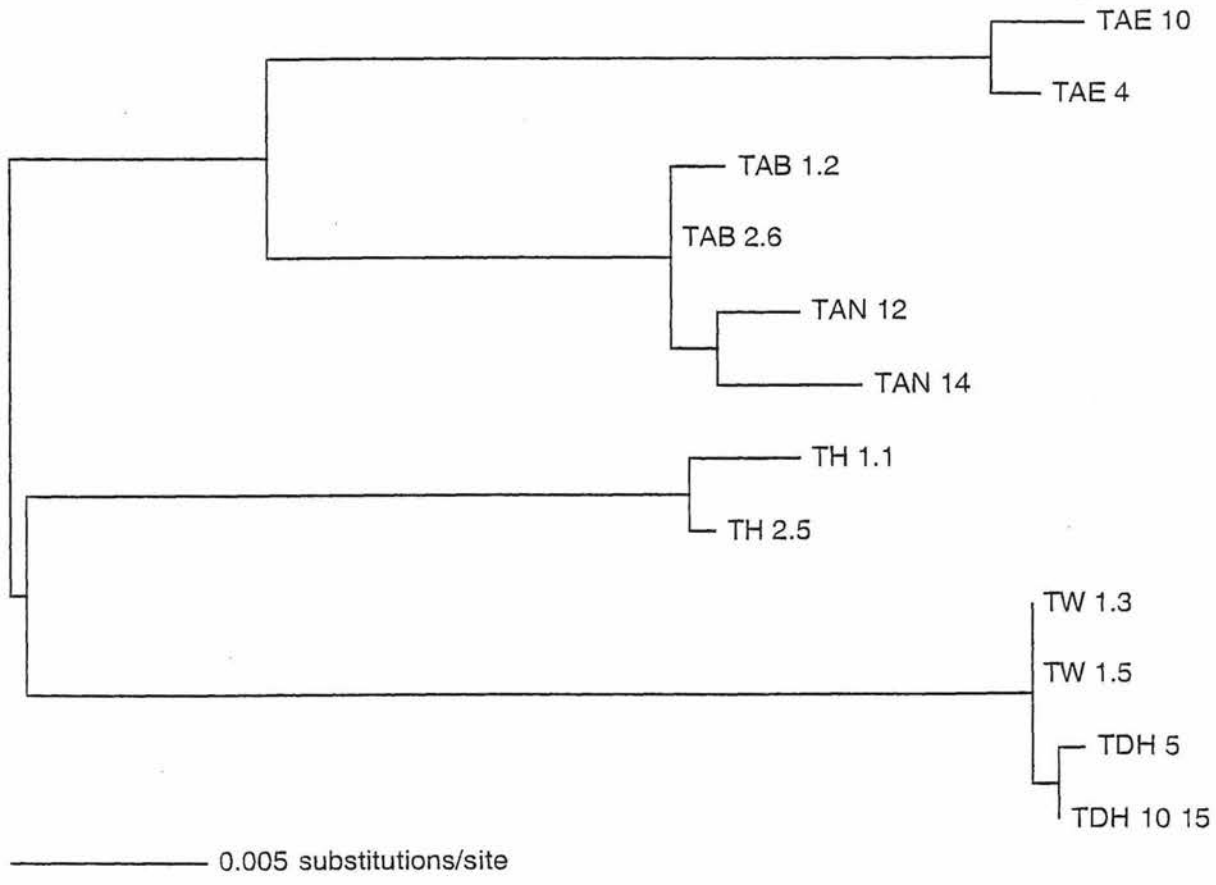

Figure 12: The single maximum likelihood tree generated for combined ITS and *trn*L-*trn*F data presented as a phylogram. The resolution of relationships within recently diverged clades (TAB/TAN & TW/TDH) is vastly improved than analyses based on either data set alone.

Conclusions:

- *Tetratheca* (Die Hardy Range) collections cannot be distinguished from those of *T. paynterae* in their nuclear ribosomal ITS sequences, but they can be distinguished at three base positions in their chloroplast *trn*L-*trn*F sequences. Variation amongst individuals of *Tetratheca* (Die Hardy Range) is negligible compared with the unambiguous variation between these samples and *T. paynterae*, and they are therefore recognised as different taxa.
- Cladistic analyses of these data place *T. paynterae* and *Tetratheca* (Die Hardy Range) as very closely related sister taxa with the short branch lengths between them suggesting they are recently diverged from one another. Comparatively they are separated from the other taxa by very long branches, indicating a distant evolutionary relationship.
- Although only cpDNA sequence was available for comparison, *Tetratheca* (Die Hardy Range) and *T. paynterae* would seem to have a closer relationship to *T. rupicola*, from New South Wales, than to *T. aphylla* and *T. harperi* from the same geographical area. This would suggest that *T. paynterae*, *Tetratheca* (Die Hardy Range) and *T. rupicola* have diverged from a separate lineage to that which gave rose to *T. aphylla*, *T. harperi* and the collections from Newdegate and Eneabba. The long branch lengths indicate that the split between *T. paynterae/Tetratheca* (Die Hardy Range) and *T. rupicola* is ancient.
- The *Tetratheca* aff. *aphylla* (Newdegate) collections cannot be distinguished from those of *T. aphylla* in their ITS sequences, but they can be distinguished at one base position in their *trn*L-*trn*F sequences. Variation amongst individuals of *T.* aff. *aphylla* (Newdegate) is negligible compared with the unambiguous variation between these samples and *T. aphylla*, and they are therefore recognised as different taxa. Cladistic analyses place these two taxa as very closely related sisters and indicate them to be most closely allied to *T. harperi* and *Tetratheca* (Eneabba) within the study group.
- The *Tetratheca* (Eneabba) collections are highly divergent in both ITS and *trn*L-*trn*F sequences from all other species examined and they clearly represent a distinct taxon.

MORPHOLOGY

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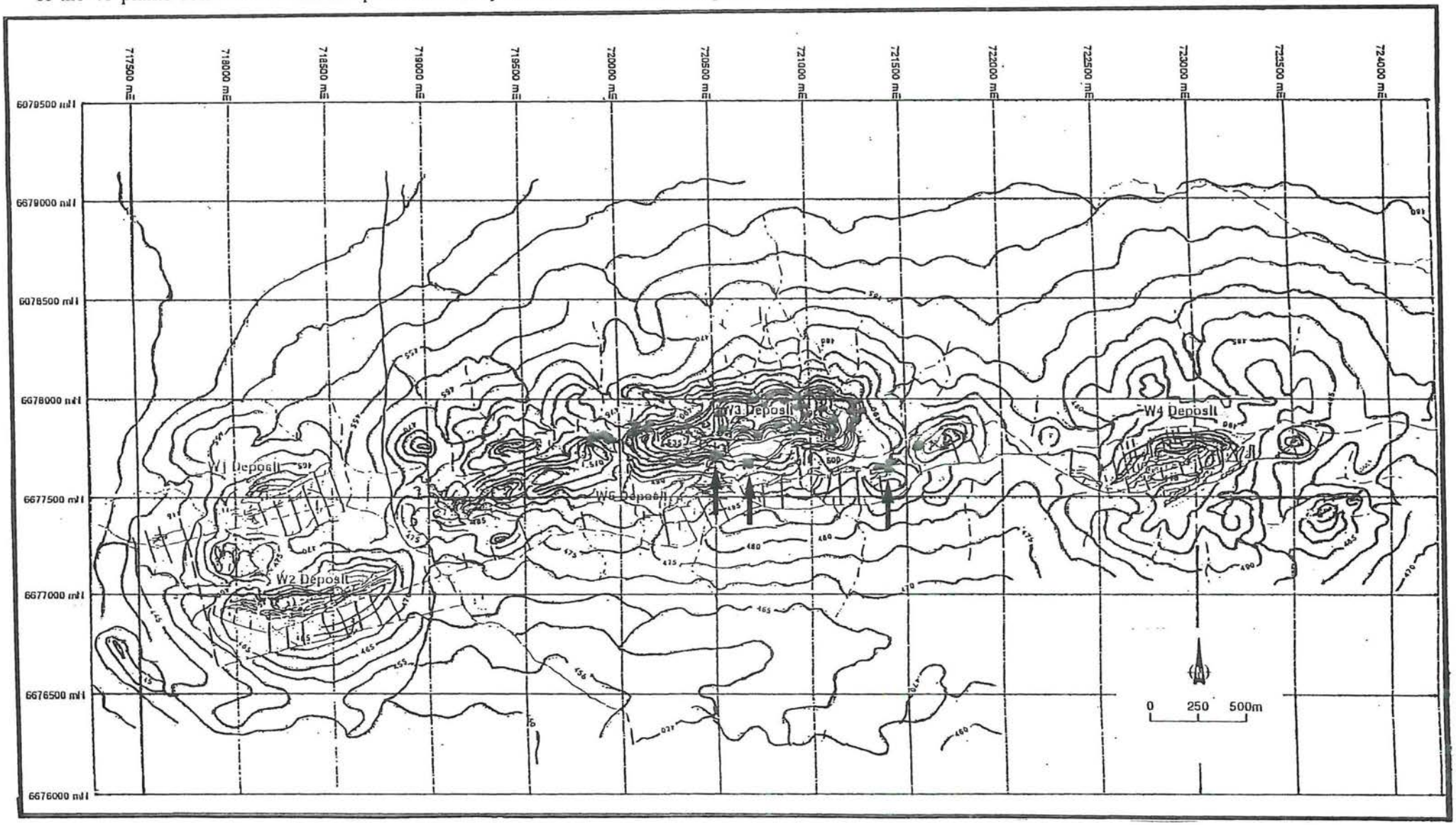
Methods:

Collection of material and characters:

Forty one individuals of *T. paynterae* were collected from across the range of this species, including 36 from along the 'W3 Deposit' and five from along the length of the disjunct 'W5 Deposit', including one individual from a newly located subpopulation of 30 plants at the far western end of 'W5' (Figure 13). Of the 36 'W3' collections, measurements were made from 29 individuals such that the total number of *T. paynterae* measured was 34 plants. Thirty six individuals of *Tetratheca* (Die Hardy Range) were collected from throughout the Die Hardy Range, including 20 from Population 1 (southernmost population; including one individual (DH 3) from a small sub-population), 12 from Population 2 (with nine and three individuals, respectively, collected from different sides of a broad valley) and four from the newly located, northernmost, Population 3 (see Figure 4). All 36 individuals were used in the morphometric study.

For each individual sampled in the field, at least three fully open flowers and three portions of leaf-bearing stem were collected and preserved in 70% ethanol for measurement, with flowers selected to represent both the largest and smallest evident on each plant. An exception to this was the *T. paynterae* collection W5-3 where only two open flowers were present on the plant. In addition to spirit preserved material, fresh flowers were collected where possible for assessment of colour variation and have been pressed as vouchers for the study. These samples are currently housed at UWA along with the spirit material.

Figure 13: Map showing the location of plants of *Tetratheca paynterae* at 'Windarling Range'. Approximately 2000 plants occur on the large 'W3 Deposit' and approx. 60 plants occur along the entire length of the low 'W5 Deposit'. Survey of other hills in the Range has not located additional plants. The locations of the 41 plants collected for the morphometric study are indicated on the map with red dots. Plants from the 'W5' deposit are marked on the map with →



Forty nine characters incorporating size, shape, colour and pubescence of vegetative and floral parts were measured and/or calculated for all individuals of each taxon, with three replicate measures per character being made for each individual where possible. Of these 49 characters, 33 were quantitative characters, 9 were ratios and 7 were binary coded qualitative characters (see Appendix 4; Figure **21** for characters/character states). Colour characters could not be coded for individuals for which insufficient flowering material was available to allow fresh collections to be made, or where flowers had come into contact with ethanol resulting in the colour being leached and altered from normal.

Morphometric Analysis & Results:

Analysis of variance (ANOVA):

Prior to statistical analyses, means of the three replicate measures per individual were calculated and leaf length and ovary width measurements were log transformed. Characters which were invariable or perfect, or near-perfect, discriminators between the two taxa were excluded as one-way analysis of variance (ANOVA) can only be calculated for characters which display both normality and homoscedasticity. Although not useful for statistical analysis, the characters which were perfect or near-perfect discriminators have excellent taxonomic value and are highlighted in the following morphological discussion. Based on the Shapiro-Wilk statistic ($P < 0.05$), 27 variables were identified as suitable for one way ANOVA. This analysis indicates that, of these 27 characters, 15 are statistically significant at the $P < 0.005$ level and can be considered good indicators of consistent morphological difference between *T. paynterae* and *Tetratheca* (Die Hardy) (Table 4). The five variables with the highest F values were used in a canonical discriminant analysis.

Table 4: ANOVA of the 27 normally distributed morphometric characters with the five most variable between the taxa, based on F values, emboldened. LWR= length/width ratio.

Variable	Univariate ANOVA F	P
Stem diameter	**54.09**	**0.0001**
Leaf length	15.02	0.0002
Leaf width	27.31	0.0001
Leaf LWR	0.04	0.8413
Leaf # abaxial hairs	**83.92**	**0.0001**
Calyx segment length	28.44	0.0001
Calyx segment width	11.53	0.0011
Calyx segment LWR	4.72	0.0333
Calyx segment length to widest point	0.94	0.3356
Calyx # hairs	**248.58**	**0.0001**
Calyx # resin hairs	**74.84**	**0.0001**
Petal length	21.36	0.0001
Petal width	4.62	0.0352
Petal LWR	6.71	0.0117
Petal length to widest point	16.8	0.0001
Peduncle length	10.56	0.0018
Receptacle diameter	**41.07**	**0.0001**
Ovary length	4.82	0.0315
Ovary width	22.95	0.0001
Ovary LWR	3.65	0.0601
Ovary length to widest point	0.71	0.4029
Ovary # hair	0.66	0.419
Style length	12.77	0.0007
Stamen total length	2.39	0.1266
Anther tube length	6.67	0.012
Anther body length	5.61	0.0208
Anther filament length	39.82	0.0001

Canonical discriminant analysis (CDA):

Canonical discriminant analysis was performed using the SAS computer program and the overall test of separation, Wilks' Lambda, is highly significant. The canonical discriminant function values are presented in Table 5. The total canonical structure for the five best characters shows that the number of hairs on the calyx segments is the single best discriminator between the two taxa (Table 6). Because there are only two groups being investigated, only one discriminant function (CAN1) can be calculated hence it is not possible to construct a plot using different canonical variates as the axes; but this single function clearly illustrates that these groups are distinct with all *T. paynterae* individuals returning a positive score and all *Tetratheca* (Die Hardy) individuals returning a negative score (Table 7). It is, however, possible to construct pair-wise plots of characters to illustrate the morphological distinctness of these two taxa and Figures 14-17 provide some examples of the variation evident in the best five normally distributed discriminators, with calyx hair density plotted against the other four characters.

Table 5: Canonical discriminant analysis results indicating multivariate statistics and exact F statistic scores where S=1, M=1.5 and N=31.

Statistic	Value	F	Num DF	Den DF	Pr > F
Wilks' Lambda	**0.10937082**	**104.2330**	**5**	**64**	**0.0001**
Pillai's Trace	0.89062918	104.2330	5	64	0.0001
Hotelling-Lawley Trace	8.14320661	104.2330	5	64	0.0001
Roy's Greatest Root	8.14320661	104.2330	5	64	0.0001

Table 6: Total canonical structure for the five best characters. The number of hairs on the calyx segments is identified as being the single best discriminator between the two taxa.

Total Canonical Structure	
Variable	**CAN 1**
Stem diameter	0.705312
Leaf # abaxial hairs	0.787552
Calyx segment # hairs	**0.938949**
Calyx # resin hairs	-0.766998
Receptacle diameter	0.650199

Table 7: Canonical scores for each individual used in the morphometric study. All individuals of *T. paynterae* have a positive score whilst all individuals of *Tetratheca* (Die Hardy Range) have a negative score.

T. paynterae			*T.* Die Hardy Range	
Observation	CAN1		Observation	CAN1
1	2.71222		35	-4.07567
2	4.28718		36	-2.49396
3	3.13074		37	-1.52332
4	3.53011		38	-0.96646
5	2.93641		39	-1.6206
6	2.93926		40	-3.69074
7	3.15627		41	-1.74507
8	2.20837		42	-3.15591
9	2.98259		43	-2.84534
10	1.14436		44	-2.03895
11	3.19141		45	-2.35019
12	3.39091		46	-2.06975
13	3.806		47	-3.20234
14	3.27807		48	-3.47346
15	4.36478		49	-1.80455
16	2.19606		50	-1.0874
17	1.06935		51	-1.42031
18	2.80591		52	-1.01487
19	2.15163		53	-2.95906
20	0.61789		54	-2.90565
21	4.66595		55	-1.75519
22	3.67281		56	-2.20823
23	2.6849		57	-3.95988
24	2.13475		58	-4.32599
25	3.60647		59	-3.66748
26	3.25533		60	-3.9539
27	1.51364		61	-4.08535
28	3.79107		62	-4.77732
29	3.85503		63	-2.18737
30	1.60692		64	-3.9506
31	2.23868		65	-3.46982
32	2.26803		66	-2.53126
33	3.56408		67	-3.32161
34	3.64257		68	-2.75104
			69	-2.95545
			70	-2.05562

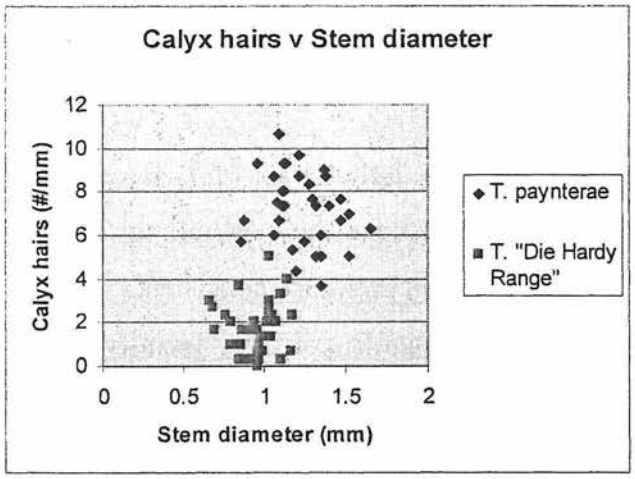


Figure 14: Pairwise plot of calyx hair density means against stem diameter means for all individuals of *T. paynterae* and *Tetratheca* (Die Hardy Range).

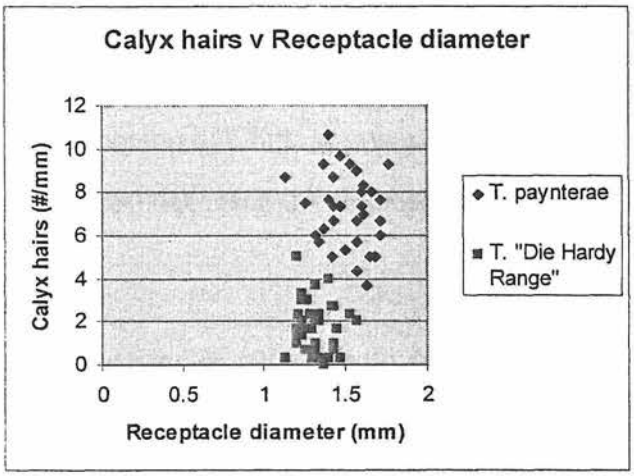


Figure 15: Pairwise plot of calyx hair density means against receptacle diameter means for all individuals of *T. paynterae* and *Tetratheca* (Die Hardy Range).

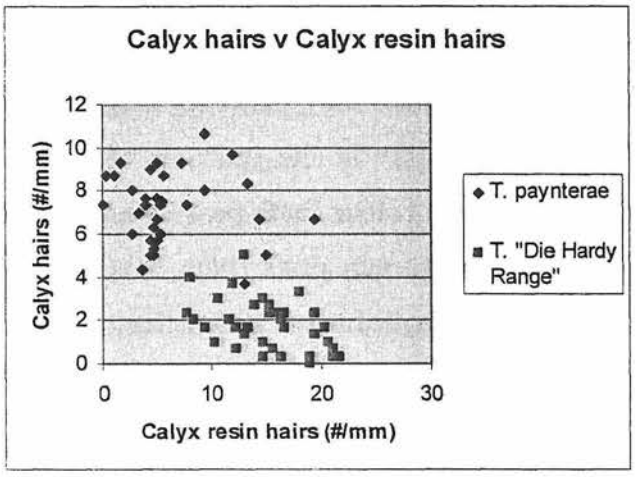


Figure 16: Pairwise plot of calyx hair density means against calyx resin hair density means for all individuals of *T. paynterae* and *Tetratheca* (Die Hardy Range).

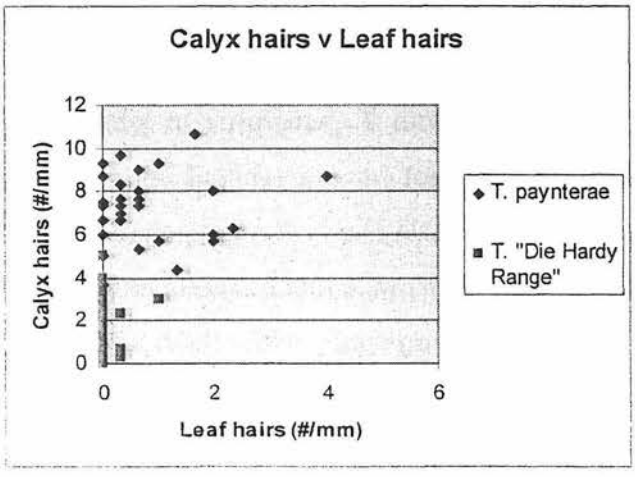


Figure 17: Pairwise plot of calyx hair density means against abaxial leaf hair density means for all individuals of *T. paynterae* and *Tetratheca* (Die Hardy Range).

Assessment of Morphological Variation:

Examination of flowering material for numerous individuals of *T. paynterae* and *Tetratheca* (Die Hardy Range) clearly showed that despite their overall similarity these two taxa are distinct and that the differences between them are consistent. There are a number of characters which can be used on their own to correctly identify each taxon (e.g. pubescence of the ovary, shape of the receptacle, pubescence of the adaxial leaf surface) as well as features which are generally reliable for taxon discrimination (e.g. colour of the anther tube and style end, number of glandular hairs on various parts) but which may be subject to change depending on the age of flowers and environmental influences. The morphological features which unite *T. paynterae* and *Tetratheca* (Die Hardy Range) as sister species, as well as those which separate them as different taxa are discussed below:

Characters highlighted as taxonomically and evolutionarily significant by Thompson (1976) and Alford (1995), and that are shared uniquely by *T. paynterae* and *Tetratheca* (Die Hardy Range) within the broad *T. aphylla* group, include stem pubescence and ornamentation (both are glabrous with broad, rounded tubercules), ovule number (both possess two ovules per locule rather than one), peduncle length and curvature (both of similar length and terminating abruptly at the junction with the receptacle), length and width of the calyx segments, petal colour (both possess a yellow spot at the base of the petal), relative lengths of the stamen parts (both have short filaments relative to the anther body) and floral scent (both have a distinctive, strong musky scent).

However, *Tetratheca* (Die Hardy Range) is distinguishable from *T. paynterae* in the field by its habit, with plants frequently hanging downwards from rock fissures and having an intricately branched appearance compared with *T. paynterae* in which the stems are erect and even when highly branching, do not have a tangled appearance. Although there is overlap in stem diameter, *Tetratheca* (Die Hardy Range) generally has more slender stems than *T. paynterae* with measurements made just below open flowers ranging from 0.47-1.69 mm (mean=1.05 mm) compared with 0.62-1.96 mm

(mean=1.23 mm). This character was statistically significant (ANOVA F= 54.09; P= 0.0001) as a discriminator between these two taxa.

The pubescence of the leaves is an excellent discriminator between these two taxa with variation evident in hair density on both the abaxial and adaxial surfaces. The number of hairs on the abaxial surface (as standardised for morphometric data collection; see Appendix 4) of the leaf was found to be a statistically significant character (ANOVA F= 83.92; P= 0.0001) for taxon discrimination with *T. paynterae* having sparse hairs over the abaxial surface (0-8 hairs per mm @ 25 x magn.; mean=0.683) and *Tetratheca* (Die Hardy Range) being almost glabrous (0-3 hairs per mm @ 25 x magn; mean=0.061). As data for ANOVA were required to display normality, the binary coded qualitative character for 'adaxial leaf surface hair density' (0= glabrous-few hairs at apex; 1= densely pubescent) was discarded prior to analysis as it provided a perfect discriminator between the two taxa i.e. all *Tetratheca* (Die Hardy Range) possessed character state 0 whilst all *T. paynterae* possessed state 1.

There is very little variation in peduncle length between *Tetratheca* (Die Hardy Range) and *T. paynterae* (0.8-8 mm v. 1.5-8.3 mm respectively), and both of these taxa have occasional glandular hairs as well as rounded tubercules on the peduncle, but the difference in the distribution of simple hairs provides an excellent discriminator between these two species; *Tetratheca* (Die Hardy Range) being +/- glabrous (0-4 hairs per mm @ 25 x magn., mean= 0.55) and *T. paynterae* having a moderate number of short hairs (0-13 hairs per mm @ 25 x magn., mean=5.29). In both taxa the peduncle has an abrupt transition into the receptacle, and the receptacle is slightly narrower in *Tetratheca* (Die Hardy Range) (1-1.7 mm, mean=1.44) than in *T. paynterae* (1-1.85, mean=1.51) and is a statistically significant (ANOVA F=41.07; P= 0.0001) character for the discrimination of these species. The shape of the receptacle is highly diagnostic and in *Tetratheca* (Die Hardy Range) it is almost circular to slightly hexagonal/angular at the edges, whereas in *T. paynterae* it is noticeably thicker in-between the calyx segments such that it has a prominently angular to lobulate appearance. The 'lobes' are evident when calyx segments fall, and give the edge of the receptacle an undulate appearance. This character was binary coded and excluded from ANOVA and CDA calculations.

Whilst the shape and length of the calyx segments is a very useful character for the differentiation of other species within the *T. aphylla* group (see Alford 1995; Butcher *et al.* 2001), there is no real variation evident in these features between *T. paynterae* and *Tetratheca* (Die Hardy Range), but differences in vestiture are both prominent and statistically significant. The number of hairs and resin-tipped glandular hairs on the calyx (see Appendix 4 for measurement parameters) are identified by one-way ANOVA as two of the five best discriminators between these species (Table 4, Table 6). *T. paynterae* can be differentiated from *Tetratheca* (Die Hardy Range) as it possesses sparse short hairs over the entire surface of the calyx segments (0-13 hairs per mm @ 25 x magn., mean=7.19) with glandular hairs usually concentrated along the margins (0-21 hairs, mean 6.27), whilst *Tetratheca* (Die Hardy Range) has very few short hairs (0-7 per mm @ 25 x magn., mean=1.92) on the calyx segments, but glandular hairs scattered over the calyx and receptacle, and present in greater number along calyx segment margins (4-27 hairs, mean 16.38).

There are a suite of differences in anther morphology that can be used to distinguish *T. paynterae* and *Tetratheca* (Die Hardy Range) including the statistically significant character of anther filament length (ANOVA F= 39.82; P= 0.0001) where *T. paynterae* has filaments ranging from 0.35-0.7 mm long (mean=0.49) and *Tetratheca* (Die Hardy Range) has filaments ranging from 0.35-0.9 mm long (mean=0.68). In addition to this feature, the anther filaments of *T. paynterae* are usually yellow and fused along most of their length (25-100% fusion, mean=92.8%) with hairs on the inner edge, the anther tube is yellow at the tip and scarcely lipped and the depression between the uppermost (abaxial) anther cells is prominent. Comparatively, the anther filaments of *Tetratheca* (Die Hardy Range) are red and usually fused in the lower half (0-100% fusion, mean 49.1%) with hairs rarely present on the inner edge, the anther tube is dull reddish-purple (though can fade with age) and slightly lipped at the apex and the depression between the abaxial anther cells is less pronounced. The colour and hair distribution characters were scored as binary quantitative characters in the morphometric data set and excluded as they were near-perfect discriminators.

A character which clearly discriminates between these species, but was unsuitable for inclusion in CDA is the pubescence of the ovary and style, where *T. paynterae* has the ovary covered with dense, short, erect hairs (8-28 hairs per mm @ 25 x magn., mean=16.19) which obscure the ovary surface and extend up the style for *c.* half its' length (range is 28-72%, mean=49%) as well as scattered glandular hairs over the ovary surface. In comparison, the ovary of *Tetratheca* (Die Hardy Range) appears shiny and red and has scattered glandular hairs over its' surface with short hairs only in a small patch at the base of the style and sometimes at the base of the ovary (0-4 hairs per mm @ 25 x magn., mean=0.316). The style is usually glabrous also, but occasionally has hairs at the base (0-36% of length, mean=9.78%) and these are usually glandular (rarely short). Another near perfect discriminator between these taxa is the colour of the style tip (coded as a binary quantitative character in this study and excluded prior to analysis), which is usually bright yellow in *T. paynterae* and dull red-purple in *Tetratheca* (Die Hardy Range). In older flowers this character can be more difficult to discern as the style ages and becomes paler in *Tetratheca* (Die Hardy Range) and it is not useful for distinguishing specimens which have been preserved in ethanol as the colours of all parts disappear.

Conclusions:

- The large number of morphological characters which can be shown to be perfect, or near-perfect, discriminators between *Tetratheca* (Die Hardy) and *T. paynterae*, as well as the statistical significance of differences between the two in more variable characters clearly indicates that they are different taxa.

~..~..~..~..~..~..~..~..~..~..~..~..~..~..~

General Discussion and Taxonomic Implications:

The use of multivariate morphometric analyses to distinguish between groups is commonplace and in this case it demonstrates that individuals of *Tetratheca* from populations at 'Windarling Range' and the Die Hardy Range display statistically significant variation in their morphology, such that they can be classified as different taxa. However, this finding does not address the issue of taxonomic rank i.e. whether *Tetratheca* (Die Hardy Range) should be recognised as a separate species or as a subspecies of *T. paynterae*. Many species concepts have been proposed and these fall primarily into two types; those that emphasise the processes of evolution through gene flow, and those that adopt a more pattern-based, operational approach, focussing on morphological difference as the basis for species recognition. Below the level of species the use of terms such as subspecies, variety and form to classify different degrees of similarity between taxa is subject to continual disagreement: there are no hard-and-fast rules for their application and terms are seemingly interchangeable. Krauss (1996) discusses the use of species and subspecies concepts and their practical application in some detail and highlights the importance of reproductive isolation in phenetically distinct groups through genetic rather than geographic factors as being a major determinant of appropriate ranking.

These two tetrathecas are morphologically distinct and each can be identified by a number of characters used either alone or in combination. As such, an operational definition of species based on phenetic distinctness can be employed and *Tetratheca* (Die Hardy Range) can reasonably be called a new species. However, this taxon could also be called a subspecies of *T. paynterae* as the two have an extremely close relationship, as indicated by molecular cladistic analyses and they share a number of significant morphological features. The proposed recognition of *Tetratheca* (Die Hardy Range) at species rank stems from a more utilitarian approach to taxon recognition as well as repeated observations of the morphological variation within and between this taxon and *T. paynterae* in the field. As is stated by Thompson (1976), and evident from the morphometric data, some characters (e.g. the presence or absence and density of glandular hairs) are more sensitive to environmental factors than others, but significant morphological features (e.g. pubescence of the ovary, shape of the receptacle, pubescence of the adaxial surface of the leaves) are consistently different between *Tetratheca* (Die Hardy Range) and *T. paynterae* and allow for their immediate distinction.

The same cannot be said for *T. aphylla* and *T.* aff. *aphylla* (Newdegate), for which the amount of variation in the cpDNA *trn*L-*trn*F data is lower than, but comparable to, that between *T. paynterae* and *Tetratheca* (Die Hardy Range) (one base, compared with three bases, different), but which are distinguishable only by small differences in the curvature of the anthers and the length and thickness of the anther filaments. In conjunction with the single base change, this minute amount of morphological difference between these two ecologically and geographically disjunct (*c.* 300 km) taxa has led us to recognise *T.* aff. *aphylla* (Newdegate) as a subspecies of *T. aphylla*. Comparatively, the recognition of *Tetratheca* (Eneabba) at species rank can be easily qualified based on the large morphological and molecular differences evident between this and the other study species. But it must be noted that the leafless habit, which lead to it being misidentified as *T. aphylla* initially, is not unique to this group, and its closest relatives probably lie elsewhere in the genus (possibly *T. pauciflora* J. Thompson, also from near Eneabba, with which it shares the distinctive pubescence of the calyx and peduncles), as might those of *T. paynterae* and *Tetratheca* (Die Hardy Range) (possibly *T. efoliata* F. Muell., a more widespread species also growing in the Koolyanobbing area, with which they share the possession of two ovules per locule).

Without a genus-wide examination of morphological and sequence variation through cladistic analysis, it is really not possible to comment in detail on the relationships between these study species, but further analysis of this *T. aphylla* group using both molecular and morphological characters in conjunction would probably lead to greater resolution of the phylogenetic relationships between the taxa.

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APPENDIX 1:

Methods for DNA extraction, and ITS and *trn*L-F amplification and sequencing utilised by Butcher *et al.* (2001) for *Tetratheca harperi, T. aphylla* and *T. paynterae*. Where methodology has differed in the examination of *Tetratheca* (Die Hardy Range) material and *T.* aff. *aphylla* collections from Eneabba and Newdegate, changes have been noted in the body text.

DNA Extractions:

Extractions were performed using a Qiagen DNeasy® Mini Prep Kit according to the manufacturer's specifications, with 0.01-0.1g of starting material yielding generally less than 25 ng of DNA per µl. To test whether extractions were successful, 5 µl of each extraction elution were run out on an 8% agarose gel for 2 hours at 80 V against a 100 ng molecular ladder and quantitative markers representing 25, 50 and 100 ng standards; the gel was stained for 20 minutes with ethidium bromide then examined under UV light. Successful DNA extractions were indicated by a discrete, high molecular weight band.

ITS:

Amplification of the ITS region was performed through PCR using a Hybaid Touchdown Thermal Cycler operating under the following parameters: 95° C for 5 minutes, followed by 30 cycles of 95° C for 1 minute, 56° C for 1 minute and 72° C for 1 minute, followed by 7 minutes at 72° C. The reaction volume consisted of 5 µl 5x PCR buffer, 1 µl 50 mM $MgCl_2$, 2.5 µl each forward and reverse primers (at 2pmol/µl concentration), 1 unit (0.2 µl) *Taq* polymerase, 1-2 µl DNA (at *c.* 25 ng/µl) and *n* µl dH_2O to a total of 25 µl. Two sets of primers were trialled and both were found to successfully amplify the ITS region in *Tetratheca*. The first primer pairs were ITSLeu1 and ITS4 (Mast 1998, modified from White *et al.* 1990), whilst the second set, P3L and P2R, were designed at the Royal Botanic Gardens, Sydney for work on Proteaceae. The annealing position and directionality of P3L and P2R with regards to the ITS region is shown in Figure 1 in the main text and the compositions of all primers are listed below. To test the success of the PCR, between 2-5 µl of product were run out on an 8%

agarose gel for 2 hours at 80 V against a 100 ng molecular ladder; the gel was stained for 20 minutes with ethidium bromide then examined under UV light. Successful PCR was indicated by a single band on the gel of *c.* 700 base pairs length.

The remaining 20-23 µl of amplified DNA were purified using a HighPure® PCR Purification Kit according to the manufacturer's specifications. DNA was precipitated out of the final 75 µl elution through the addition of 7.5 µl of sodium acetate (3M; pH 5.2) and 150µl of freezer-stored 100% ethanol in a 30 minute spin at 13 000 RPM in a standard table-top centrifuge. The supernatant was removed and the pellet washed with 200 µl of 70% ethanol in a 5 minute centrifugation at 13 000 RPM. The supernatant was again removed and the DNA pellet air dried then resuspended in 20 µl of sterile distilled water.

Sequence reaction of the ITS region was performed through PCR under the following parameters: 96° C for 4 minutes, followed by 25 cycles of 95° C for 30 seconds, 43° C for 15 seconds and 60° C for 4 minutes, utilising two 10 µl reaction volumes for each individual so that the ITS region was sequenced in both directions. Sequencing reaction volumes comprised 4 µl purified DNA, 4 µl Big Dye Terminator (BDT) and 2 µl of either P3L/ITSLeu1 OR P2R/ITS4. DNA was precipitated out through the addition of 1 µl of sodium acetate (3 M; pH 5.2) and 30 µl of freezer-stored 100% ethanol in a 30 minute centrifugation at 13 000 RPM. The supernatant was removed and the pellet washed with 40 µl of 70% ethanol in a 5 minute centrifugation at 13 000 RPM. The supernatant was again removed and the DNA pellet air dried and submitted to the Department of Clinical Immunology at Royal Perth Hospital for gel separation on an ABI-Prism 373 automated sequencer.

ITS primer compositions:

P3L:	5'-TTG AAT GGT CCG GTG AAG TGT TCG G-3'
P2R:	5'-CTT TTC CTC CGC TTA TTG ATA-3'
ITSLeu1:	5'-GTC CAC TGA ACC TTA TCA TTT AG-3'
ITS 4:	5'-TCC TCC GCT TAT TGA TAT GC-3'

*trn*L-*trn*F:

Amplification and sequencing of the *trn*L-*trn*F region utilised universal primers designed by Taberlet *et al.* (1991; compositions shown below and annealing positions indicated in Figure 2 in main text) and the same PCR protocols as outlined for ITS (above), with the following modifications in reaction volume composition and product assessment:

- 25 µl PCR amplification mix: 5 µl 5x PCR buffer, 1.5 µl 50mM $MgCl_2$, 2 µl 2 mM dNTPs, 0.5 µl each 10 mM *trn*C and *trn*F, 1 unit (0.2 µl) *Taq* polymerase, 2 µl DNA, 13.3 µl dH_2O.

- 2- 10 µl PCR sequencing mix: 4 µl DNA, 2 µl BDT, 1 µl either *trn*C or *trn*F and 3 µl dH_2O.

- When testing the preliminary PCR by gel electrophoresis, successful amplification of *trn*L-*trn*F was indicated by a band of *c.* 900 bp length.

*trn*L-*trn*F primer compositions:

*trn*C:	5'-CGA AAT CGG TAG ACG CTA CG-3'
*trn*F:	5'-ATT TGA ACT GGT GAC ACG AG-3'

APPENDIX 2:

Aligned ITS sequence data for all species of *Tetratheca* used in this study. Taxon abbreviations are as used in the main text.

```
[                 10        20        30        40        50        60        70        80        90       100       110       120]
[                  .         .         .         .         .         .         .         .         .         .         .        .]
TW_1.3      TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCCGAACGACCCGTGGACTTGTTCAAGAACCAAACCGCACGGCGCGGCGTCCCKCCTTGGTGGGCCCRCGTCRTC
TW_1.5      TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCCGAACGACCCGTGGACTTGTTCAAGAACCAAACCGCACGGCGCGGCGTCCCGCCTTGGTGGGCCCCGCGTCGTC
TW_2.2      TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCCGAACGACCCGTGGACTTGTTCAAGAACCAAACCGCACGGCGCGGCGTCCCGCCTTGGTGGGCCCCGCGYCRTC
TW_2.3      TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCCGAACGACCCGTGGACTTGTTCAAGAACCAAACCGCACGGCGCGGCGTCCCKCCTTGGTGGGCCCRCGTCRTC
TDH_5       TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCCGAACGACCCGTGGACTTGTTCAAGAACCAAACCGCACGGCGCGGCGTCCCGCCTTGGTGGGCCCRCGTCRTC
TDH_15      TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCCGAACGACCCGTGGACTTGTTCAAGAACCAAACCGCACGGCGCGGCGTCCCGCCTTGGTGGGCCCRCGTCRTC
TH_1.1      TTTCNGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCCGAACGACCCGAGGACTTGTTCAAGAACCGAACCGCACGGCGCGGCGTCCCGCCTTGTGGGGCCCCGCGTCGTC
TH_1.3      NNCCCGNAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCCGAACGACCCGAGGACTTGTTCAAGAACCGAACCGCACGGCGCGGCGTCCCGCCTTGTGGGGCCCCGCGTCGTC
TH_2.1      NNT-CGNAGGNGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCCGAACGACCCGAGGACTTGTTCAAGAACCGAACCGCACGGCGCGGCGTCCCGCCTTGTGGGGCCCCGCGTCGTC
TH_2.5      TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCCGAACGACCCGAGGACTTGTTCAAGAACCGAACCGCACGGCGCGGCGTCCCGCCTTGTGGGGCCCCGCGTCGTC
TAB_1.2     TTTTCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCTGAACGACCCGTGGACTTGTTCGAGAACCTAACAGCACGGCGGCGYGTCCCRCCTTGGYGGGCCCCRYRTCATC
TAB_1.8     TTTYYRTAGGTGAACCTGYGGAAGGATCATTGTCGAGACCTGCCCGGCTGAAYGACCCGTGGACTTGTTCGAGAACCTAACAGCACGGCGGCGYGTCCCRCCTTGGYGGGCCCRYRTCRTC
TAB_2.1     TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCTGAACGACCCGTGGACTTGTTCGAGAACCTAACAGCACGGCGGCGGTCCCGCCTTGGCGGGCCCCCCNTCATC
TAB_2.6     TTTYYRTAGGTGAACCTGYGGAAGGATCATTGTCGAGACCTGCCCGGCTGAACGACCCGTGGACTTGTTCGAGAACCTAACAGCACGGCGGCGYGTCCCRCCTTGGYGGGCCCRYRTCRTC
TAB_3.1     TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCTGAACGACCCGTGGACTTGTTYGAGAACCTAACAGCACGGCGGCGGTCCCGCCTTGGCGGGCCCCGYGTCATC
TAB_3.3     TTTCCRTAGGTGAACCTGCGGAAGGATCATTGTCGARACCTGCCCGGCTGAACGACCCGTGGACTTGTTCGAGAACCTAACAGCACGGCGGCGGTCCCRCCTTGGCGGGCCCCGYRTCATC
TAN_12      TTTCYGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCTGAACGACCCGTGGACTTGTTCGAGAACCTAACAGCACGGCGGCGYGTCCCRCCTTGGYGGGCCCCRYRTCATC
TAN_14      TTTCYGTAGGTGAACCTGYGGAAGGATCATTGTCGAGACCTGCCYGGCTGAACGACCCGTGGACTTGTTCGAGAACCTAACAGCACGGCGGCGYGTCCCGCCTTGGTGGGCCCCGGRTCRTC
TAE_2.10    TTTYCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCYGGCCGAACGACCCGTGGACTTGTTCAAGAACCAAAYCGCACGGCGGCGTGTCCYGCCTTGGTGRGCCYGTGYCATC
TAE_1.4     TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCCGAACGACCCGTGGACTTGTTCAAGAACCAAACTGCCCGGCGGCGYGTCCCGCCTTGGTGGGCCCCGTGYCRTY
TAE_1.8     TTTCCGTWGGTGAACCTGCGGAAGGATCATTGTCGAGACCTGCCCGGCCGAACGACCCGTGGACTTGTTCAAGAACCAAACTGCCCGGCGGCGYGTCCCGCCTTGGTGGGCCCCGTGYCGTC

[                130       140       150       160       170       180       190       200       210       220       230       240]
[                 .         .         .         .         .         .         .         .         .         .         .        .]
TW_1.3      CCGGACCTAACAACCTAACCCGGCGCGGTCTGCGCCAAGGAAGTACGAAGAAGGGG-TCACTTGTCACCGGCGCGAGCGGGTGGCTGACCTCTGTACA-TACATATACATAA---TATAT
TW_1.5      CCGGACCTAACAACCTAACCCGGCGCGGTCTGCGCCAAGGAAGTACGAAGAAGGGG-TCACTTGTCACCGGCGCGAGCGGGTGGCTGACCTCTGTACA-TACATATACATAA---TATAT
TW_2.2      CCGGACCTAACAACCTAACCCGGCGCGGTCTGCGCCAAGGAAGTACGAAGAAGGGG-TCACTTGKCACCGGCGCGAGCGGGTGGCTGACCTCTGTACA-TACATATACATAA---TATAT
TW_2.3      CCGGACCTAACAACCTAACCCGGCGCGGTCTGCGCCAAGGAAGTACGAAGAAGGGG-TCACTTGTCACCGGCGCGAGCGGGTGGCTGACCTCTGTACA-TACATATACATAA---TRTAT
TDH_5       CCGGACCTAACAACCTAACCCGGCGCGGTCTGCGCCAAGGAAGTACRAAGAAGGGG-TCACTTGTSWCCGGCGCGAGCGGGTGGCTGAYCTCTGTACA-TACATATACATAA---TATAT
TDH_15      CCGGACCTAACAACCTAACCCGGCGCGGTCTGCGCCAAGGAAGTACRAAGAAGGGG-TCACTTGTSRCCGGCGCGAGCGGGTGGCTGACCTCTGTACA-TACATATACATAA---TATAT
TH_1.1      CCGGACCTAACAACCTAACCCGGCGCGGTCTGCGCCAAGGAAGTACGAAGAAGGGGGTCACTTGTCACCGGCGCGAGCGGGTGGCTGACCTCCATATA-TATATTTTTATA----TATAT
TH_1.3      CCGGACCTAACAACCTAACCCGGCGCGGTCTGCGCCAAGGAAGTACGAAGAAGGGGGTCACTTGTCACCGGCGCGAGCGGGTGGCTGACCTCCATATA-TATATTTTTATA----TATAT
TH_2.1      CCGGACCTAACAACCTAACCCGGCGCGGTCTGCGCCAAGGAAGTACGAAGAAGGGGGTCACTTGTCACCGGCKCGAGCGGGTGGCTGACCTCCATATAATATATTTTTATA----TATAT
TH_2.5      CCGGACCTAACAACCTAACCCGGCGCGGTCTGCGCCAAGGAAGTACGAAGAAGGGGGTCACTTGTCACCGGCGCGAGCGGGTGGCTGACCTCCATATA-TATATTTTTATA----TATAT
TAB_1.2     ACGGACCTAACAACCTAACCCGGCGYGGTCTGYGCCAAGGAAGTACGAAGAAGGGG-TCACTTGTCACCGGCGYGAGCGGGTGGCCGACCTCCATACA-TACATATATATCTATGTATGT
TAB_1.8     RYGGACCTAACAACCTAACCCGGCGCGGTCTGCGCCAAGGAAGTACGAAGAAGGGG-TCACTTGTCACCGGCGCGAGCGGGTGGCCGACCTCCATACA-TACATATATATCTATGTATGT
TAB_2.1     ACGGACCTAACAACCTAACCCGGCGCGGTCTGCGCCAAGGAAGTACGAAGAAGGGG-TCACTTGTCACCGGCGCGAGCGGGTGGCCGACCTCCATACA-TACATATATATCTATGTATGT
TAB_2.6     RYGGACCTAACAACCTAACCCGGYGYGGTCTGYGCCAAGGAAGTACGAAGAAGGGG-TCACTTGTCACCGGCGYGAGCGGGTGGCCGACCTCCATACA-TACATATATATCTATGTATGT
TAB_3.1     ACGGACCTAACAACCTAACCCGGCGYGGTCTGYGCCAAGGAAGTACGAAGAAGGGG-TCACTTGTCACCGGCGCGAGCGGGTGGCCGACCTCCATACA-TACATATATATCTATGTATGT
TAB_3.3     ACGGACCTAACAACCTAACCCGGCGCGGTCTGYGCCAAGGAAGTACGAAGAAGGGG-TCACTTGTCACCGGCGYGAGCGGGTGGCCGACCTCCATACA-TACATATATATCTATGTATGT
TAN_12      ACGGACCTAACAACCTAACCCGGGGYGGTCTGCGCCAAGGAAGTACGAAGAAGGGG-TCACTTGTCACCGGCGCGAGCGGGTGGCCGACCTCCATACA-TACATATATATSTATGKATGK
TAN_14      AYGGACCTAACAACCTAACCCGGYGTGGTCTGTGCCAAGGAAGTAYRAAGAAGGGG-TCACTTGTCACCGGCGYRAGCGGGTGGCCGACCTCCATACA-TACATATATATCTATGTATGT
TAE_2.10    RYGGACCTAACAACCTAACCYGGSGSGGTCTGCGMCAAGGAAGTACGAAGAAGGGG-TCACTTRTTACCNGCGSGAGSGGGTGGYC-ACCCCTATACA-TACATRTATAKGM--GTATGT
TAE_1.4     RYGGACCTAACAACCTAACCCGGCGCGGTCTGCGCCAAGGAAGTACGAAGAAGGGG-TCACTTGTCACCAGCGCGAGCGGGTGGCCRMCCYNNNNNNNNNNNNNNNNNNNNNNNNNN
TAE_1.8     GYGGACCCAACAACCTAACCCGGCGCGGTCTGCGCCAAGGAAGTWCGAAGAAGGGG-TCMCTTGTCACCGGCGCGGAGCGGGTGGCCGACCTYTWTMCA-TNNNNNNNNNNNNNNNNNNN
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[                  250        260        270        280        290        300        310        320        330        340        350        360]
[                    .          .          .          .          .          .          .          .          .          .          .          .]
TW_1.3      ATGT--------ATGTATATATATTTGACAGAACGACTCTYGGCAACGGATATCTCGGCTCTTGCATCGATGAARAACGTA-GCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGT
TW_1.5      ATGT--------ATGTATATATATTTGACAGAACGACTYTYGGCAACGGATATCTCGGCTCTTGCATCGATGAAGAACGTA-GCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGT
TW_2.2      ATGT--------ATGTATATATATTTGACAGAACGACTCTCGGCAACGGATATCTCGGMTCTTGMATCGATRAAGAACGTA-GCGAAATGCGATACTTGGTGTGAATTGCARAATCCCGT
TW_2.3      ATGT--------ATGTATATATATTTGACAGAACGACTCTCGGCAACGGATATCTCGGCTCTTGCATCGATGAAGAACGTA-GCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGT
TDH_5       ATGT--------ATGTATATATATTWGACAGAACGACTCTCGGCAACGGATATCTCGGCTCTTGCATCGATGAARAACGTA-GCGAAATGCGATACTTGGTGTGAATTGCADAATCCCGT
TDH_15      ATGT--------ATGTATATATATTWGACAGAACGACTCTYGGCAACGGATATSTMGGCTCTTGCATCGATGAARAACGTA-GCGAAATGCGATACTTGGTGTGAATTGCADAATCCCGT
TH_1.1      ATAT--------GCTCGAAGAGAGCCAACAGAACGACTCTCGGCAACGGATATCTCGGCTCTYGCATMGATGAARAACGTA-GCGAAATGCGATACTTGGTGTGAATTGCANAATCCCGT
TH_1.3      MTAT--------GCTCGAAGAGAGCCAACAGAACGACTCTCGGCAACGGATATCTCGGCTCTTGCATCGATGAAGAACGTA-GCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGT
TH_2.1      ATAT--------GCTCGAAGAGAGCCAACAGAACGACTCTCGGCAACGGATATCTCGGCTCTTGCATMGATGAARAACGTA-GCGAAATGCGATACTTGGTGTGAATTGCANAATCCCGT
TH_2.5      ATAT--------GCTCGAAGAGAGCCAACAGAACGACTCTCGGCAACGGATATCTCGGCTCTTGCATCGATGAAGAACGTA-GCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGT
TAB_1.2     ATATTTTTATTAGCTCGAAGAGAGCCAACAGAAYGACTCTNGGCAACGGATATCTNGGCTCTTGCATCGATGAAGAACGTA-GCAAAATGCGATACTTGGTGTGAATTGYAGAATCCCGT
TAB_1.8     ATATTTTTATTAGCTCGAAGAGAGCCAACAGAAYGACTCTYGGCAACGGATATYTYGGCTYTTGCATCGATGAARAACGTA-GCGAAATGYGATACTTGGTGTGAATTGYAGAATCCCGT
TAB_2.1     ATATTTTTATTAGCTCGAAGAGAGCCAACAGAATGACTCTCGGCAACGGATATCTCGGCTCTTGCATCGATGAAGAACGTA-GCNAAATGCGATACTTGGTGTGAATTGCAGAATCCCGT
TAB_2.6     ATATTTTTATTAGCTCGAAGAGAGCCAACAGAAYGACTCTCGGCAACGGATATCTYGGCTCTTGCATCGATGAAGAACGTA-GCGAAATGYGATACTTGGTGTGAATTGYARAATCCCGT
TAB_3.1     ATATTTTTATTAGCYCGAAGAGAGCCAACARAACGACTTTYGGGAACGGATATCTCRGYTCTYGCATCRATGAANAACGTA-CCGAAATGTGATACTTGGTGTNAATTNCAGAATCCHGT
TAB_3.3     ATATTTTTATTAGCTCRAAGAGAGCCAACAGAATGACTCTCGGCAAYGGATATYTYGGCTCTTGCATCGATGAAGAACGTAGCCGAAATGYGATACTTGGTGTGAATTGCAGAATCCCGT
TAN_12      ATATTTTTATTAGCTCGAAGAGAGSCAACAGAAYGACTCTYGGGAACGGATATTTYGGCTCTTGCATCGATGAAGAACGTA-GCGAAATKYGATACTTGGTGTGAATTGCAGAATCCCGT
TAN_14      ATATTTTTATTAGCTCRAAGAGAGCCAACAGAATGACTCTCGGYAACGGATATCTYGGCTCTTGCATCGATGAAGAACGTA-GCGAAATGYGATACTTGGTGTGAATTGCAGAATCCCGT
TAE_2.10    --------ATTAGCTCAAAGAAAGCCWACAGAAYGMCTCTCRMCAACGRATATCTSGGYTCTTGCATCRATAAAGAASGWA-GCGAAATCCRATACTTGGTATGAATTSCAGAATCCTGT
TAE_1.4     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
TAE_1.8     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNSAARAWYCCKKK

[                  370        380        390        400        410        420        430        440        450        460        470        480]
[                    .          .          .          .          .          .          .          .          .          .          .          .]
TW_1.3      GAACCATTGAGTCTTTGAACGCAAGTTGCGCCCTAAGCCTTTAGGCCGAGGGCAMGTCCGCCTGGGTGTCACGCATCGTCGACCCCCCACCCCGGAGAACCTCGCCGCCRCGYGGGGCTT
TW_1.5      GAACCATTGAGTCTTTGAACGCAAGTTGCGCCCTAAGCCTTTAGGCCGAGGGCACGTCCGCCTGGGTGTCACGCATCGTCGACCCCCCACCCCGGAGAACCTCGCCGCCRCGYGGGGCTT
TW_2.2      GAACCATTGAGTCTTTGAACGCAAGTTGCGCCCTAAGCCTTTAGSCCRAGGGCACGTCCGCCTGGGTGTCACGCATCGTCGACCCCCCACMCYRGAGAACCTCGCCGCCRCGYGAGGCTT
TW_2.3      GAACCATTGAGTCTTTGAACGCAAGTTGCGCCCTAAGCCTTTAGGCCGAGGGCAMGTCCGCCTGGGTGTCACGCATCGTCGACCCCCCACCCYRGAGAACCTCGCCGCCRCGYGRGGCTT
TDH_5       GAACCATTGAGTCTTTGAACGCAWGTTGCGCCCTAAGCCTTTAGGCCGAGGGCACGTCCGWCTGGGTGTCACGCATCGTCGACCCCCCACMCYRGAGAACCTCGCCRCCGCGYGGGGCTT
TDH_15      GAACCATTGAGTCTTTGAACGCAWGTTGCGCCMTAAGCCTTTAGGCCGAGGGCACGTSCGCCTGGGTGTCACGCATCGTCGACCCCCCACMCYRGAGAACCTCGCCGCCRCGYGRGGCTT
TH_1.1      NAACCATTGAGTCTTNGAACGCAAGTTGCGCCCTAAGCCNCTAGGCCNAGGGCACGTNCCGNTGGGTGTCANGCTTCGTCGNCCCCCCCCCCTGTAGAACCTCTCCGCGA---GGGGCTT
TH_1.3      GAACCATTGAGTCTTTGAACGCAAGTTGCGCCCTAAGCCGTTAGGCCGAGGGCACGTCCGCCTGGGTGTYACGCTTYSTCSACCCCCCCCCCTGTAGAACCTCTCCGCGA---GGGGCTT
TH_2.1      GAACCATTGAGTCTTTGAACRCAAGTTGCGCTCTAAGCCGATAGGCCGAGGGCACGTCCCCCTGGGKKTCACNGTTTGGCNVCCCCCCCCCCTGTAGAACCTCTCCGCGA---GGGGCTT
TH_2.5      GAACCATTGAGTCTTTGAACGCAAGTTGCGCCCTAAGCCGTTAGGCCGAGGGCACGTCCGCCTGGGTGTCACGCTTTGTCGACCCCCCCCCCTGTAGAACCTCTCCGCGA---GGGGCTT
TAB_1.2     GAACCATTGAGTCTTTGAACGCAAGTTGCGCCCTAAGCCGTTAGGCCGAGGGCACGTCCGCCTGGGTGTCACGCATCGTYGACCCCCCACCCCGGAGAACCTCACCAYGY---GGGGCTT
TAB_1.8     GAACCATTGAGTCTTTGAACGCAAGTTGCGCCCTAAGCCGTTAGGCCGAGGGCACGTCCGCCTGGGTGTCACGCATCRTYGACCCCCCACCCYGGAGAACCTCACCAYGY---GGGGCTT
TAB_2.1     GAACCATTGAGTCTTTGAACGCAAGTTGCGCCCTAARCCGTTAGGCCRAGGGCACGTCCGCCTGGGTGTCACGSATCGTTGACCCCCCACCCCGGAGAACCTCACCRYGY---GGGGCTT
TAB_2.6     GAACCATTGAGTCTTTGAACGCAAGTTGCGCCCTAAGCCGTTAGGCCGAGGGCACGTCCGCCTGGGTGTCACGCATYRTYGACCCCCCACCCCGGAGAACCTCACCRYGY---GGGGCTT
TAB_3.1     GAACCATTGAGTCTTTGAACGCAAGTTRCGCCCTAARCCGTTAGGCYRAGGGCACGTCCGCCTGGGTGTCACGSATCGKTGACCCCCCACCCHGGAGAACCTCKCCRYGY---GGGGCTT
TAB_3.3     GAACCATTGAGTCTTTGAACGCAAGTTGCGCCCTAAGCCGTTAGGCCGAGGGCACGTCCGCCTGGGTGTCACGCATCGTYGACCCCCCACCCCGGAGAACCTCACCRYGY---GGGGCTT
TAN_12      GAACCATTGAGTCTTTGAACGCAAGTTGCGCCCTAAGCCGTTAGGCCGAGGGCACGTCCGCCTGGGTGTCACGCATYGTYGACCCCCCACCCCGGAGAACCTCRCCRYRT---GGGGCTT
TAN_14      GAACCATTGAGTCTTTGAACGCAAGTTGCGCCCTAAGCCGTTAGGCCGAGGGCACGTCCGCCTGGGTGTCACGCATTGTTGACCCCCCACCCCGGAGAACCTYAYYRTGT---GGGGCTT
TAE_2.10    GAMCCATTRAGTCTTTGAACSCAAGTTGCSCCTTATGYKGTTAGSCCAAKGGCASGTCCGCGTGGGTGTCATGCATSGTSGACMCCCCACCCSGRARAMCCTCRCSATGG---KGGGYTT
TAE_1.4     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGGYTT
TAE_1.8     AA-CCMWTKAGTCTTTKAACMCMAGTTGCCCCCTWAACTGTTARGCCSAAGGCMMGKCCSCCTKGGTKTYMMGSATYGTCGACCCCCCMCCCCGGAAAACCTCSCCSMKS---GGGGCTT
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[                490       500       510       520       530       540       550       560       570       580       590       600]
[                .         .         .         .         .         .         .         .         .         .         .         .]
TW_1.3      CGGAGGGGGGCAAGCTGGCCTCCCGTGAGCC-TCRCGGGCRCGGTTGGCCCAAAATCGAGCCCCACAAGCGCTGGAGCGCAACGGCAAAYGGTGGTTGGCCTGATCCAAAGACCACGG
TW_1.5      CGGAGRGGGGCAAGCTGGCCTCCCGTGAGCC-TCRCGGGCRCGGTTGGCCCAAAATCGAGCCCCACAAGCGCTGGAGCGCAACGGCAAAYGGTGGTTGGCCTGATCCAAAGACCACGG
TW_2.2      CGGAGRGGGGCAAGCTGGCCTCCCGTGAGCC-TCRCGSGGCRCGGTTGGCCCAWAATCGAGCCCCACAAGCGCTGGAGCGCAACGGCAAAYGGTGGTTGGCCTGATCCAAAGACCACGG
TW_2.3      CGGAGGGGGCGYAAGCTGGCCTCCCGTGAGCC-TCRCGSGGCRCGGTTGGCCCAAAATCGAGCCCCACAAGCGCTGGAGCGCAACGGCAAAYGGTGGTTGGCCTGATCCAAAGACCACGG
TDH_5       SRGAGGGGGGCAAGCTGGCCTCCCGTGAGCC-TCRCGGGCGCGGTTGGCCCAAAATCGAGCCCCACAAGCGCTGGAGCGCAACGGCAAAYGGTGGTTGGCCTGATCCAAAGACCACGG
TDH_15      CGGAGGGGGGCAAGCTGGCCTCCCGTGAGCC-TCRCGGGCGCGGTTGGCCCAAAATCGAGCCCCACAAGCGCTGGAGCGCAACGGCAAAYGGTGGTTGGCCTGATCCAAAGACCACGG
TH_1.1      CGGAGGGGGCGTAAGCTGGCCTCCCGTGCGCC-TTGCCGGCGCGGTTGGCCCAAAACCGAGCCCCACAAGCGCTGGAGCGCAACTGCAAACGGTGGTTGGCCTGATCCAAAGACCGCGG
TH_1.3      CGGAGGGGGCGTAAGCTGGCCTCCCGTGCGCC-TTGCCGGCGCGGTTGGCCCAAAACCGAGCCCCACAAGCGCTGGAGCGCAACTGCAAACGGTGGTTGGCCTGATCCAAAGACCGCGG
TH_2.1      CGGAGGGGGCGTAAGCTGGCCTCCCGTGCGCC-TTGCCGGSGCGGTTGGCCCAAAACCGAGCCCCACAAGCGCTGGAGCGCAACTGCAAACGGTGGTTGGCCTGATCCAAAGACCGCGG
TH_2.5      CGGAGGGGGCGTAAGCTGGCCTCCCGTGCGCC-TTGCCGGCGCGGTTGGCCCAAAACCGAGCCCCACAAGCGCTGGAGCGCAACTGCAAACGGTGGTTGGCCTGATCCAAAGACCGCGG
TAB_1.2     YRGAGGGGGCGTAAGCTGGCCTCCCGTGCTCC-TTRYGGGCRCGGTTGGCCCAAAATYGAGCCCCACAAGCGCTGCAGYGCAAGTGCAAATGGTGGTTGGCCTGATCCAAAGACCACGG
TAB_1.8     YRGAGGGGGCGTAAGCTGGCCTCCCGTGCTCC-TTRCGGGGCGCGGTTGGCCCAAAATYGAGCCCCACAAGCGCTGCAGYGCAAGTGCAAAYGGTGGTTGGCCTGATCCAAAGACCACGG
TAB_2.1     YRGAGGGGGCRTAAGCTGGCCTCCCGTGCTCC-TTRYGGGCRCGGTTGGCCCAAAATYGAGCCCCACAAGCGCTGCAGYGCAAGTGCAAATGGTGGTTGGCCTGATCCAAAGACCACGG
TAB_2.6     YRGAGGGGGCGTAAGCTGGCCTCCCGTGCTCC-TTRCGGGGCRCGGTTGGCCCAAAATYGAGCCCCACAAGCGCTGCAGYGCAAGTGCAAATGGTGGTTGGCCTGATCCAAAGACCACGG
TAB_3.1     YRGAGGGGGCGTAAGCTGGCCTCCCGTGCTCC-TTRYRGGGSGYGGTTGGCCCAAAATYGAGCCCCACAAGYRCTGCAGYGCAAGTGCAAATGGTGGTTGGCCTGATCCAAAGACCAYGG
TAB_3.3     YRGAGGGGGCRTAAGCTGGCCTCCCGTGCTCC-TTRYRGGGCRYGGTTGGCCCAAAATYGAGCCCCACAAGCGCTGCAGYGCAAGTGCAAATGGTGGTTGGCCTGATCCAAAGACCACGG
TAN_12      YGGAGGGGGCGTANGCTGGCCTCCCGTGCTCC-TTGCGGGGCRCGGTTGGCCCAAAATCGAGCCCCACAAGCGCTGCAGCGCAAGTGCAAAYGGTGGTTGGCCTGATCCAAAGACCACGG
TAN_14      TGGAGGGGGCGTAAGCTGGCCTCCCGTGCTCC-TTGCGGGGCRCGGTTGGCCCAAAATYGAGCCCCACAAGCGCTGCAGYGCAAGTGCAAATGGTGGTTGGCCTGATCCAAAGACCACGG
TAE_2.10    SRAAGGGGGCGTAAGTTGSCYTYYCRTGCTCC-TTGCGGAGCGGSGATTGGCCCAAAAACGAGCCCCACAAGCGCTGGAGCGCAACTGCAAAAGGTGGTTRGCCTGATCCAAAGACCACGG
TAE_1.4     SGAAGGGGGCDTAASTKGSCTTCCC-TGYYCC-TTSSGMRGCGKGATTRGCCCAAAAAYGAGCCCCACAAGCGCTGGAGCGCAACTGCAAAHGGTGGTTGGCCTGATCCAAAGACCACGR
TAE_1.8     SGAAGGGGCGTWAACTGGCCTYCCS-TKCTCCCTTCSGCRSCSGGCTKGCCCMAAAAACGAGCCCCACAAGCGCTGGAGCGCAACTGCAAACGGTGGTTGGCYTGATCCAAAGACCACGR

[                610       620       630       640       650       660       ]
[                .         .         .         .         .         .         ]
TW_1.3      AACACCGTCGTGCAAAGCGTCMGCGAGGTGGGTGCTCCACGACCACGAACGCTGGCAIMGYGACCCCAG
TW_1.5      AACACCGTCGTGCAAAGCGTCCGCGAGGTGGGTGCTCCACGACCACGAACGCTGGCATCGYGACCCCAG
TW_2.2      AACACCGTCGTGCAAAGCGTCMGCGAGGTGGGTGCTCCACGACCACGAACGCTGGCAIMGYGACCCCAG
TW_2.3      AACACCGTCGTGCAAAGCGTCCGCGAGGTGGGTGCTCCACGACCACGAACGCTGGCATCGYGACCCCAG
TDH_5       AACACCGTCGTGCAAAGCGTCCGCGAGGTGGGTGCTCCAYGACCACGAACGCTGGCATCGCGACCCCAG
TDH_15      AACACCGTCGTGCAAAGCGTCCGCGAGGTGGGTGCTCCACGACCACGAACGCTGGCATCGCGACCCCAG
TH_1.1      AACACCGTCGTGCAAAGCGCCTGCGAGGTGGGTGCGTCACGACCACGAACGCTGGCATCGCGACCCCAG
TH_1.3      AACACCGTCGTGCAAAGCGCCTGCGAGGTGGGTGCGTCACGACCACGAACGCTGGCATYGCGACCCCAG
TH_2.1      AACACCGTCGTGCAAAGCGCCTGCGAGGTGGGTGCGTCACGACCACGAACGCTGGCATCGCGACCCCAG
TH_2.5      AACACCGTCGTGCAAAGCGCCTGCGAGGTGGGTGCGTCACGACCACGAACGCTGGCATCGCGACCCCAG
TAB_1.2     AACACCGTCGTGCAAAGCGTCTGYGAGGTGGGTGCTCCAYGACCAYGAACGCTGGCATCGCGACCCCAG
TAB_1.8     AACACCGTCGTGCAAAGCGTCTGYGAGGTGGGTGCTCCAYGACCAYGAACGCTGGCATCGCGACCCCAG
TAB_2.1     AACACCRTCRTGCAAAGCGTCTRYGAGGTGGGTGCTCCAYGACCAYGAACGCTGGYATCGYGACCCCAG
TAB_2.6     AACACCRTCGTGCAAAGCGTCTGYGAGGTGGGTGCTCCAYGACCAYGAACGCTGGCATCGYGACCCCAG
TAB_3.1     AACACCGTMRTGYAAAGCGTCTGTGAGGTGGGTGCTCCAYGACCAYGAACGCTGGCATCGTGACCCCAG
TAB_3.3     AACACCRTCRTGYAAAGCGTCTGYGAGGTGGGTGCTCCAYGACCAYGAACGCTGGCATCGYGACCCCAG
TAN_12      AACACCGTCGTGCAAAGCGTCTGYGAGGTGGGTGCTCCAYGACCAYGAACGCTGGCATCGCGACSCCAG
TAN_14      AACACCGTCGTGGAAAGCGTCTGTGAGGTGGGTGCTCCATGACCATGAACGCTGGCATCRNNNNNNNNN
TAE_2.10    AACACCGTCGTGCAAAGCGTCTGCGAGGTGGGTGCTCCAYGACCAYGAACGCTGGCATCGCGANNNNNN
TAE_1.4     AACACCGTCGTGCAAAGCGTCTGCGAGGTGGGTGCTCCAYGACCAYGAACGCTGGCATCGCGACCNNNN
TAE_1.8     AAAACCGTCGTGCAAAGCGTCTGCGAGGTGGGTGCTCCAYGACCAYGAACGCTGGCATCGCGACCNNNN
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APPENDIX 3:

Aligned *trnL-trnF* sequence data for all the *Tetratheca* species used in this study and including sequence of *T. rupicola*. Taxon abbreviations are as used in the main text.

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[              10        20        30        40        50        60        70        80        90       100       110       120]
[               .         .         .         .         .         .         .         .         .         .         .        .]
TDH2      GAAACTTACTAAGIGATAACTTTCAAATTCAGAGAAACCCTGGAATAAAAAATGGGCAATCCTGAGCCAAATCCTGTTTTCTGAAAACAAACGAAGGTTCAGAAAGCGAGAATCAAAAAA
TDH10     GAAACTTACTAAGIGATAACTTTCAAATTCAGAGAAACCCTGGAATAAAAAATGGGCAATCCTGAGCCAAATCCTGTTTTCTGAAAACAAACGAAGGTTCAGAAAGCGAGAATCAAAAAA
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TW1.3     GAAACTTACTAAGIGATAACTTTCAAATTCAGAGAAACCCTGGAATAAAAAATGGGCAATCCTGAGCCAAATCCTGTTTTCTGAAAACAAACGAAGGTTCAGAAAGCGAGAATCAAAAAA
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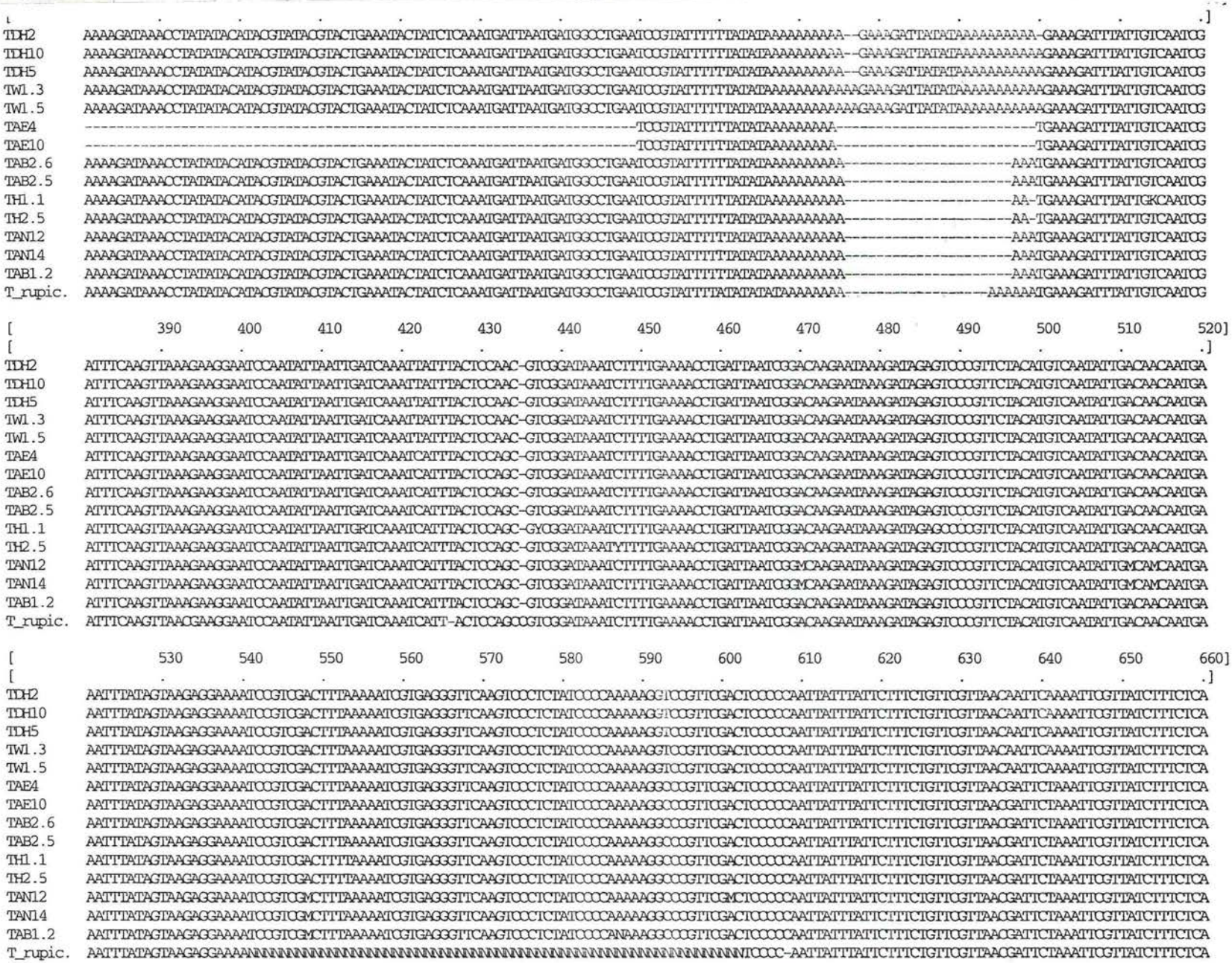
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TAE4
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TH1.1
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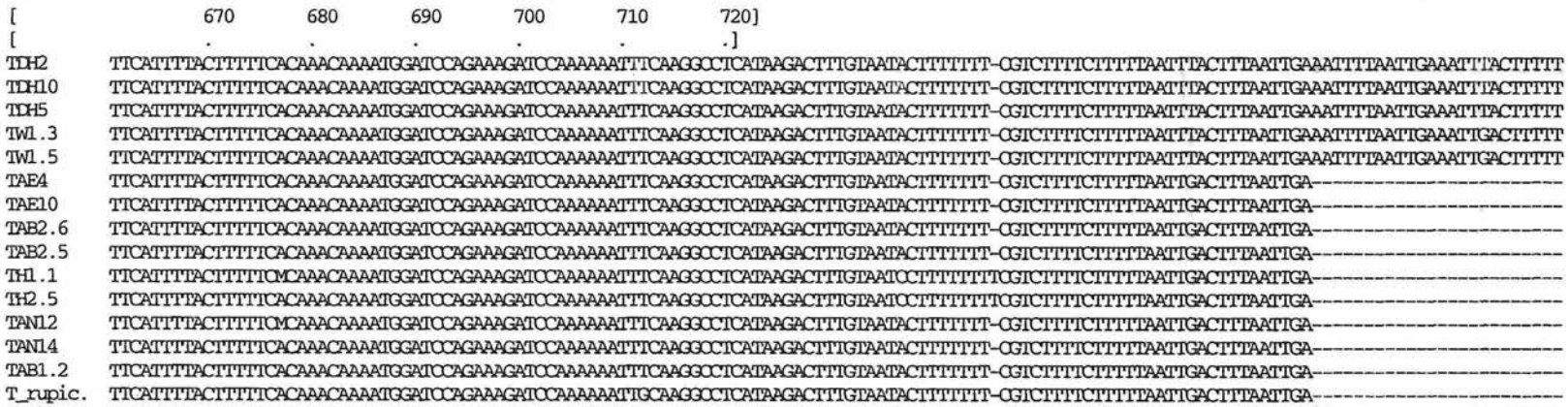
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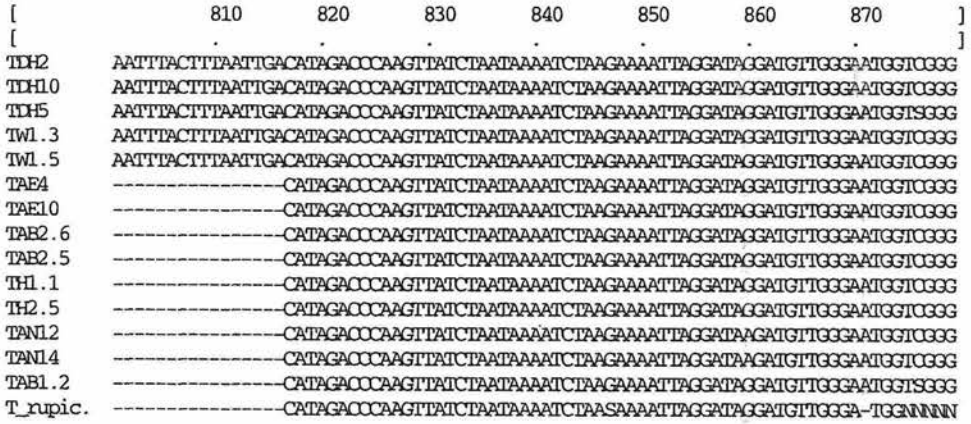
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APPENDIX 4:

A complete list of characters measured for the morphometric analysis of variation between *Tetratheca paynterae* and *Tetratheca* (Die Hardy Range). All measurements were recorded in millimetres (mm).

Quantitative and ratio characters:

1. **Stem diameter:** measured with digital callipers just below each flower.
2. **Leaf length:** see Figure 18 .
3. **Leaf width:** see Figure 18 .
4. **Leaf L:W ratio:** Leaf length/Leaf width.
5. **Leaf length to widest point:** see Figure 18 .
6. **Leaf -position of widest point:** Leaf length to widest point/Leaf length.
7. **Leaf # hairs on adaxial surface:** The total number of hairs in the middle of the leaf were counted along a 1 mm transect as seen at x25 magnification using a microscope with a 1 cm graticule eyepiece. See Figure 18 .
8. **Leaf number of resin hairs:** The total number of resin-tipped hairs occurring along the margin of the leaf were counted. See Figure 18 .
9. **Calyx length:** see Figure 18 .
10. **Calyx width:** see Figure18 .
11. **Calyx L:W ratio:** Calyx length/Calyx width.
12. **Calyx length to widest point:** see Figure 18 .
13. **Calyx-position of widest point:** Calyx length to widest point/Calyx length.
14. **Calyx hairs:** The total number of hairs in the middle of the calyx segment were counted along a 1 mm transect as seen at x25 magnification using a microscope with a 1 cm graticule eyepiece. See Figure 18 .
15. **Calyx resin hairs:** The total number of resin-tipped hairs occurring along the margin of the calyx segment were counted.
16. **Petal length:** see Figure 18 .
17. **Petal width:** see Figure 18 .
18. **Petal L:W ratio:** Petal length/Petal width.
19. **Petal length to widest point:** see Figure 18 .

20. **Peduncle length:** see Fig 18 .
21. **Peduncle hairs:** The total number of hairs in the middle of the peduncle were counted along a 1 mm transect as seen at x25 magnification using a microscope with a 1 cm graticule eyepiece. See Figure 18 .
22. **Peduncle resin hairs:** The total number of resin-tipped hairs in the middle of the peduncle were counted along a 1 mm transect as seen at x25 magnification using a microscope with a 1 cm graticule eyepiece. See Figure 18 .
23. **Receptacle diameter:** see Figure 18 .
24. **Ovary length:** see Figure 18 .
25. **Ovary width:** see Figure 18 .
26. **Ovary L:W ratio:** Ovary length/Ovary width.
27. **Ovary length to widest point:** see Fig 18 .
28. **Ovary-position of widest point:** Ovary length to widest point/Ovary length.
29. **Ovary # hairs:** The total number of hairs to the side of the centre-line of the ovary were counted along a 1 mm transect as seen at x25 magnification using a microscope with a 1 cm graticule eyepiece. See Figure 18 .
30. **Ovary # resin hairs:** The total number of resin-tipped hairs to the side of the centre-line of the ovary were counted along a 1 mm transect as seen at x25 magnification using a microscope with a 1 cm graticule eyepiece. See Figure 18 .
31. **Style length:** see Figure 18 .
32. **Style- extension of hairs:** see Figure 18 . This value included both simple and resin-tipped hairs.
33. **Style-proportion covered with hairs:** Style extension of hairs/Style length.
34. **Stamens total length:** see Figure 18 .
35. **Anther tube length:** see Figure 18 .
36. **Anther body length:** see Figure 18 .
37. **Anther filament length:** see Figure 18 .
38. **Anther filament fusion:** see Figure 18 .
39. **Anther filament- proportion fused:** Anther filament fusion/Anther filament length.
40. **Calyx segment #:** Numeric value.
41. **Petal #:** Numeric value.
42. **Anther #:** Numeric value.

Binary coded qualitative characters:

1. **Leaf hairs on adaxial surface:** 0- glabrous or with few hairs at apex; 1- densely hairy throughout.
2. **Peduncles tuberculate:** 0- rounded tubercules absent; 1- rounded tubercules present.
3. **Receptacle shape:** 0- receptacle appearing distinctly angular or lobulate with thickenings between the calyx segments; 1- receptacle appearing almost circular to slightly hexagonal and not thickened between the calyx segments.
4. **Style end colour:** 0- creamy yellow; 1- dull purple
5. **Stamens anther tube colour:** 0- creamy yellow; 1- dull purple
6. **Anther filament colour:** 0- yellow; 1- red
7. **Hairs on inner surface of anther filament:** 0- absent; 1- present.

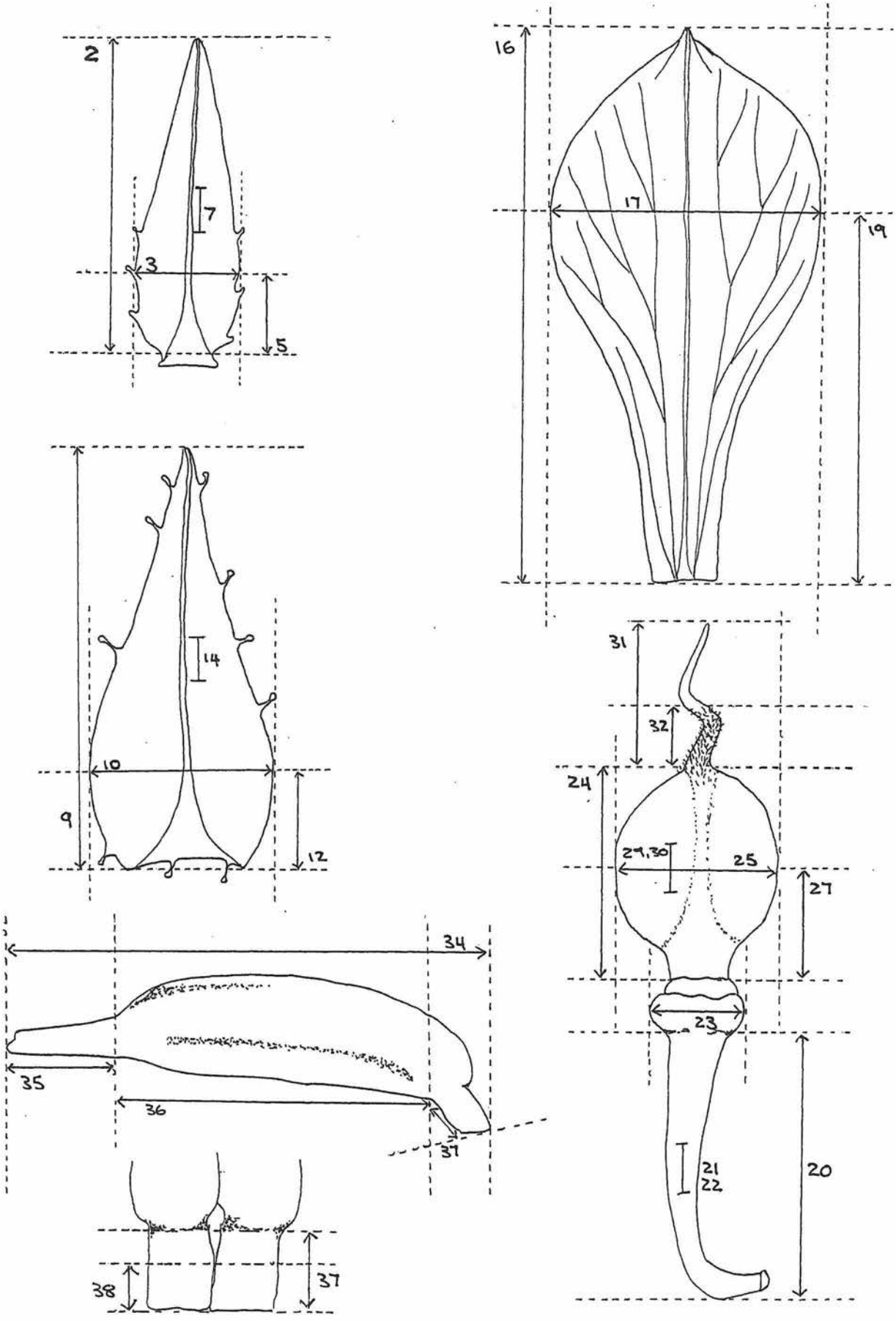

Figure 18 : Measurement standards for vegetative and floral characters examined in the morphometric analysis of variation in *Tetratheca paynterae* and *Tetratheca* (Die Hardy Range). Numbers on diagrams correspond to characters listed in Appendix 4.