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Molecular phylogenetics of the clover genus (*Trifolium*—Leguminosae)

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Abstract

Trifolium, the clover genus, is one of the largest genera of the legume family. We conducted parsimony and Bayesian phylogenetic analyses based on nuclear ribosomal DNA internal transcribed spacer and chloroplast *trnL* intron sequences obtained from 218 of the ca. 255 species of *Trifolium*, representatives from 11 genera of the vicioid clade, and an outgroup *Lotus*. We confirm the monophyly of *Trifolium*, and propose a new infrageneric classification of the genus based on the phylogenetic results. Incongruence between the nrDNA and cpDNA results suggests five to six cases of apparent hybrid speciation, and identifies the putative progenitors of the allopolyploids *T. dubium*, a widespread weed, and *T. repens*, the most commonly cultivated clover species. Character state reconstructions confirm $2n = 16$ as the ancestral chromosome number in *Trifolium*, and infer a minimum of 19 instances of aneuploidy and 22 of polyploidy in the genus. The ancestral life history is hypothesized to be annual in subgenus *Chronosemium* and equivocal in subgenus *Trifolium*. Transitions between the annual and perennial habit are common. Our results are consistent with a Mediterranean origin of the genus, probably in the Early Miocene. A single origin of all North and South American species is hypothesized, while the species of sub-Saharan Africa may originate from three separate dispersal events.

Keywords: *Trifolium*; Clover; Leguminosae; Fabaceae; Phylogeny; Biogeography; Hybrid speciation; Chromosome evolution; Life history evolution; DNA barcoding

1. Introduction

The Leguminosae (= Fabaceae) is the third largest family of flowering plants (727 genera and ca. 19,325 species; Lewis et al., 2005) and the clover genus, *Trifolium* L., is one of the largest genera in the family, with ca. 255 species (Gillett and Taylor, 2001; Zohary and Heller, 1984). The genus name refers to the distinctive leaves usually composed of three leaflets (trifoliolate). All species are herbaceous perennials or annuals, often prostrate and rarely more than 50 cm tall. The small to medium-sized flowers (ca. 0.3–2.5 cm) are usually arranged in capitate to spicate heads. The four lower petals (wing and keel) are partially connate and their claws are adnate to the staminal tube; the upper petal (banner) may also be connate to the lower petals, and sometimes to the free stamen (Hossain, 1961; Zohary and Heller, 1984). The corolla and calyx are generally persistent after anthesis, with one or sometimes both functioning in fruit dispersal (Zohary, 1972). Fruits are usually only 1–2 seeded, but may contain up to nine seeds. The pods may be regularly dehiscent, or lacking sutures and irregularly dehiscent. In the latter case, the pod is often of papery texture and wholly contained within the persistent and often highly modified corolla or calyx.

The native distribution of *Trifolium* (Table 1) encompasses the temperate and, to a lesser extent, subtropical

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Table 1
Proposed subgeneric classification of *Trifolium* and corresponding taxa in Zohary and Heller (1984)

New classification	Zohary and Heller taxonomy	Native distribution	Number of species	Figure
subgenus *Chronosemium* (Ser.) Reichenb.	sect. *Chronosemium*	Mediterranean region	20	Fig. 2
subgenus *Trifolium*				
sect. *Glycyrrhizum* Bertol.	*T. alpinum* and *T. polyphyllum* of sect. *Lotoidea*	European Alps—Caucasus Mts.	2	Fig. 3
sect. *Paramesus* (C. Presl) Berchtold and J. Presl	sect. *Paramesus*	Mediterranean region	2	Fig. 3
sect. *Lupinaster* (Fabricius) Ser.	*T. eximium*, *T. gordejevii* and *T. lupinaster* of sect. *Lotoidea*	E Europe—Siberia	3	Fig. 3
sect. *Trifolium*	sect. *Trifolium*	Mediterranean region, S Africa (1)	73	Fig. 4
sect. *Trichocephalum* Koch	sect. *Trichocephalum*	Mediterranean region	9	Fig. 4
sect. *Vesicastrum* Ser.	sects. *Mistyllus*, *Vesicaria*, and *Lotoidea* in part	Mediterranean region, sub-Saharan Africa, Madagascar (1)	54	Fig. 5
sect. *Trifoliastrum* S.F. Gray	sect. *Lotoidea* in part	Mediterranean region	20	Fig. 6
sect. *Involucrarium* Hooker	sects. *Lotoidea* and *Lotoidea* in part	N and S America	72	Fig. 7

Nomenclatural priority for new infrageneric taxa follows Hendrych (1988). Species numbers and geographic distributions are based on Zohary and Heller (1984) with the addition of seven species described since 1984 and 11 species treated by them as synonyms or subspecific taxa. The Mediterranean region includes the area bordering the Mediterranean Sea, extending to Europe and Southwest Asia.

regions of the Northern and Southern Hemispheres. Native clovers are absent from southeast Asia and Australia. The greatest species diversity is found in three geographic regions: (1) the Mediterranean basin, (2) western North America, and (3) the highlands of eastern Africa. *Trifolium* species occur in a wide range of habitats, including meadows and prairies, open woodlands, semi-deserts, mountains, and alpine peaks. A common feature of these diverse habitats is high solar radiation; few clover species tolerate shade.

Chromosome numbers are known for at least 184 species of *Trifolium* (summarized in Taylor et al., 1979; Zohary and Heller, 1984; see also Goldblatt and Johnson, 2003). Over 80% of the examined species are $2n = 16$, and $x = 8$ is the inferred base number of the genus (Goldblatt, 1981). Aneuploidy ($2n = 10$, 12, or 14) is known from 31 species, 11 of which have both aneuploid and diploid ($2n = 16$) or polyploid counts. Polyploidy is known from 24 species, of which six are exclusively tetraploid, two are hexaploid, and one is dodecaploid (12×). Eleven species have both diploid and polyploid counts, while three have multiple polyploid counts at the tetraploid level and above. Nitrogen-fixing root nodules have been reported from over 125 species of clover (Sprent, 2001). Among those species that have been studied, nodulation is by *Rhizobium leguminosarum* biovar. *trifolii* (Sprent, 2001).

Clovers are widely grown as livestock forage and green manure crops, and introduced species have become extensively naturalized worldwide. At least 16 species of *Trifolium* are actively cultivated (Gillett and Taylor, 2001), a fairly large number for a single genus. Many native species are also heavily utilized by grazing animals (Crampton, 1985). Fertile interspecific hybrids are difficult to achieve in *Trifolium* (Taylor et al., 1980), and generally only succeed between closely related taxa (Taylor and Quesenberry, 1996). This limitation has spurred interest in the evaluation of the agronomic potential of locally utilized and currently uncultivated species (Morris and Greene, 2001).

The comprehensive monograph of Zohary and Heller (1984) summarizes the extensive taxonomic history of *Trifolium* and provides detailed descriptions and illustrations of all recognized species. They classify the genus into eight sections (Table 1). Although most authors treat *Trifolium* as a single genus, several segregates have been proposed (see Section 4.2.), indicating the need for a critical evaluation of the monophyly of the genus.

Trifolium is a member of the large clade of legumes lacking one copy of the chloroplast inverted repeat, the IRLC (Lavin et al., 1990; Liston, 1995). Molecular phylogenetic studies have identified a strongly supported "vicioid clade" within the IRLC composed of the tribes Trifolieae and Fabeae (Table 2), and the genera *Cicer*, *Galega*, and *Parochetus* (Liston and Wheeler, 1994; Sanderson and Wojciechowski, 1996; Wojciechowski et al., 2000, 2004). Within the vicioid clade, Fabeae and Trifolieae comprise a monophyletic group. Steele and Wojciechowski (2003) conducted a phylogenetic analysis of the Trifolieae and Fabeae based on cpDNA *matK*. Their analysis provided strong support for the monophyly of *Trifolium*. Surprisingly, the genus was resolved (with moderate bootstrap support) as sister lineage to the Fabeae, making Trifolieae paraphyletic.

No previous molecular phylogenetic analysis of *Trifolium* has sampled the taxonomic and geographic breadth of the genus. In a study of 59 Old World *Trifolium* species based on nrDNA ITS sequences and restriction site

Table 2
Classification and geographic distribution of the vicioid clade and the outgroup genus, *Lotus*

Tribe genus	Native distribution	Species number
Trifolieae		
Medicago L.	Eurasia, E and S Africa	83
Melilotus Mill.	Eurasia, E Africa	20
Ononis L.	Mediterranean region—Central Asia, E Africa	75
Trifolium L.	see Table 1	255
Trigonella L.	Mediterranean region—Central Asia, S Africa, Australia	55
Fabeae		
Lathyrus L.	Eurasia, E Africa, N and S America	160
Lens Mill.	Mediterranean region, Africa	4
Pisum L.	Mediterranean region	3
Vicia L.	Eurasia, E Africa, N and S America, Hawaii	160
Other vicioids		
Cicer L.	Mediterranean region—Central Asia, E Africa	43
Galega L.	Eurasia, E Africa	6
Parochetus D. Don	Mountains of tropical Asia, E Africa	2
Outgroup		
Lotus L.	Eurasia, N Africa, N and S America	100

Species numbers and distribution data from Lewis et al. (2005).

analysis of PCR-amplified cpDNA, Watson et al. (2000) provided the first molecular phylogenetic evidence that most of the sections recognized by Zohary and Heller (1984) are not monophyletic. A novel finding was the resolution of a clade defined by geography comprising the nine sampled African species. Steele and Wojciechowski (2003) sampled 23 species of *Trifolium*, including six North American species, in their cpDNA *matK* analysis. They obtained a clade of North American species and found sect. *Lotoidea* sensu Zohary and Heller to be polyphyletic. In a study focused on three subspecies of *Trifolium nigrescens*, Williams et al. (2001) demonstrated the utility of nrDNA ITS sequences to resolve closely related taxa of the genus.

The goal of this study was to develop a molecular phylogenetic framework for *Trifolium* based on comprehensive taxonomic sampling and sequences of two loci, one nuclear and one organellar. In addition to evaluating the classification of the genus, we use the phylogenetic results to obtain evidence for hybrid speciation and to examine patterns of life history (annual vs. perennial) and chromosome number change in *Trifolium*. Zohary and Heller (1984) considered the perennial growth form to be ancestral in clover, a conclusion supported by the phylogenetic results of Watson et al. (2000). Although several previous authors have described patterns of chromosome evolution in *Trifolium* (Cleveland, 1985; Goldblatt, 1981; Taylor et al., 1979; Zohary and Heller, 1984), their hypotheses have not been examined in a phylogenetic context.

Our phylogenetic analyses are based on data from the nuclear and chloroplast (cpDNA) genome. The rate and pattern of ITS sequence mutation are typically appropriate for resolving relationships among species and genera (Baldwin et al., 1995; Hershkovitz et al., 1999). Although thousands of copies of the ITS exist in angiosperm genomes, they are generally homogenized by concerted evolution, and thus can be treated as a single locus (Baldwin et al., 1995). However, phylogenetic estimates based on ITS may be compromised by paralogy (due to polyploidy or incomplete concerted evolution), compensatory base changes, and problems in alignment due to length variation (Alvarez and Wendel, 2003; Bailey et al., 2003). To complement the nrDNA ITS data set, we sequenced the intron-containing chloroplast tRNA (UAA) for leucine (*trnL*). Like the nrDNA ITS, cpDNA introns are widely used in the phylogenetic analysis of land plants (Kelchner, 2000). However, numerous studies have observed that lineage sorting, introgressive hybridization, and cytoplasmic sharing among unrelated sympatric species (Belahbib et al., 2001) can confound phylogenetic analysis based on plastid loci (Cronn et al., 2002; Wendel and Doyle, 1998).

Combining data from multiple loci is an effective approach for avoiding problems associated with single locus estimates of phylogeny (Gatesy and Baker, 2005; Rokas et al., 2003). Because organellar and nrDNA sequences are prone to different types of potential bias, any resulting "noise" should be randomly distributed and phylogenetic signal should be reinforced in a combined analysis (Wiens, 1998). We also analyzed the nuclear and organellar sequences in separate analyses, to detect incongruence that could be the result of interspecific hybridization (Wendel and Doyle, 1998). Both maximum parsimony and Bayesian approaches to phylogenetic estimation were used.

2. Materials and methods

2.1. Materials

A total of 218 species of *Trifolium*, representing ca. 86% of the genus, was sampled (Appendix 1). Five *Trifolium* species were represented by two (*T. longipes*, *T. montanum*, *T. rusbyi*) or three (*T. nigrescens*, *T. subterraneum*) subspecies. Nineteen species from 10 other genera in the vicioid clade were also included (Table 2). The outgroup was chosen from the genus *Lotus*, represented by 11 species. Although this genus is relatively distant from *Trifolium* (Wojciechowski et al., 2004), previous cpDNA (Hu et al., 2000) and ITS (Hu et al., 2002) analyses have demonstrated that *Lotus* is an appropriate outgroup for the clade of legumes that lacks the cpDNA inverted repeat. To facilitate communication, we use our proposed infrageneric classification of *Trifolium* throughout the results and discussion (Table 1; see Section 4.2, for justification of the classification).

Germplasm collections were the primary source of *Trifolium* samples, and represent a valuable resource for studies of this genus (Taylor et al., 1979). Most of the accessions are deposited in the USDA-ARS National Plant Germplasm System (Appendix 1), and are readily available from this source. Additional seed accessions, including most of the species of other genera, were obtained from the Margot Forde Forage Germplasm Centre, Palmerston North, New Zealand, and the N.L. Taylor Clover Germplasm Center, University of Kentucky. These accessions are available upon request. To date, seed-grown plants from 135 accessions have been deposited as voucher specimens at the Dame Ella Campbell Herbarium, Massey University, Palmerston North, New Zealand (MPN). Vouchers are also maintained at the University of Kentucky, Department of Plant and Soil Sciences Herbarium. Twenty-two samples were obtained from herbarium specimens (Appendix 1).

In addition to the accessions included in the present study, 167 of the 218 sampled *Trifolium* species were represented by 1–34 additional accessions (Ellison and Williams, unpublished data). A total of 686 accessions were sequenced for both the nrDNA ITS and cpDNA *trnL* intron, and an additional 196 were sequenced for only ITS. These sequences were included in a series of neighbor-joining analyses. In most cases, multiple accessions of a single species clustered together, and a single accession was chosen for inclusion in our study. When multiple accessions of the same species did not cluster together, plants were grown from seed to confirm identification, and when possible, additional accessions were sequenced. This process revealed that most discrepancies resulted from labeling errors, mixed seed collections or misidentifications, and these accessions were excluded. Two species endemic to Ethiopia, *Trifolium abyssinicum* and *T. decorum*, are represented by two accessions with divergent sequences, since a single accession could not be determined as "representative" of the species.

For initial experiments, total genomic DNA was isolated using the method of Thompson and Henry (1995) as described in Williams et al. (2001). Subsequently, the method of Lefort and Douglas (1999), with minor modifications, was found to be more reliable. Using this method, DNA was isolated from ca. 10 mg seed, 5 mg fresh leaf, or 2 mg dried leaf. Seeds were first placed between two sheets of weighing paper, crushed by striking with a small hammer, and then transferred to a 1.5 mL microcentrifuge tube; a piece of fresh or dried leaf was placed directly into a 1.5 mL microcentrifuge tube. A 400 μL volume of extraction buffer (50 mM Tris–HCl, pH 8.0, 20 mM EDTA, 0.7 M NaCl, 0.4 M LiCl, 1% w/v CTAB, 1% w/v PVP 40, 2% w/v SDS) was added to each tube. Leaf samples were ground with a plastic pestle, while seed samples required only brief vortexing. Samples were digested at 65 °C for 20 min to 1 h, and then extracted once with 300 μL chloroform–isoamyl alcohol (24:1). DNA was precipitated from the aqueous phase by the addition of an equal volume of isopropanol, with centrifugation for 15 min. DNA pellets were rinsed with 750 μL of 70% ethanol and resuspended in 50 μL H_2O at 65 °C for 5–10 min. The ITS region was amplified using the primers EC-1 and EC-2 (Williams et al., 2001), and the *trnL* (UAA) intron was amplified using the primers "c" and "d" (Taberlet et al., 1991). PCR amplification reactions were performed in a volume of 20 μL containing 2 μL of the prepared DNA, 1× PCR buffer (75 mM Tris–HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.1% v/v Tween 20), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.4 μM of each primer, and 1 U of *Taq* DNA polymerase. Amplification conditions consisted of a single cycle of 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by a final extension of 72 °C for 7 min. PCR products were electrophoresed directly on a low-melting point agarose gel (Cambrex, Rockland, ME) and subsequently purified from the agarose gel as described in Williams et al. (2001). Sequencing was performed on both strands using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) in a volume of 10 μL containing 1 μM of primer, 5 μL of purified PCR product, and 10% DMSO. Sequencing reactions were purified on mini spin columns prepared with Sephadex G-50 (Amersham Biosciences, Sweden) and electrophoresed on either an AB310, AB3700, or AB3100 automated sequencer (Applied Biosystems, Foster City, CA).

2.2. Data analysis

For each sequence, the sequence data for both strands were assembled into a contig and manually edited using AutoAssembler (Applied Biosystems, Foster City, CA). Multiple sequence alignments were generated with the Clustal implementation within the MegAlign software (DNASTAR, Madison, WI), with manual optimization. Maximum parsimony analyses were implemented with PAUP* 4.0b10 (Swofford, 2002). Gaps were treated as missing data. Heuristic searches were conducted on the ITS, cpDNA, and combined ITS and cpDNA data. Fifty shortest trees were held at each of 1000 random addition sequences with TBR swapping. The resulting trees were then used as the starting trees for a second round of TBR swapping, retaining a maximum of 50,000 trees. Bootstrap values were obtained from 100 resamplings analyzed with 1000 random addition sequence replicates and TBR swapping, holding a single shortest tree at each replicate. Although we had a priori decided to combine data sets, we evaluated the degree of conflict between the sequence partitions using the relatively conservative incongruence length difference (ILD) test (Farris et al., 1995; Hipp et al., 2004), and a more liberal criterion, a comparison of clades based on bootstrap support values (Binder and Hibbett, 2002). To identify potential conflict, plots were made of bootstrap values for all clades with $\geqslant$70% support in the combined or separate analyses. The ILD test was implemented in PAUP* with 100 replicates of the partition homogeneity test using the above heuristic search parameters. ILD tests were also conducted excluding taxa with conflicting placement in the separate nrDNA ITS and cpDNA *trnL* analyses.

Bayesian analyses of the separate and combined data were conducted with MrBayes 3.0b4 (Huelsenbeck et al., 2002; Ronquist and Huelsenbeck, 2003). The best-fit models of sequence evolution were chosen using the hierarchical Likelihood Ratio Test (hLRT) and Akaike Information Criterion (AIC), calculated with MrModeltest 2.0 (Nylander, 2004). A model with six variable substitution types, gamma-distributed among site rate variation, and an estimated proportion of invariant sites (GTR + Γ + I) was chosen for nrDNA ITS. A transition/transversion model (two variable substitution types), gamma-distributed among site rate variation, and an estimated proportion of invariant sites (HKY + Γ + I) was chosen for cpDNA *trnL*. These models were applied to their respective partitions in the separate and combined analyses. In the combined analysis, three runs of 1,000,000 generations, and one run of 5,000,000 generations were conducted. In the separate analyses, a single run of 5,000,000 generations was conducted. In each run, trees were sampled every 100 generations and burn-in was determined by inspection of the log-likelihoods of sampled trees. Branch length information was recorded and averaged across all retained trees, and majority rule consensus trees were computed to obtain posterior probabilities. Clades with >85% bootstrap support and >0.95 posterior probability are considered well supported.

Chromosome numbers (missing for 41 species) and plant life history (annual or perennial) were recorded for *Trifolium* following Gillett and Taylor, 2001 (Appendix 1). For the other genera, life history information was obtained from the LegumeWeb database (ILDIS, 2002) and chromosome numbers were obtained from the TROPICOS database (Goldblatt and Johnson, 2003). Parsimony optimization of character states was performed in Mesquite 1.05 (Maddison and Maddison, 2004). Character states were unordered and all changes were equally probable.

3. Results

3.1. Combined analyses

Sequences of the nrDNA ITS and cpDNA *trnL* intron were obtained from 257 accessions (Appendix 1). The ITS alignment was 828 bp, with 452 variable and 377 parsimony informative sites. The *trnL* intron alignment was 783 bp, with 340 variable and 241 parsimony informative sites. All DNA sequences used in this analysis were generated by us and are deposited in GenBank (Appendix 1). Sequence alignments are available from TreeBase as Accession S1387.

Four Bayesian analyses (three times 1,000,000 and one of 5,000,000 generations; burn-ins of 2000 and 5000 trees, respectively) of the combined sequence data set consistently converged on the same narrow range of log-likelihoods and tree topologies. The maximum parsimony (MP) analyses resulted in 50,000 trees (the maxtrees limit) of length 2977 (consistency index = 0.44, retention index = 0.84). The topology of the parsimony strict consensus tree (not shown) is very similar to the Bayesian tree, differing primarily in the larger number of polytomies. Bayesian posterior probabilities (PP) and parsimony bootstrap (BP) values are well correlated, with the Bayesian values consistently higher (Figs. 1–7). In the MP analysis, 119 clades have BP support ⩾70%, and 78 have ⩾90% support.

Two key relationships, the monophyly of *Trifolium* and the sister group relationship between *Trifolium* and *Trigonella* + *Melilotus*, have relatively low parsimony BP (62% and <50%) and moderate to low Bayesian PP support (0.85 and 0.69, respectively). Both analyses resolve an initial subdivision of *Trifolium* between subgenus *Chronosemium* and the rest of the genus (Fig. 1). Subgenus *Chronosemium* is split into two well-supported clades, characterized by different chromosome numbers (Fig. 2).

Subgenus *Trifolium* (Fig. 3) has a paraphyletic grade at its base, comprising sect. *Paramesus* and five Eurasian perennials that we place in sect. *Glycyrrhizum* and sect. *Lupinaster* (Table 1). These five species are sometimes placed in two segregate genera (see Section 4.2.). There is strong support for a sister group relationship between sect. *Lupinaster* and the remaining five sections of the subgenus (Fig. 3). The monophyly of these five sections, and their inter-relationships, are well supported by the Bayesian analysis (PP ⩾0.95) but most have <85% BP support.

Sections *Trifolium* and *Trichocephalum* are resolved as monophyletic sister taxa (Fig. 4). Section *Trichocephalum* contains two well-supported clades, excluding *T. israeliticum*. This species, that was originally described as a variety of *T. subterraneum*, is distinct from that species, but its position in the section is poorly resolved. Section *Trifolium* can be divided into two large clades: one that is well supported (clade A in Fig. 4) and one that has little statistical support (clade B in Fig. 4). In contrast, interspecific relationships are better resolved within clade B than within clade A.

Section *Vesicastrum* (Fig. 5) unites all the sampled sub-Saharan species of *Trifolium* (clades E and H) together with four Eurasian species (clade C) previously classified in sect. *Lotoidea* and all species previously classified in sects. *Mistyllus* (clades D and J) and *Vesicaria* (clades F and G). The latter two taxa are thus polyphyletic. The clovers of sub-Saharan Africa (clades E and H) are also not monophyletic, and their relationships are generally unresolved or weakly supported. With the exception of three species formerly placed in sect. *Mistyllus* (clade J) and one in sect. *Trifolium* (not sampled here), all sub-Saharan *Trifolium* had been previously classified in sect. *Lotoidea*. Two Ethiopian species (*Trifolium abyssinicum*, *T. decorum*) represented by two accessions are each resolved as polyphyletic. Whether this results from misidentifications or unrecognized taxa remains to be determined. The sub-Saharan clovers are not well studied, and this result may reflect the uncertain taxonomy of this group.

Section *Trifoliastrum* is restricted to ca. 20 Eurasian species (Fig. 6) formerly classified in sect. *Lotoidea*. This clade includes the economically important white clover,

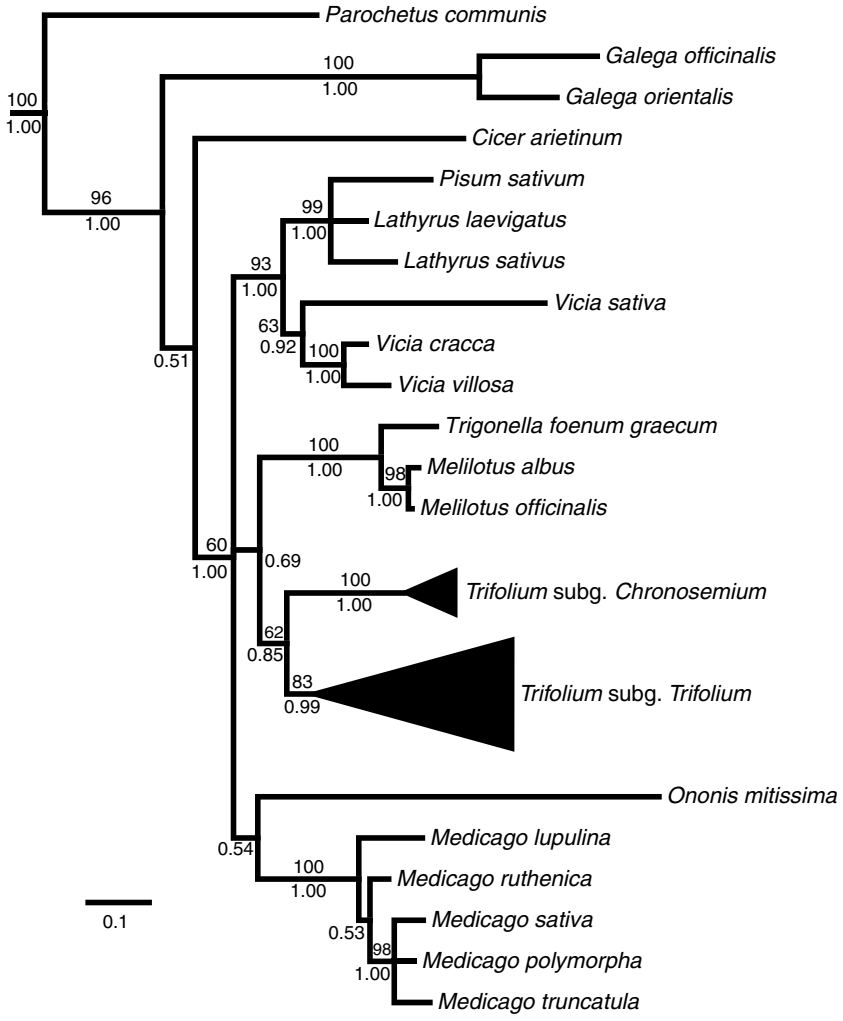

Fig. 1. The position of *Trifolium* among the genera of the vicioid clade. The outgroup *Lotus* is not shown. The area of the triangles is proportional to the number of species in each *Trifolium* subgenus. In Figs. 1–7, the combined analysis Bayesian majority rule consensus tree with average branch lengths are represented; scale bars are substitutions per site. Bayesian posterior probabilities are below branches, parsimony bootstrap values are above. Values below 0.50 or 50% are not shown. Symbols placed after species names represent an autapomorphic occurrence of the character state.

T. repens. Many relationships within this clade are resolved in the Bayesian analysis, but have BP values below 50%. Likewise, the sister-group relationship between this clade and the American clovers (sect. *Involucrarium*) has <50% BP, but PP = 0.99 (Fig. 3).

All of the species in sect. *Involucrarium* are native to the western Hemisphere. Although there are several well-supported clades, relationships among them are not resolved (Fig. 7). The first dichotomy has no BP support and a low PP (0.60), thus it is equivocal whether this clade has a North (*T. breweri*) or South American (*T. amabile*, *T. peruvianum*) origin. A polytomy of two species (*T. brandegei*, *T. dasyphyllum*) and seven clades (K–Q) make this the most poorly resolved portion of the phylogenetic results. The species of this section were previously classified in sect. *Involucrarium* (clades K, L, and *T. oliganthum*) or sect. *Lotoidea*. The species of clades K and L are characterized by an involucre of fused bracts below the inflorescence. Clade M includes the western North American annuals that lack an involucre, plus the involucrate *T. oliganthum*. All of the eastern North American species of *Trifolium* form a clade (N) in which the three annual (sometimes biennial) species are monophyletic (Fig. 7). Three South American species comprise a well-supported clade (O). The remaining species (clades P, Q, *T. brandegei*, and *T. dasyphyllum*) are primarily distributed in the Intermountain Region of western North America. Neither large clade, nor the majority of inter-relationships among these species are well supported (Fig. 7).

3.2. Separate analyses

The Bayesian and MP analyses of the cpDNA *trnL* intron sequences result in poorly resolved topologies (not shown). In the majority rule consensus of 50,000 MP trees (897 steps, CI = 0.56, RI = 0.43) only 43 clades have BP support $\geqslant$70%, and 22 have $\geqslant$90% support. The Bayesian and MP analyses of the nrDNA ITS sequences result in well resolved and similar topologies (not shown). For this partition, 101 clades have BP support $\geqslant$70%, and 60 have

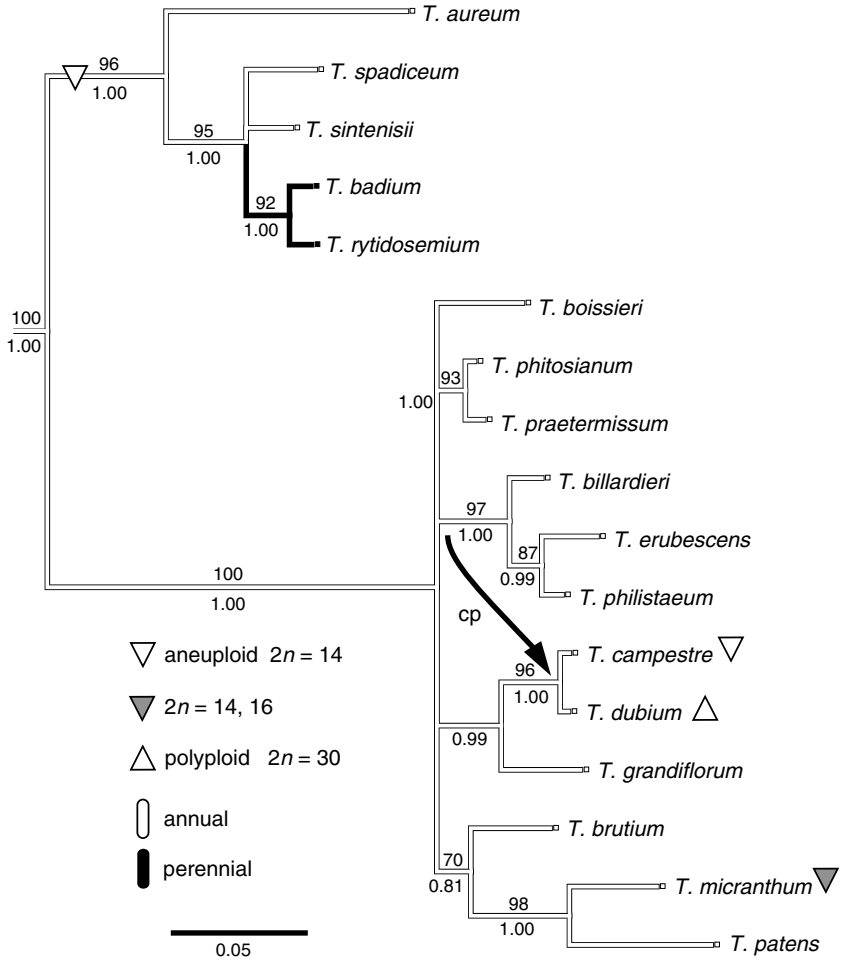

Fig. 2. Subgenus *Chronosemium*. Branch shading corresponds to life history. All species are $2n = 16$ or unknown, except as noted. The arrow denotes the inferred origin of the chloroplast in *T. campestre* and *T. dubium*.

$\geqslant$90% support in the majority rule consensus of 50,000 MP trees (2056 steps, CI = 0.39, RI = 0.81). Based on the ILD test, the two partitions are significantly different ($P = 0.01$). Removal of all non-*Trifolium* sequences (where several topological discrepancies were observed) and 12 species of *Trifolium* with well supported but conflicting positions, still results in significantly different partitions ($P = 0.01$).

Despite extensive topological incongruence, few of the conflicting clades have high BP support in the separate partitions (Fig. 8). Among the 22 highly supported ($\geqslant$90% BP) clades in the cpDNA bootstrap tree, only seven have $\leqslant$90% BP in the nrDNA analysis. Six of these seven clades have $\geqslant$90% BP support in the combined topology. Due to poor resolution in the cpDNA topology, 45 of the highly supported nrDNA clades have $\leqslant$90% BP support with cpDNA. These bootstrap comparisons demonstrate that despite topological incongruence, the combined topology has more nodes with increased support and retains all but two of the highly supported clades found in the separate analyses. Furthermore, the poorly resolved cpDNA topology does not reduce resolution in the combined vs. nrDNA analyses.

The comparison of the cpDNA and nrDNA trees identifies several cases of potential reticulate evolution in *Trifolium*:

(1) *Trifolium campestre* and *T. dubium* have identical *trnL* intron and ITS sequences (Fig. 2). The two species are morphologically similar but readily distinguishable, and differ in chromosome numbers: $2n = 14$ and $2n = 30$, respectively (H. Ansari, pers. comm.). In the cpDNA topology they form a sister group to *T. billardieri*, *T. erubescens*, and *T. philistaeum* (all $2n = 16$), while in the nrDNA and combined topology (Fig. 2) they are sister to *T. grandiflorum* ($2n = 16$). This is the only example of a conflict where the alternative single gene topologies are both well supported (cpDNA BP = 87% and PP = 1.0; nrDNA BP = 97% and PP = 1.0).

(2) *Trifolium pannonicum* is a polyploid, with a range of reported chromosome numbers (see Section 4.4.). With cpDNA, this species is placed in clade B (Fig. 4) of sect. *Trifolium* (BP = 70%), while the nrDNA results place it in clade A (BP = 100%). The actual ancestry of *T. pannonicum* may be complex. In

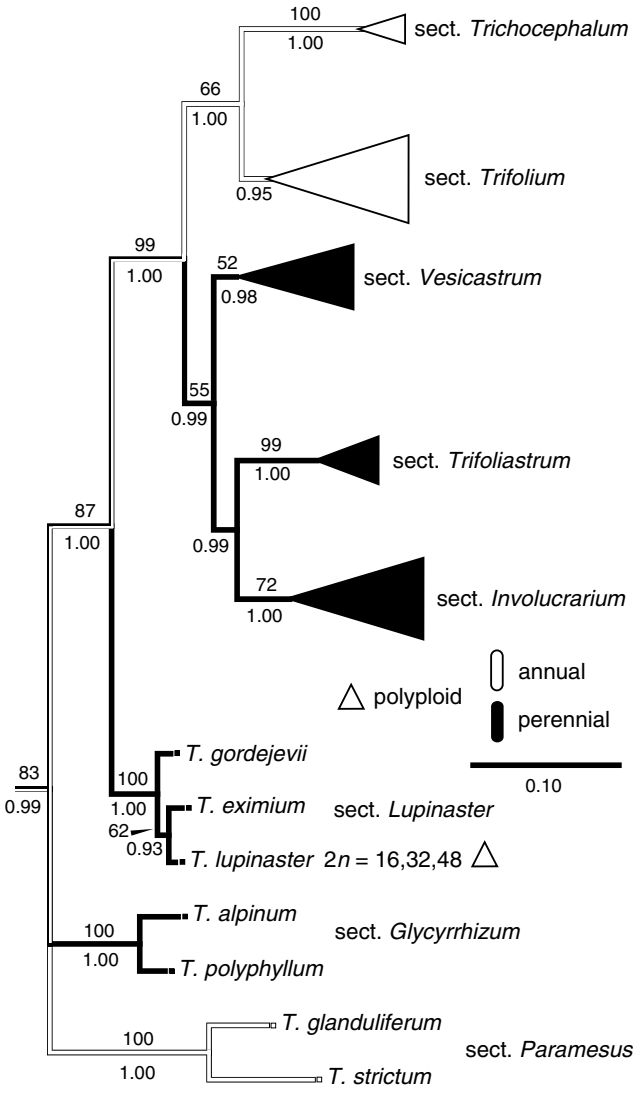

Fig. 3. The major clades of subgenus *Trifolium*. The area of the triangles is proportional to the number of species in each clade. Branch shading corresponds to life history. All species shown here are $2n = 16$, except for *T. lupinaster*; *T. gordejevii* is uncounted.

the cpDNA tree, there is weak BP support (65%) but a high PP (0.97) for a clade with *T. patulum* and *T. squamosum*, two species that are not united in the nrDNA or combined results. The nrDNA tree places *T. pannonicum* in a polytomy at the position seen in the combined Bayesian tree (Fig. 4).

(3) *Trifolium repens*, a $2n = 32$ tetraploid, is sister to *T. pallescens* ($2n = 16$) in the cpDNA analysis (BP = 67%), and to *T. occidentale* ($2n = 16$) in the nrDNA analysis (BP = 79%). It must be noted that neither relationship is obtained in the combined analysis (Fig. 6). The separate Bayesian analyses result in high support (PP = 1.0) for the nrDNA placement, but no support (PP = 0.58) for the cpDNA placement. This is one of the few instances where the posterior probability is lower than the bootstrap value.

(4) Several cases of conflict involve a group of East African species. *Trifolium cheranganiense* and *T. usambarense* share identical *trnL* sequences (Fig. 5); *T. stolzii* is united with *T. rueppellianum* and *T. cryptopodium* (BP = 88%); and the positions of *T. masaiense* and *T. semipilosum* are not resolved. With nrDNA, *T. cheranganiense*, *T. masaiense*, and *T. semipilosum* are monophyletic (Fig. 5, clade E, BP = 79%); *T. usambarense* and *T. stolzii* comprise another (BP = 100%); and *T. rueppellianum* and *T. cryptopodium* are unresolved. The same clades are seen in the nrDNA and combined Bayesian analyses with higher posterior probabilities. The chromosome number for *T. stolzii* is uncounted, *T. cryptopodium* is reported as both $2n = 16$ and $2n = 48$, and the other species are $2n = 16$.

(5) One case of weakly supported conflict involves *T. cyathiferum* ($2n = 16$), a North American species. With cpDNA, *T. cyathiferum* has the same *trnL* intron sequence as *T. buckwestiorum*, *T. variegatum*, and *T. polyodon* (Fig. 7). In the nrDNA and combined analyses, *T. cyathiferum* is placed in a clade of annual species that share the morphological feature of inflated fruiting corollas. Bootstrap support is <50% and the Bayesian PP is only 0.70 (nrDNA and combined analyses) for a clade with *T. physanthum*, *T. barbigerum*, and *T. jokerstii*.

3.3. Character evolution

3.3.1. Life history

Due to limited sampling of species diversity in other genera of the vicioid clade (Table 2), the ancestral life history for *Trifolium* cannot be inferred. The annual habit is the ancestral state reconstruction for subgenus *Chronosemium* (Fig. 2), while the ancestral state for subgenus *Trifolium* is equivocal (Fig. 3). Within this subgenus, the common ancestor of sects. *Trifolium* and *Trichocephalum* is inferred to be annual, while the common ancestor of the three other large sections is inferred to be perennial (Fig. 3). Within subgenus *Chronosemium* there is one change to the perennial habit involving two species (Fig. 2), and within sect. *Trifolium* there are seven changes (Fig. 4). Five of these are autapomorphic, while the others involve clades of three and seven species, respectively. Among the sections with a perennial ancestral state, there are at least five changes to the annual habit in sect. *Vesicastrum* (Fig. 5), three changes in sect. *Trifoliastrum* (Fig. 6), and five changes in sect. *Involucrarium*, including three in clade K (Fig. 7). In sect. *Vesicastrum*, character state reconstruction within the large African clade H is hampered by limited phylogenetic resolution, taxonomic uncertainty, and the presence of several species that are polymorphic for life history. However, there appears to be several reversals back to the perennial habit (Fig. 5). There is also evidence for two reversals back to the perennial habit within sect. *Trifoliastrum* (Fig. 6), but no reversals are reconstructed in sect. *Involucrarium* (Fig. 7).

3.3.2. Chromosome numbers

The ancestral chromosome number in *Trifolium* is diploid $2n = 16$. Our sampling included 23 of the 24 species with

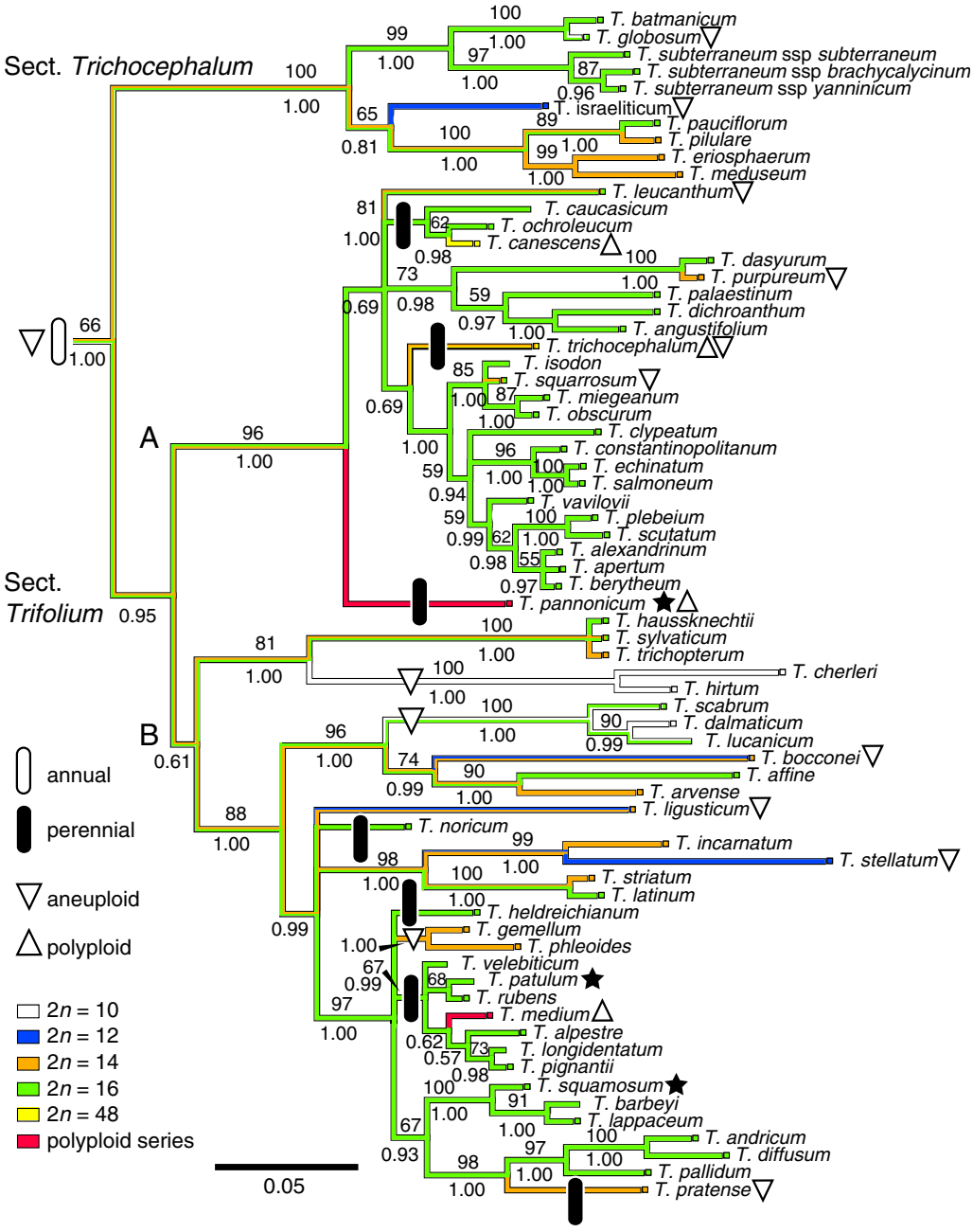

Fig. 4. Sections *Trifolium* and *Trichocephalum*. Branch color corresponds to chromosome numbers. The chloroplast results place *T. pannonicum* in a clade with *T. patulum* and *T. squamosum*, these three species are marked with stars. See text for clades A–B.

known polyploid counts, including 11 species with both diploid and polyploid records. Ancestral state reconstruction (Figs. 2–7) infers at least 22 independent origins of polyploidy (not counting subsequent chromosome number increases in species with multiple ploidy levels). Although all of the large clades have at least one inferred origin of polyploidy, the largest number (10) occurs in sect. *Involucrarium* (Fig. 7).

Aneuploidy, unlike polyploidy, is not evenly distributed across the phylogeny. It is very common in sect. *Trifolium* (Fig. 4, at least 12 times), present in subgenus *Chronosemium* (Fig. 2, three times) and sect. *Trichocephalum* (Fig. 4, two times), restricted to single species in sect. *Vesicastrum* (Fig. 5, *T. resupinatum*) and sect. *Trifoliastrum* (Fig. 6, *T. glomeratum*), and absent in the remaining sections. Within subgenus *Chronosemium*, there have been three reductions to $2n = 14$ (Fig. 2). In sects. *Trifolium* and *Trichocephalum* the situation is more complex (Fig. 4). The common ancestor of both sections is potentially polymorphic for $2n = 14$ and $2n = 16$. There are two clades in sect. *Trifolium* characterized by $2n = 10$, but it is equally parsimonious for their ancestors to have been $2n = 14$ or $2n = 16$. Counts of $2n = 12$ are reported from three species (two of which are polymorphic for $2n = 12$ and $2n = 14$) in sect. *Trifolium* and one in sect. *Trichocephalum*. Each case of $2n = 12$ appears to have evolved independently from the $2n = 14$ condition (Fig. 4). Although it is possible that reversals from $2n = 14$

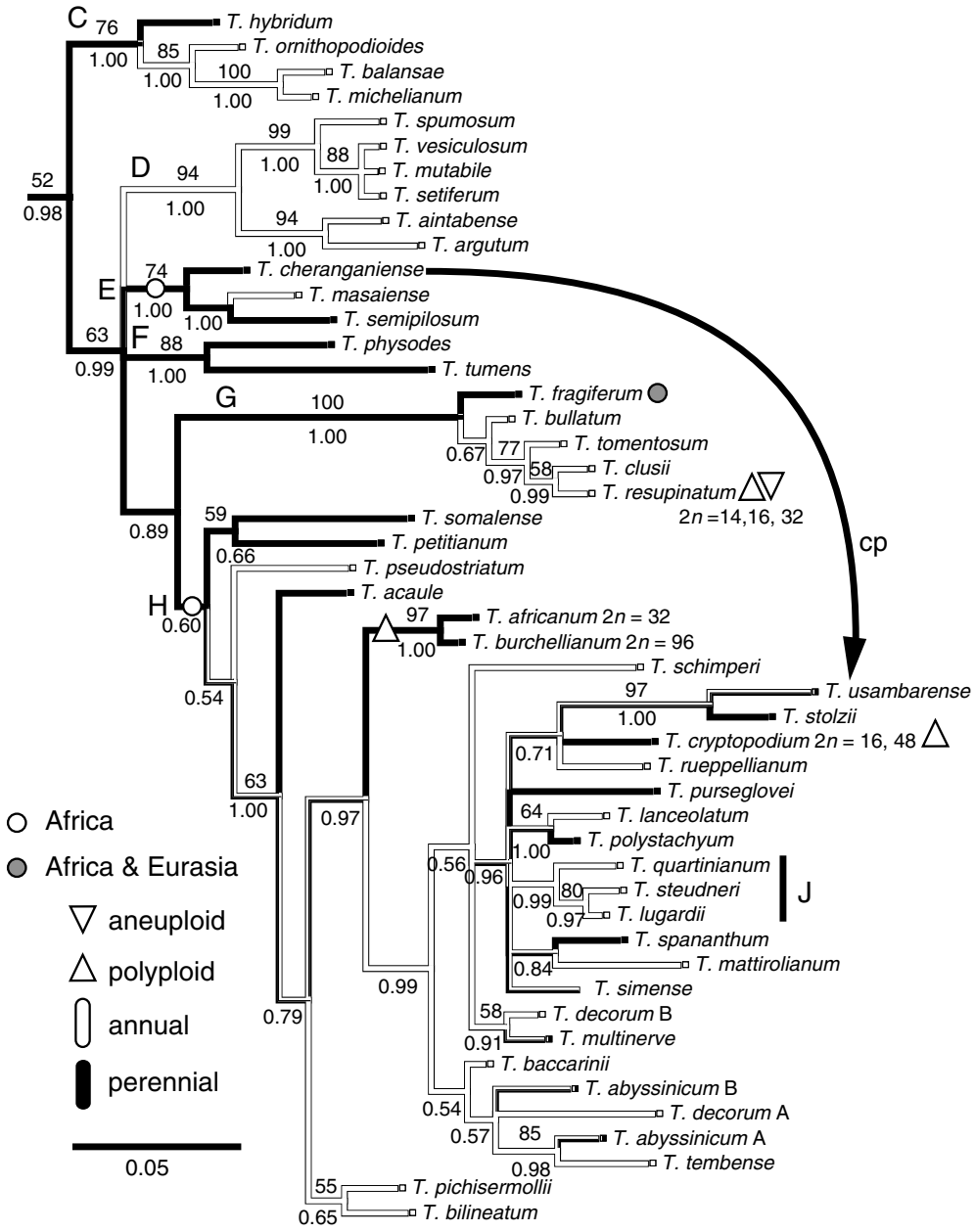

Fig. 5. Section *Vesicastrum*. Branch shading corresponds to life history. All species are $2n = 16$ or unknown, except as noted. The arrow denotes the inferred origin of the chloroplast in *T. usambarense*. See text for clades C–H and J.

to $2n = 16$ have occurred in both sections (*T. affine*, *T. haussknechtii*, *T. latinum*, and *T. pauciflorum*) all such cases can also be explained by lineage sorting of ancestral chromosome number polymorphisms (Fig. 4). Across the genus, eight species are reported to have both aneuploid and diploid counts, and two have two different aneuploid counts, suggesting that polymorphic ancestry is possible.

4. Discussion

4.1. Comparison to prior studies

Our results show both concordance and conflict with previous molecular phylogenetic studies of *Trifolium* (Steele and Wojciechowski, 2003; Watson et al., 2000). A novel finding of Steele and Wojciechowski (2003) was the sister group relationship between *Trifolium* and the Fabeae (Table 2). In contrast, our combined nrDNA ITS and *trnL* intron analysis resolved *Trifolium* and *Trigonella* + *Melilotus* of Trifolieae as sister groups (Fig. 1). Although this relationship is very poorly supported, it is more consistent with traditional classification (Heyn, 1981). Limited sampling of the other genera of the vicioid clade (Table 2) has limited our ability to better resolve the sister group of *Trifolium*, and the phylogenetic relationships among Trifolieae and Fabeae genera. In accordance with our study, Steele and Wojciechowski (2003) resolved *Trifolium* as monophyletic, however they obtained greater support. Among the 23 *Trifolium* species they sampled, many of the same

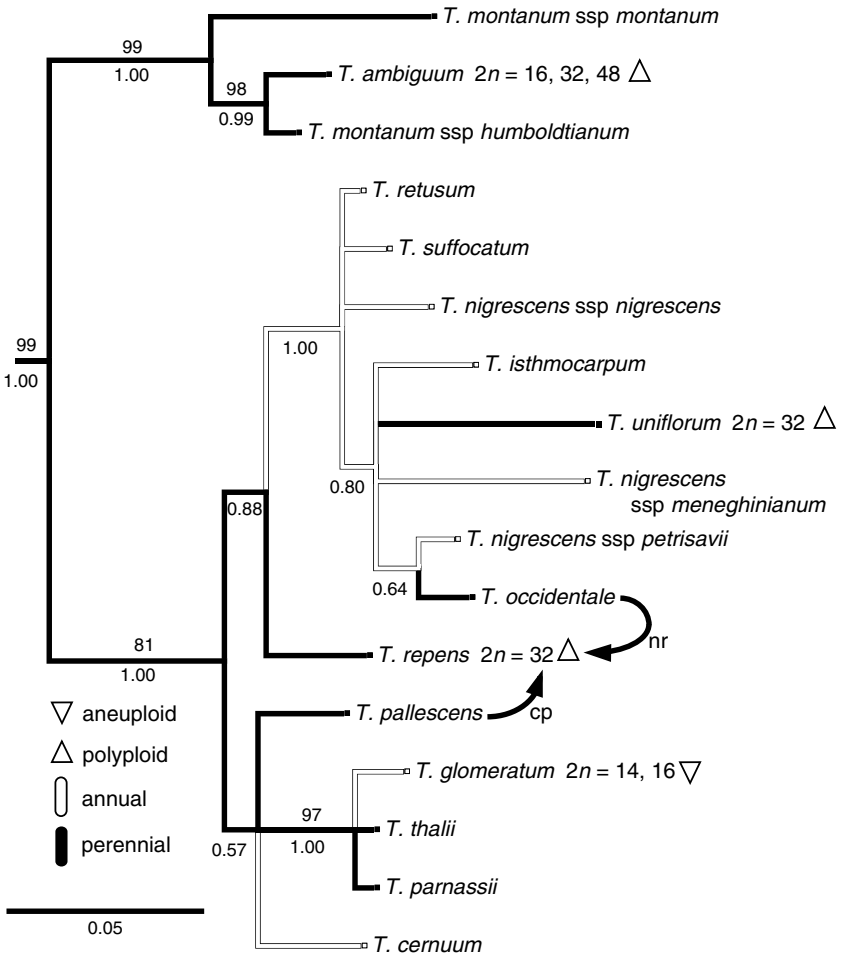

Fig. 6. Section *Trifoliastrum*. Branch shading corresponds to life history. Arrows denote the inferred ancestry of *T. repens*. All species are $2n = 16$, except as noted.

relationships reported here were resolved (including the sister group status of subgenus *Chronosemium* to the remainder of the genus and monophyly of the American species), but often with lower bootstrap support.

In contrast to the agreement between our results and Steele and Wojciechowski (2003), the Watson et al. (2000) study contains several conflicts, including a nested position of subgenus *Chronosemium* and the polyphyly of sect. *Trifolium*. Relationships in their separate and combined cpDNA restriction site and nrDNA ITS sequence analyses were generally poorly resolved. Comparisons of published nrDNA ITS sequences suggest that some of the Watson et al. (2000) accessions (e.g., *T. alpinum*, *T. cherleri*, *T. scabrum*, and *T. subterraneum*) were misidentified or mislabeled. As a result, these species were resolved in unexpected positions and apparently contributed to the discrepancies between our studies. In fact, none of the Watson et al. (2000) ITS sequences match any of ours, and for this reason we did not integrate their sequences into this study.

4.2. Implications for the classification of Trifolium

Like other large genera of Leguminosae (e.g., *Astragalus*, *Indigofera*, and *Mimosa*), speciation in *Trifolium* is accompanied by diversification of structures associated with seed dispersal. In contrast to those genera, the clover fruits are fairly unmodified, but the more variable corolla and calyx are responsible for the diversity of dispersal mechanisms (Zohary, 1972). This floral diversity has led to classical (Presl, 1831) and recent proposals to split *Trifolium* into a series of smaller genera (Hendrych, 1976, 1978; Khokhrjakov, 1998; Roskov, 1990; Soják, 1986; see also Small, 1987). Based on our molecular phylogenetic results, most of these segregates are either polyphyletic (*Amoria* C. Presl, *Lupinaster* Fabr., *Xerosphaera* Soják) or create paraphyletic groups (*Bobrovia* A.P. Khokhrjakov = sect. *Glycyrrhizum*) and *Ursia* I.T. Vasil'chenko (= sect. *Lupinaster*). The only segregate that might be considered useful is elevating subgenus *Chronosemium* (commonly known as hop clovers) to the genus *Chrysaspsis* Desv. (Hendrych, 1976, 1978). To maintain nomenclatural stability, we do not advocate this change, but propose that the hop clovers should be considered one of two subgenera of *Trifolium*. Following this classification, the remaining species of *Trifolium* are placed in subgenus *Trifolium*. We further propose recognizing eight of its clades as sections (Table 1 and Fig. 3). The designation of this infrageneric classification is greatly facilitated by the thorough nomenclatural work of Hendrych (1988). The larger clades

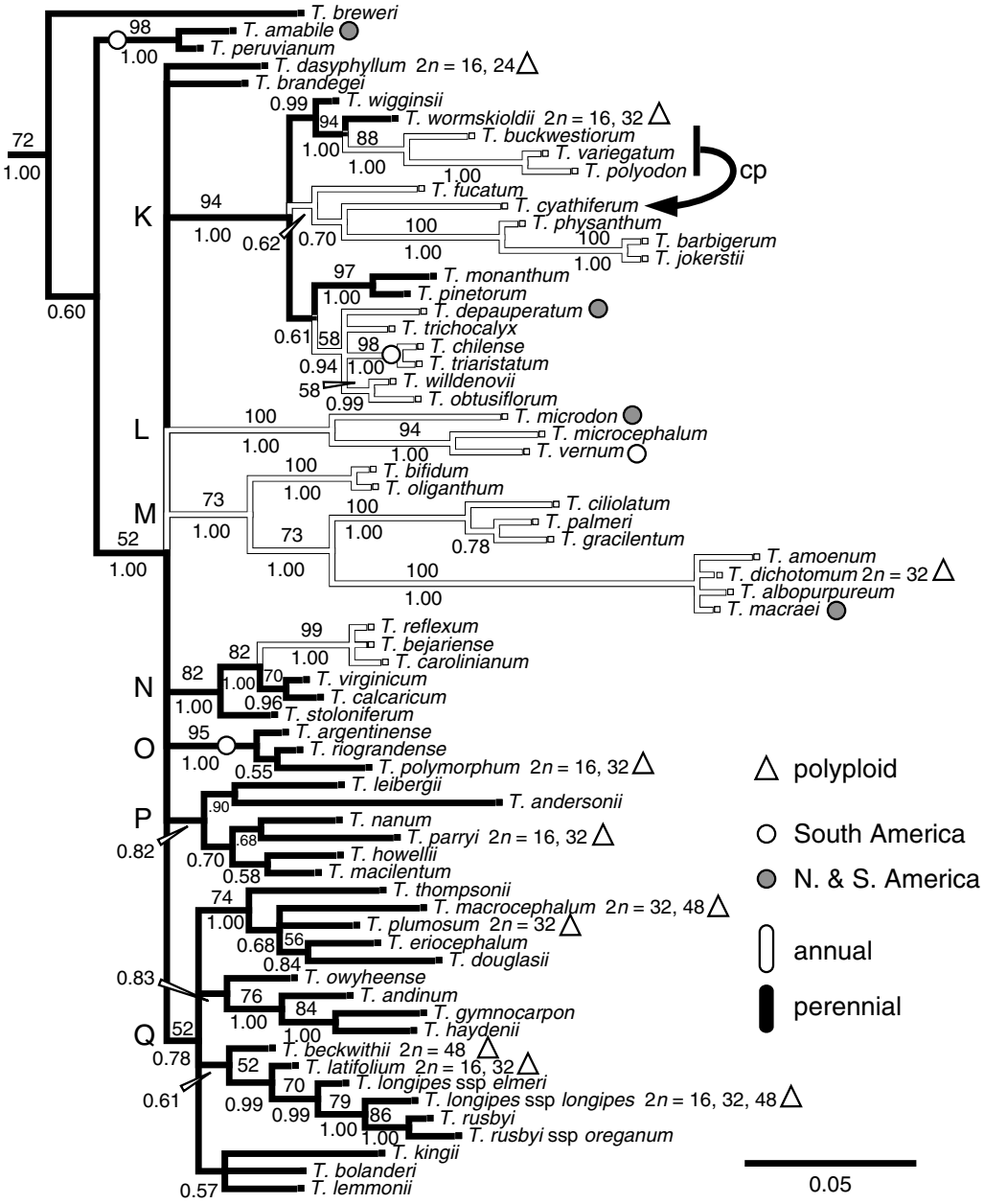

Fig. 7. Section *Involucrarium*. Branch shading corresponds to life history. The arrow denotes the inferred origin of the chloroplast in *T. cyathiferum*. All species are $2n = 16$ or unknown, except as noted. See text for clades K–Q.

could be further divided into subsections, but this is premature pending more intensive sampling and better phylogenetic resolution at this taxonomic level. With few exceptions, the subsections and series recognized by Zohary and Heller (1984) are not monophyletic.

Subgenus *Chronosemium* and some of the proposed sections of subgenus *Trifolium* can be characterized morphologically, following the descriptions of Zohary and Heller (1984). Species of subgenus *Chronosemium* are characterized by persistent banner petals that function in the dispersal of the small one-seeded fruit, usually yellow corollas, and pinnate trifoliolate leaves. The latter two characters are rare, or absent, in subgenus *Trifolium* and may be symplesiomorphic in Trifolieae. The two species of sect. *Paramesus* possess gland-tipped stipules, a unique feature in the genus. In sect. *Trifolium* the pod is included in the calyx tube, which is either narrowed or closed by hairs or tissue development. Section *Trichocephalum* is characterized by inflorescences with a mixture of fertile and sterile, apetalous flowers. The remaining five sections include species that were classified by Zohary and Heller (1984) in sect. *Lotoidea*. They characterized this section by the absence of morphological traits observed elsewhere in the genus. Thus, no putative synapomorphies for these five sections are apparent. The character of inflated bladder-like calyces is restricted to sect. *Vesicastrum*; however, these species (Fig. 5, clades D, F, G, and J) are polyphyletic. Likewise, sect. *Involucrarium* contains numerous species with an invo-

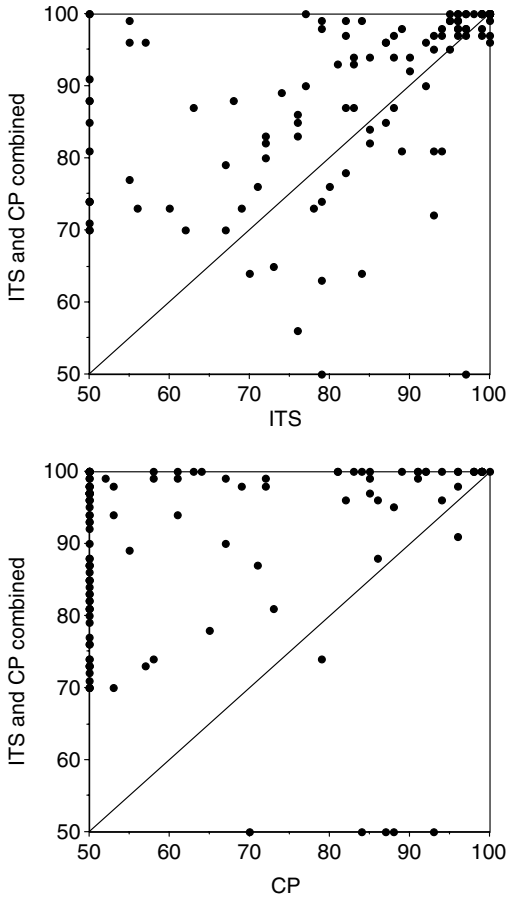

Fig. 8. Bootstrap percentages for clades with ⩾70% support in the combined and/or separate analyses. The *y*-axis gives the values from the combined analysis, the *x*-axis gives the values for the separate nrDNA ITS (above) and cpDNA *trnL* intron (below) analyses. Values <50% were not determined, and are treated as equivalent to 50%.

lucre of fused bracts below the inflorescence (Fig. 7, clades K, L, and *T. oliganthum*), but these do not comprise a clade. The above generalizations remain unproven pending an explicit phylogenetic analysis of morphological traits in *Trifolium*.

4.3. Biogeography

In agreement with previous phylogenetic analyses of *Trifolium*, two largely geographic clades are recognized involving the African (sect. *Vesicastrum*, Fig. 5) and American (sect. *Involucrarium*, Fig. 7) species, respectively. The derived position of the African and American clades argues against a North American (Zohary, 1972) or African (Pritchard, 1962) origin of the genus. Subgenus *Chronosemium* and the early diverging clades of subgenus *Trifolium* (sects. *Paramesus* and *Glycyrrhizum*) are currently restricted to the Mediterranean basin (and adjacent regions of Eurasia), consistent with a hypothesized Mediterranean origin of *Trifolium* (Gillett, 1952; Taylor et al., 1979). Based on a fossil-calibrated penalized likelihood approach and the Bayesian analysis of two cpDNA genes (*matK* and *rbcL*), Lavin et al. (2005) estimated the origin of the crown clade of Trifolieae + Fabeae at 17.1–30.2 million years ago (mya). They did not sample subgenus *Chronosemium*, thus their *matK* chronogram places the origin of subgenus *Trifolium* at 12.4 mya. Because the genus must have originated between these dates, we hypothesize that *Trifolium* originated in the Early Miocene, 16–23 mya.

Watson et al. (2000) sampled nine African species, and resolved them as monophyletic. With our increased taxonomic sampling, 29 sub-Saharan African species comprise the majority of sect. *Vesicastrum*, but are not monophyletic (Fig. 5). The most parsimonious reconstruction implies two dispersal events (clades E and H) from the Mediterranean region to sub-Saharan Africa. A third event is needed to account for *T. stipulaceum* Thunb., not sampled here, a South African species of sect. *Trifolium*. We also did not include the poorly known *T. ankaratrense* Bosser, endemic to Madagascar (Bosser, 1959). No *Trifolium* species currently grow in the Saharan or Arabian deserts. However, it is noteworthy that the sister group (clade G) to the large African clade H includes *T. clusii* and *T. tomentosum*, two Mediterranean species that occur further into the northern sections of these deserts than any other *Trifolium*. Furthermore, *T. fragiferum* has been reported for Ethiopia (Zohary and Heller, 1984). If this species is indeed native to that country (Thulin, 1983), it would suggest that the remaining species of clade G represent a secondary dispersal out of Africa (Fig. 5).

All of the North and South American species of *Trifolium* comprise a monophyletic group (Fig. 7). No morphological synapomorphies are known for this clade and it has not been recognized in any previous classification. This result parallels the discovery of species-rich, morphologically diverse "cryptic American clades" in other large temperate genera (Noyes and Rieseberg, 1999; Wojciechowski et al., 1993). In *Aster*, the North American species had been classified in 14 genera and were assumed to represent at least five independent colonization events (Noyes and Rieseberg, 1999). Like American *Trifolium*, their monophyly had not been previously hypothesized. In *Astragalus*, the North American clade shares aneuploid chromosome numbers (Spellenberg, 1976), but no morphological synapomorphies are known. Their monophyly was not confirmed until molecular phylogenetic studies were conducted (Wojciechowski et al., 1993). Like *Trifolium*, the *Astragalus* clade has both North and South American representatives. In contrast, *Astragalus* has non-aneuploid boreal and montane North American species that are derived from Eurasian lineages, while the montane species of *Trifolium* are not closely related to ecologically similar Eurasian species. *Trifolium* differs from both of these genera in the absence of Eurasian representatives in the "American" clade (contra *Astragalus echinatus* Murr. and *Erigeron uniflorus* L.).

Due to poor resolution at the base of sect. *Involucrarium* (Fig. 7), a North or South American origin cannot be confidently resolved. Assuming a North American origin, seven independent dispersals to South America can be inferred (Fig. 7), with no clade larger than three species. Unlike the

situation in Africa, there is not a strong geographic barrier to contemporary dispersal. In fact, *T. amabile* occurs discontinuously at high elevations (1600–3400 m) from southern Arizona to northern Argentina. The Texas endemic *T. amphianthum* Torr. and Gray (previously considered a synonym of *T. polymorphum*) apparently belongs to clade O (M. Vincent, pers. comm.). In both of these cases, the direction of dispersal cannot be inferred. The remaining five intercontinental disjunctions are reconstructed as dispersal events from North to South America (Fig. 7). All involve annual species, including three (*T. depauperatum*, *T. microdon*, and *T. macraei*) that are considered conspecific on both continents. These five cases can be interpreted as California–Chile "amphitropical" disjunctions (Raven, 1963). The North American origin hypothesized here has also been found in other examples of this disjunction (Vargas et al., 1998).

4.4. Potential cases of reticulate evolution

Integrating chromosome number changes and cpDNA/nrDNA conflict provides evidence for several examples of hybrid speciation in *Trifolium*. However, some uncertainty exists in each case, indicating the need for more intensive taxonomic sampling, and incorporation of additional sequence data (and chromosome counts) to confirm the hypotheses of reticulate evolution presented here.

Trifolium dubium (subgenus *Chronosemium*), a native of western Eurasia, is widely introduced in North America, and it is particularly common along the west coast of the continent (Gillett and Taylor, 2001). The species has long been considered a $2n=28$ tetraploid, but recent studies have documented $2n=30$ (H. Ansari, pers. comm.). The new number is consistent with a hybrid origin between $2n=14$ *T. campestre* and a $2n=16$ common ancestor of *T. billardieri*, *T. erubescens*, and *T. philistaeum* (Fig. 2). The only discrepant finding is that *T. campestre* also shares the cpDNA of this latter clade. Cytoplasmic introgression (also known as chloroplast capture) is a common phenomenon (Rieseberg et al., 1996), and is a likely explanation for this observation. It remains to be determined whether such introgression involved the *T. billardieri* clade, or occurred directly between *T. campestre* and *T. dubium*. The latter is considered more likely, since these two species have no divergence at the *trnL* locus.

Trifolium repens (sect. *Trifoliastrum*) is an allotetraploid (Williams et al., 1998), however attempts to identify its parental taxa have been inconclusive (Ansari et al., 2004, 1999; Badr et al., 2002). Our phylogenetic results implicate *T. occidentale* and *T. pallescens* as its diploid progenitors (Fig. 6). *Trifolium occidentale* has long been considered a potential ancestor of *T. repens* (but see Kakes and Chardonnens, 2000). The two species are morphologically similar and interfertile (Gibson and Beinhart, 1969). In contrast, *T. pallescens*, a montane species distributed in the Alps and Pyrenees of Europe, has rarely been included in crossing studies and molecular genetic investigations of the origin of *T. repens*, impeding efforts to identify the progenitors of this species. Ansari et al. (2004) included *T. pallescens* in their molecular cytogenetic survey of 16 *Trifolium* species for the distribution of a centromeric satellite sequence (TrR350) isolated from *T. repens*. The satellite sequence is found in several other species of sect. *Trifoliastrum* including *T. pallescens*, and thus cannot be used alone to identify the progenitors of *T. repens*.

Trifolium pannonicum (sect. *Trifolium*) is a perennial species native to the steppes of central and eastern Europe. A broad range of polyploid chromosome numbers ($2n=48$, 49, 60, 65, 96, 98, 126, 128, 130, and 180) has been reported (Cleveland, 1985; Gillett and Taylor, 2001; Zohary and Heller, 1984). Many of these reports are unvouchered and require confirmation. Our phylogenetic results are the first to suggest that *T. pannonicum* originated through hybridization between two distantly related species of sect. *Trifolium* (Fig. 4). Unfortunately, the parental species remain unidentified. *Trifolium pannonicum* shares an identical *trnL* intron sequence with two species in clade B: *T. patulum* (chromosome number unknown) and *T. squamosum* ($2n=16$). However, these two species do not form a clade in the nrDNA or combined analyses. The isolated position of *T. pannonicum* in the nrDNA and combined analyses suggests that the parental species from clade A may be unsampled or extinct.

Two other examples of incongruence of cpDNA data and ITS data may also result from interspecific hybridization. The patterns of cpDNA and nrDNA incongruence among several African species are suggestive of reticulate evolution (Fig. 5). The most striking example is *Trifolium usambarense*, which shares a *trnL* intron sequence with the distantly related *T. cheranganiense*. However, only diploid $2n=16$ counts are known from *T. usambarense*, suggesting that it may have acquired the chloroplast of *T. cheranganiense* via cytoplasmic introgression, and not polyploid speciation. Interspecific hybridization may also explain the fact that *Trifolium rueppellianum*, *T. cryptopodium*, and *T. stolzii* comprise a well-supported clade in the cpDNA analyses, but not in the nrDNA analyses. While *T. rueppellianum* is a diploid, both diploid and hexaploid counts are known from *T. cryptopodium*. Unfortunately, the chromosome number of *T. stolzii* is unknown. Until additional well-documented chromosome numbers are known for this group, no definitive conclusions can be drawn. A similar situation exists in the North American species *T. cyathiferum*, that is associated with different clades in the cpDNA and nrDNA analyses (Fig. 7). The only reported chromosome count, from a single individual, is diploid (Gillett and Mosquin, 1967). As in the case of the above African species, additional chromosome counts are desirable. All of the above examples of apparent reticulate evolution involve species that are sympatric in at least part of their geographic range.

It is remarkable that only 5–6 cases of apparent reticulate evolution were discovered in our analysis of 218 *Trifolium* species. This finding is consistent with the presence of strong genetic barriers to interspecific hybridization in the

genus (Taylor et al., 1980). It is noteworthy that each of the three apparent allopolyploid species (*T. dubium*, *T. repens*, and *T. pannonicum*) has putative parental species from well-separated clades. This could indicate that genetic reinforcement occurs among closely related species, and that these reproductive isolation mechanisms, that are rarely overcome in natural populations, can be surmounted in relatively distantly related species. Artificial crosses between distantly related species can produce fertile hybrids (Cleveland, 1985; Taylor et al., 1980), but generally require the technique of embryo rescue to succeed. Although natural allopolyploidy is apparently an infrequent phenomenon in *Trifolium*, two of the resulting species, *T. dubium* and *T. repens*, are evolutionary "success stories," spreading with the assistance of humans into appropriate habitats around the globe.

4.5. Life history evolution

While we can be fairly confident that the ancestor of subgenus *Chronosemium* was an annual (Fig. 2), the ancestral state of subgenus *Trifolium* is uncertain (Fig. 3). Resolution of the polytomy between the annual sect. *Paramesus* and the perennial sect. *Glycyrrhizum* should clarify this. Within subgenus *Trifolium*, two sections (*Trifolium* and *Trichocephalum*) share an annual ancestry, while three sections (*Vesicastrum*, *Trifoliastrum*, and *Involucrarium*) share a perennial ancestry. Across the genus, initial transitions to the perennial habit (at least nine) are much less common than transitions to the annual habit (at least 19). However, in sect. *Trifoliastrum* (Fig. 5) two reversals to the perennial habit are reconstructed, and at least four are inferred in sect. *Vesicastrum* (Fig. 6). A hypothesis for this observation is that the transition from annual to perennial is under more developmental constraint in the ancestrally annual sections than in the ancestrally perennial sections. In general, perennial species occur at higher elevation habitats than the annual species.

4.6. Chromosome evolution

Although several authors have reviewed chromosome evolution in *Trifolium* (Cleveland, 1985; Goldblatt, 1981; Taylor et al., 1979; Zohary and Heller, 1984), ours is the first study to examine these data from a phylogenetic perspective. In agreement with previous authors, our results support $2n = 16$ as the ancestral chromosome number in the genus.

We sampled 23 of the 24 known polyploids and all 31 known aneuploids. Despite the greater number of aneuploids, there is little difference in the inferred number of aneuploid (19) and polyploid (22) events in the genus. These results reflect the fact that polyploidy is almost always autapomorphic in *Trifolium*, while aneuploid events are more likely to demarcate clades. Apparently only one instance of polyploidy has been followed by subsequent speciation (*T. africanum* and *T. burchellianum*, Fig. 5). The majority of inferred polyploid events occur in the three large sections (Figs. 5–7) where aneuploidy is rare. Polyploidy is associated with the perennial habit in all but three species (Taylor et al., 1979), and two of the annual polyploids (*T. dichotomum* and *T. resupinatum*) occur in sections that share a perennial ancestry. Among the 22 inferred polyploid events, three are clearly allopolyploid (see Section 4.4.). There is lability in the cpDNA and nrDNA positions of *T. latifolium* (results not shown) that is perhaps also indicative of allopolyploidy. This species is part of the *T. longipes–T. rusbyi* species complex (Fig. 7). The cytogeography of this widespread western North American clade has been well documented (Gillett, 1969), and our results suggest further investigations with molecular markers and broader sampling would be very informative. The remaining hypothesized polyploidy events show no evidence of interspecific hybridization, and thus could be the result of autopolyploidy. However, despite the thoroughness of our taxonomic sampling, it is not exhaustive and unsampled taxa could have contributed to these polyploids. Furthermore, genetic evidence for autopolyploidy has only been reported for *T. uniflorum* (Chen and Gibson, 1982).

Aneuploidy is most common in sects. *Trifolium* and *Trichocephalum* (Fig. 4). These sections are characterized by apparent ancestral polymorphism for $2n = 16$ and $2n = 14$, followed by lineage sorting in descendent species. The alternative explanation, the return to the $2n = 16$ condition through aneuploid increase, cannot be ruled out. Molecular cytogenetic investigations of these clades could differentiate between the two hypotheses. Taylor et al. (1979) noted that reduced chromosome numbers are absent in the African and American species of *Trifolium*, and rare in perennial species. Our results extend their findings by demonstrating that aneuploidy is rare in the three sections (*Vesicastrum*, *Trifoliastrum*, and *Involucrarium*, Figs. 5–7) that share a perennial ancestry and contain all of the African and American species. The two instances of aneuploidy in these three sections involve annual species (*T. glomeratum* and *T. resupinatum*), providing further evidence for a correlation between these character states. Our results also suggest that aneuploid species have rarely given rise to polyploids. The only exception is *T. dubium* (Fig. 2), that apparently combines a $2n = 16$ and $2n = 14$ genome (Ansari and Ellison, unpublished data). *Trifolium trichocephalum* (Fig. 4) and *T. resupinatum* (Fig. 5) are the only species with aneuploid and polyploid counts, however in both species the polyploidy is based on $2n = 16$.

With the exception of aneuploidy in sects. *Trifolium* and *Trichocephalum*, the majority of chromosome number changes in *Trifolium* have been autapomorphic. Most instances of aneuploidy and polyploidy are known from species that are polymorphic for chromosome number, suggestive of recent evolution of these cytologically differentiated variants. Two cases were found where morphologically distinct species share identical cpDNA and nrDNA sequences, but differ in chromosome number: *T. macraei* ($2n = 16$) and *T. dichotomum* ($2n = 32$) (Fig. 7)

and *T. haussknechtii* ($2n = 16$), *T. sylvaticum* ($2n = 14$), and *T. trichopterum* ($2n = 14$) (Fig. 4). These cases can be explained by recent differentiation of the polyploid or aneuploid species.

4.7. Maximum parsimony vs. Bayesian analyses

Bayesian approaches to phylogenetic reconstruction are enjoying increasing popularity, despite lingering concerns over the robustness of these methods (Kolaczkowski and Thornton, 2004). It has been repeatedly demonstrated that Bayesian posterior probabilities are higher than parsimony bootstrap (Simmons et al., 2004; Suzuki et al., 2002), and thus it is important to not consider them as equivalent. In our interpretations of clade support, we took both the bootstrap percentages and posterior probabilities into account. However, in the comparison of the separate nrDNA and cpDNA analyses, we relied primarily on the parsimony bootstrap values since they exhibit more even distribution of support values across the resulting clades. It is notable in our combined analyses that several clades demarcated by morphological characters or geographic distributions had little or no bootstrap support but posterior probabilities >.85. Prominent examples include the monophyly of the genus *Trifolium*, sect. *Trifolium* and sect. *Involucrarium*. These examples of concordance with outside data suggest that the greater phylogenetic support and resolution obtained with the Bayesian approach better reflects evolutionary history in this genus. All of the topological differences between the MP and Bayesian results are due to the greater resolution of the latter. One such example involves the placement of a suspected interspecific hybrid, *T. cyathiferum*: the combined Bayesian topology resolves this species within a clade of species that share inflated fruiting corollas (Fig. 7), while the MP topology places it unresolved at the base of the large sect. *Involucrarium* clade. It is a common occurrence for hybrids to be "pulled" to the base of a clade (McDade, 1992). This is apparent in other cases (e.g., *T. repens*, Section 3.2). Thus, at least in this instance, the Bayesian approach may be less prone to this phenomenon than maximum parsimony.

4.8. Utility of nrDNA ITS and cpDNA trnL intron in Trifolium

The topologies resulting from the separate nrDNA and cpDNA analyses were significantly different by the ILD test, even after all genera outside of *Trifolium* and the 12 potentially discordant *Trifolium* species were removed. Furthermore, the nrDNA ITS sequences made a much greater contribution than the cpDNA *trnL* intron sequences, due to the poorly resolved cpDNA results. Nevertheless, analysis of the combined data set revealed phylogenetic information that would not be available if the cpDNA sequences were excluded. Despite the conflicting signal in the two data sets, the combined analysis was better resolved and bootstrap support increased for the great majority of the clades (Fig. 8). Most importantly, several cases of potential interspecific hybridization were identified by comparing the position of species in the separate analyses (see Section 4.4.). While hybridization for some of these species has been previously suspected, others are novel. In contrast to many other plants (Lihova et al., 2004; Nieto Feliner et al., 2004; Sang et al., 1995; Whittall et al., 2000) nrDNA additivity was rarely observed in *Trifolium* species. Although this facilitated analysis of these sequences, it provided limited opportunity for the identification of hybrids using this single locus. In contrast, artificial hybrids do show clear nrDNA additivity (N. Ellison, unpublished data).

4.9. Potential for DNA barcoding of Trifolium species

The use of DNA sequences to identify organisms has been proposed as a more efficient approach than traditional taxonomic practices (Blaxter, 2004; Tautz et al., 2003). Kress et al. (2005) have demonstrated the effectiveness of such "DNA barcoding" in angiosperms using nrDNA and non-coding cpDNA sequences. In *Trifolium*, extensive germplasm collections of most wild-collected species exist (Morris and Greene, 2001); however rates of misidentification may exceed 5–10% (R. Morgan, pers. comm.). Growing samples to maturity is time consuming, and many of the perennial species are difficult to bring to flower and fruit outside of nature. A DNA barcoding approach would be a useful supplement to existing identification methods. In our study, 94% of the sampled *Trifolium* species had unique combinations of nrDNA ITS and cpDNA *trnL* intron sequences. When indels are considered, only five species pairs and one triplet lack any sequence divergence. Thus, DNA-based identification of *Trifolium* species is quite feasible. It is important to note that DNA-based identification in *Trifolium* would be much more challenging without the availability of a comprehensive global monograph (Zohary and Heller, 1984) and biological information for most of the genus (Gillett and Taylor, 2001). Such a robust taxonomic foundation is lacking for the great majority of the world's species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.01.004.

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Appendix 1. DNA sources, voucher, GenBank information, life history and chromosome numbers. Germplasm sources: Margot Forde Forage Germplasm Centre, Palmerston North, New Zealand (NZ), USDA-ARS National Plant Germplasm System (US), N.L. Taylor Clover Germplasm Center, University of Kentucky (KY). Specimens grown from seed are deposited at the Dame Ella Campbell Herbarium, Massey University, New Zealand (MPN). Other herbarium acronyms: Botanical Museum Berlin-Dahlem (B), Royal Botanic Garden Edinburgh (E), The Hebrew University (HUJ), Royal Botanic Garden Kew (K), New York Botanical Garden (NY), Oregon State University (OSC), and Rancho Santa Ana Botanic Garden (RSA). Life history: annual (A), perennial (P). Life history and chromosome numbers are from the literature and online databases (Gillett and Taylor, 2001; ILDIS, 2002; Goldblatt and Johnson, 2003).

Species	Source / Voucher	Accession or Specimen	nrDNA ITS	*trnL* intron	Life history	Somatic chromosome number (2*n*)
Loteae						
Lotus angustissimus L.	NZ	S2065	**DQ311970**	**DQ311698**	A	12,24
Lotus corniculatus L.	NZ	S3343	**DQ311971**	**DQ311699**	P	24
Lotus edulis L.	US	PI 283627	**DQ311972**	**DQ311700**	A	14
Lotus glaber Mill.	NZ	S3221	**DQ311979**	**DQ311707**	P	12
Lotus glaucus Sol.	US	PI 239945	**DQ311973**	**DQ311701**	P	14,28
Lotus glinoides Del.	US	PI 246736	**DQ311974**	**DQ311702**	A	14
Lotus japonicus (Regel) K.Larsen	NZ	S1482	**DQ311975**	**DQ311703**	P	12
Lotus parviflorus Desf.	NZ	S1364	**DQ311976**	**DQ311704**	A	12,14
Lotus pedunculatus Cav.	NZ	S3307	**DQ311977**	**DQ311705**	P	12
Lotus suaveolens Pers.	NZ	S2044	**DQ311978**	**DQ311706**	A	12,24
Lotus unifoliolatus (Hook.) Benth.	NZ	S3230	**DQ311969**	**DQ311697**	A	14
Trifolieae						
Medicago lupulina L.	NZ	AL4657	**DQ311980**	**DQ311708**	A	16,32
Medicago polymorpha L.	NZ	AL4513	**DQ311981**	**DQ311709**	A	14
Medicago ruthenica (L.)Ledeb.	NZ	AL4073	**DQ311982**	**DQ311710**	P	16
Medicago sativa L.	NZ	AF2958	**AF053142**	**DQ311711**	P	16,32
Medicago truncatula Gaertner	NZ	AL1125	**DQ311983**	**DQ311712**	A	16
Melilotus albus Medikus	NZ	AL1850	**DQ311984**	**DQ311713**	A,P	16
Melilotus officinalis (L.)Pallas	NZ	AL4383	**DQ311985**	**DQ311714**	A,P	16
Ononis mitissima L.	NZ	AL4630	**DQ311986**	**DQ311715**	A	?
Trigonella foenum-graecum L.	NZ / MPN	AL4437	**DQ312196**	**DQ311952**	A	16
Vicieae						
Lathyrus laevigatus (Waldst. & Kit.) Gren.	NZ	AL4059	**DQ311967**	**DQ311695**	P	14
Lathyrus sativus L.	NZ	AL1684	**DQ311968**	**DQ311696**	A	14
Pisum sativum L.	NZ	AL4997	**DQ311988**	**DQ311717**	A	14
Vicia cracca L.	NZ	AL4988	**DQ312197**	**DQ311953**	P	14,28
Vicia sativa L.	NZ	AL3794	**DQ312198**	**DQ311954**	A	12
Vicia villosa Roth	NZ	AL4595	**DQ312199**	**DQ311955**	A	14

other vicioid genera						
Cicer arietinum L.	NZ	AL4995	**DQ312219**	**DQ315487**	A	16
Galega officinalis L.	NZ	AL4305	**DQ311965**	**DQ311693**	P	16
Galega orientalis Lam.	NZ	AL4789	**DQ311966**	**DQ311694**	P	16
Parochetus communis D.Don	NZ / MPN	AL4979	**DQ311987**	**DQ311716**	P	16
Trifolium* subg. *Chronosemium						
T. aureum Pollich	US / MPN	PI 108696	**DQ312005**	**DQ311738**	A	14
T. badium Schreber	NZ / MPN	AZ157	**DQ312007**	**DQ311740**	P	14
T. billardieri Spreng.	NZ	AZ4242	**DQ312015**	**DQ311750**	A	16
T. boissieri Guss.	NZ / MPN	PI 369022	**DQ312017**	**DQ311752**	A	16
T. brutium Ten.	KY / MPN	S-296-2	**DQ312201**	**DQ311756**	A	?
T. campestre Schreber	US / MPN	PI 291774	**DQ312025**	**DQ311763**	A	14
T. dubium Sibth.	NZ / MPN	AZ167	**DQ312047**	**DQ311785**	A	30
T. erubescens Fenzl	US / MPN	PI 639954	**DQ312051**	**DQ311789**	A	16
T. grandiflorum Schreber	NZ / MPN	AZ1774	**DQ312062**	**DQ311800**	A	16
T. micranthum Viv.	US / MPN	PI 516359	**DQ312091**	**DQ311836**	A	14,16
T. patens Schreber	US / MPN	PI 591675	**DQ312116**	**DQ311865**	A	16
T. philistaeum Zohary	US / MPN	PI 369090	**DQ312121**	**DQ311870**	A	16
T. phitosianum N.Böhling, W.Greuter & T.Raus	B	Böhling 8290	**DQ312122**	**DQ311871**	A	?
T. praetermissum W.Greuter, R.Pleger & T.Raus	B	Jahn s.n.	**DQ312137**	**DQ311887**	A	?
T. rytidosemium Boiss. & Hohen.	RSA	Vášak s.n.	**DQ312151**	**DQ311901**	P	?
T. sintenisii Freyn	E	Davis & Hedge 32153	**DQ312204**	**DQ315488**	A	?
T. spadiceum L.	NZ	AZ2820	**DQ312160**	**DQ311911**	A	14
Trifolium* subg. *Trifolium						
Trifolium* sect. *Glycyrrhizum						
T. alpinum L.	KY	S-106-6	**DQ311995**	**DQ311725**	P	16
T. polyphyllum C.A.Mey.	US	W6 18506	**DQ312135**	**DQ311885**	P	16
Trifolium* sect. *Lupinaster						
T. eximium Steph.ex Ser.	K	Jeffrey 1476	**DQ312052**	**DQ311790**	P	16
T. gordejevii (Kom.) Z.Wei	NY	Kharkevich & Buch 565	**DQ312059**	**DQ311797**	P	?
T. lupinaster L.	NZ	AZ1777	**DQ312083**	**DQ311827**	P	16,32,48
Trifolium* sect. *Paramesus						
T. glanduliferum Boiss.	US / MPN	PI 296666	**DQ312056**	**DQ311794**	A	16
T. strictum L.	US / MPN	PI 369147	**DQ312169**	**DQ311923**	A	16
Trifolium* sect. *Trichocephalum						
T. batmanicum Katzn.	NZ / MPN	AZ2262	**DQ312011**	**DQ311744**	A	16
T. eriosphaerum Boiss.	NZ	AZ1510	**DQ312050**	**DQ311788**	A	14
T. globosum L.	NZ / MPN	AZ2274	**DQ312057**	**DQ311795**	A	10,16

T. israeliticum D.Zohary & Katzn.	US / MPN	PI 292501	**DQ312069**	**DQ311810**	A	12
T. meduseum Blanche ex Boiss.	US / MPN	PI 369049	**DQ312090**	**DQ311834**	A	14
T. pauciflorum Urv.	NZ / MPN	AZ1556	**DQ312118**	**DQ311867**	A	16
T. pilulare Boiss.	NZ / MPN	AZ2295	**DQ312129**	**DQ311878**	A	14
T. subterraneum L. ssp. *brachycalycinum* Katzn. & F.H.W.Morley	NZ	AK808	**DQ312170**	**DQ311924**	A	16
T. subterraneum L. ssp. *subterraneum*	NZ	AK640	**DQ312171**	**DQ311925**	A	16
T. subterraneum L. ssp. *yanninicum* Katzn. & F.H.W.Morley	NZ	AK1226	**DQ312172**	**DQ311926**	A	16
Trifolium* sect. *Trifolium						
T. affine C.Presl	NZ / MPN	AZ925	**DQ311990**	**DQ311719**	A	16
T. alexandrinum L.	US / MPN	PI 163315	**DQ311993**	**DQ311723**	A	16
T. alpestre L.	US / MPN	PI 314116	**DQ311994**	**DQ311724**	P	16
T. andricum P.Lassen	KY / MPN	81-S-268-1	**DQ311999**	**DQ311730**	A	16
T. angustifolium L.	KY / MPN	S-1-21	**DQ312200**	**DQ311732**	A	16
T. apertum Bobrov	NZ / MPN	AZ4248	**DQ312000**	**DQ311733**	A	16
T. arvense L.	US	PI 494713	**DQ312004**	**DQ311737**	A	14
T. barbeyi Gibelli & Belli	B	Raus 9890	**DQ312009**	**DQ311742**	A	?
T. berytheum Boiss. & Blanche	NZ / MPN	AZ1487	**DQ312013**	**DQ311747**	A	16
T. bocconei Savi	US / MPN	PI 369021	**DQ312016**	**DQ311751**	A	12,14
T. canescens Willd.	KY	S-9-19	**DQ312026**	**DQ311764**	P	48
T. caucasicum Tausch	NZ / MPN	AZ2802	**DQ312028**	**DQ311766**	P	?
T. cherleri L.	NZ / MPN	AZ2267	**DQ312031**	**DQ311770**	A	10
T. clypeatum L.	US / MPN	PI 202804	**DQ312034**	**DQ311774**	A	16
T. constantinopolitanum Ser.	US / MPN	PI 369028	**DQ312035**	**DQ311959**	A	16
T. dalmaticum Vis.	US / MPN	PI 516292	**DQ312038**	**DQ311777**	A	10
T. dasyurum C.Presl	US / MPN	PI 369030	**DQ312040**	**DQ311779**	A	16
T. dichroanthum Boiss.	KY / MPN	S-84-2	**DQ312044**	**DQ311782**	A	16
T. diffusum Ehrh.	US / MPN	PI 204517	**DQ312045**	**DQ311783**	A	16
T. echinatum M.Bieb.	US	PI 516304	**DQ312048**	**DQ311786**	A	16
T. gemellum Willd.	US / MPN	PI 287963	**DQ312055**	**DQ311793**	A	14
T. haussknechtii Boiss.	US	PI 591662	**DQ312064**	**DQ311802**	A	16
T. heldreichianum Hausskn.	KY / MPN	S-148-6	**DQ312066**	**DQ311804**	P	16
T. hirtum All.	NZ / MPN	AZ2277	**AF053158**	**DQ311805**	A	10
T. incarnatum L.	NZ	AZ3280	**AF053160**	**DQ311808**	A	14
T. isodon Murb.	NZ / MPN	AZ181	**DQ312068**	**DQ311809**	A	?
T. lappaceum L.	US / MPN	PI 107120	**DQ312073**	**DQ311814**	A	16
T. latinum Sebast.	NZ / MPN	AZ2283	**DQ312075**	**DQ311816**	A	16
T. leucanthum M.Bieb.	US / MPN	PI 292826	**DQ312078**	**DQ311819**	A	14,16

T. ligusticum Lois.	US / MPN	PI 419415	**DQ312079**	**DQ311820**	A	12,14
T. longidentatum Nabelek	US / MPN	PI 419348	**DQ312080**	**DQ311821**	P	?
T. lucanicum Guss.	NZ / MPN	AZ1531	**DQ312081**	**DQ311825**	A	?
T. medium L.	NZ	AZ150	**DQ312089**	**DQ311833**	P	48,49, 63,64, 68,70, 72,80
T. miegeanum Maire	NZ / MPN	AZ1780	**DQ312094**	**DQ311839**	A	16
T. noricum Wulfen	US	PI 516372	**DQ312104**	**DQ311849**	P	16
T. obscurum Savi	NZ	AZ2281	**DQ312105**	**DQ311850**	A	16
T. ochroleucum Huds.	US	PI 258449	**DQ312107**	**DQ311853**	P	16
T. palaestinum Boiss.	US / MPN	PI 292476	**DQ312110**	**DQ311858**	A	16
T. pallidum Waldst. & Kit.	US / MPN	PI 419296	**DQ312112**	**DQ311860**	A	16
T. pannonicum Jacq.	NZ / MPN	AZ641	**DQ312113**	**DQ311861**	P	48,49, 60,65, 96,98, 126,128 130,180
T. patulum Tausch	US / MPN	PI 253200	**DQ312117**	**DQ311866**	P	?
T. phleoides Willd.	US / MPN	PI 208727	**DQ312123**	**DQ311872**	A	14
T. pignantii Fauche & Chaub.	US	G 31423	**DQ312128**	**DQ311877**	P	16
T. plebeium Boiss.	NY	Samuelsson 4724	**DQ312131**	**DQ311880**	A	16
T. pratense L.	NZ	F2086	**DQ312138**	**DQ311888**	P	14
T. purpureum Lois.	KY / MPN	S-76-10	**DQ312140**	**DQ311890**	A	14
T. rubens L.	KY / MPN	S-72-11	**DQ312147**	**DQ311897**	P	16
T. salmoneum Mout.	US	PI 179056	**DQ312152**	**DQ311902**	A	16
T. scabrum L.	NZ / MPN	AZ2299	**DQ312153**	**DQ311903**	A	10,16
T. scutatum Boiss.	US / MPN	PI 369115	**DQ312155**	**DQ311906**	A	16
T. squamosum L.	US	PI 419428	**DQ312163**	**DQ311915**	A	16
T. squarrosum L.	NZ	AZ485	**DQ312164**	**DQ311917**	A	14,16
T. stellatum L.	US / MPN	PI 419374	**DQ312165**	**DQ311918**	A	12
T. striatum L.	NZ / MPN	AZ2672	**DQ312168**	**DQ311922**	A	14
T. sylvaticum Gerard	US / MPN	PI 369121	**DQ312174**	**DQ311929**	A	14,16
T. trichocephalum M.Bieb.	NZ / MPN	AZ2831	**DQ312181**	**DQ311936**	P	14,48
T. trichopterum Pancic	US / MPN	PI 583430	**DQ312182**	**DQ311937**	A	14
T. vavilovii Eig	US	PI 516467	**DQ312186**	**DQ311942**	A	16
T. velebiticum Degen	KY / MPN	S-236-2	**DQ312187**	**DQ311943**	P	?
Trifolium* sect. *Vesicastrum						
T. abyssinicum Fresen. A	NZ	AZ1939	**DQ312024**	**DQ311761**	A,P	?
T. abyssinicum Fresen. B	US / MPN	PI 516203	**DQ312208**	**DQ311762**	A,P	?
T. acaule A.Rich.	KY	S-272-1	**DQ311989**	**DQ311718**	P	16
T. africanum Ser.	US / MPN	PI 516206	**DQ311991**	**DQ311720**	P	32
T. aintabense Boiss. & Hausskn.	KY	S-194-2	**DQ311992**	**DQ311721**	A	?

T. argutum Sol.	NZ / MPN	AZ148	**DQ312003**	**DQ311736**	A	16
T. baccarinii Chiov.	US / MPN	PI 102063	**DQ312006**	**DQ311739**	A	16
T. balansae Boiss.	RSA	Strid et al. 26121	**DQ312008**	**DQ311741**	A	?
T. bilineatum Fresen.	US / MPN	PI 516254	**DQ312014**	**DQ311749**	A	16
T. bullatum Boiss. & Hausskn.	US / MPN	PI 516261	**DQ312021**	**DQ311758**	A	16
T. burchellianum Ser.	US / MPN	PI 516262	**DQ312022**	**DQ311759**	P	96
T. cheranganiense J.B.Gillett	NZ / MPN	AZ213	**DQ312030**	**DQ311769**	P	16
T. clusii Godron & Gren.	US / MPN	PI 516284	**DQ312033**	**DQ311773**	A	16
T. cryptopodium A.Rich.	US	PI 516290	**DQ312036**	**DQ311775**	P	16,48
T. decorum Chiov. A	NZ / MPN	AZ4202	**AF053153**	**DQ311957**	A	16
T. decorum Chiov. B	US / MPN	PI 516293	**DQ312041**	**DQ311956**	A	16
T. fragiferum L.	NZ	AO321	**DQ312053**	**DQ311791**	P	16
T. hybridum L.	NZ	AB262	**AF053159**	**DQ311807**	P	16
T. lanceolatum (J.B. Gillett) J.B. Gillett	HUJ	CPI 24977	**DQ312127**	**DQ311876**	A	16
T. lugardii Bullock	US / MPN	PI 193744	**DQ312082**	**DQ311826**	A	16
T. masaiense J.B.Gillett	US	PI 262236	**DQ312087**	**DQ311831**	A	16
T. mattirolianum Chiov.	US / MPN	PI 516346	**DQ312088**	**DQ311832**	A	16
T. michelianum Savi	NZ / MPN	AZ4323	**AF053165**	**DQ311835**	A	16
T. multinerve A.Rich.	US / MPN	PI 516369	**DQ312098**	**DQ311843**	A,P	16
T. mutabile Portenschlag	US / MPN	PI 369053	**DQ312099**	**DQ311844**	A	16
T. ornithopodioides (L.)Smith	NZ / MPN	AZ2290	**AF053169**	**DQ311856**	A	16
T. petitianum A.Rich.	K	Friis & al. 5642	**DQ312120**	**DQ311869**	P	?
T. physodes M.Bieb.	US / MPN	PI 419266	**DQ312125**	**DQ311874**	P	16
T. pichisermollii J.B.Gillett	K	Gilbert & Thulin 1000	**DQ312126**	**DQ311875**	A	?
T. polystachyum Fresen.	NZ	AZ4494	**DQ312136**	**DQ311886**	P	16
T. pseudostriatum Baker f.	NY	Rwaburindore 2250	**DQ312139**	**DQ311889**	A	16
T. purseglovei J.B.Gillett	KY	S-109-1	**DQ312141**	**DQ311891**	P	?
T. quartinianum A.Rich.	US / MPN	PI 517490	**DQ312142**	**DQ311892**	A	16
T. resupinatum L.	NZ / MPN	AZ2364	**DQ312144**	**DQ311894**	A	14,16, 32
T. rueppellianum Fresen.	KY	S-37-9	**DQ312148**	**DQ311898**	A	16
T. schimperi A.Rich.	US	PI 516429	**DQ312154**	**DQ311905**	A	?
T. semipilosum Fresen.	US / MPN	PI 262238	**DQ312156**	**DQ311907**	P	16
T. setiferum Boiss.	B	Manob s.n.	**DQ312157**	**DQ311908**	A	?
T. simense Fresen.	US	PI 517687	**DQ312158**	**DQ311909**	?	?
T. somalense Taubert	US	G 31435	**DQ312159**	**DQ311910**	P	?
T. spananthum Thulin	KY / MPN	S-266-2	**DQ312161**	**DQ311912**	P	16
T. spumosum L.	US / MPN	PI 419268	**DQ312162**	**DQ311913**	A	16
T. steudneri Schweinf.	US / MPN	PI 262239	**DQ312166**	**DQ311919**	A	16

T. stolzii Harms	US / MPN	W6 22235	**DQ312167**	**DQ311921**	P	?
T. tembense Fresen.	US / MPN	PI 225796	**DQ312175**	**DQ311930**	A	16
T. tomentosum L.	US / MPN	PI 141508	**DQ312178**	**DQ311933**	A	16
T. tumens M.Bieb.	US	PI 516459	**DQ312183**	**DQ311938**	P	16
T. usambarense Taubert	NZ / MPN	AZ147	**DQ312203**	**DQ311940**	A,P	16
T. vesiculosum Savi	NZ / MPN	AZ2011	**DQ312190**	**DQ311946**	A	16
Trifolium* sect. *Trifoliastrum						
T. ambiguum M.Bieb.	NZ	AZ3119	**AF053145**	**DQ311726**	P	16,32, 48
T. cernuum Brot.	NZ / MPN	AZ2438	**AF053150**	**DQ311768**	A	16
T. glomeratum L.	NZ / MPN	AZ2595	**DQ312058**	**DQ311796**	A	14,16
T. isthmocarpum Brot.	NZ / MPN	AZ1643	**DQ312070**	**DQ311811**	A	16
T. montanum L. ssp. *humboldtianum* (A.Br. & Asch.)Hossain	NZ / MPN	AZ2814	**DQ312096**	**DQ311841**	P	?
T. montanum L. ssp. *montanum*	NZ	AZ2871	**DQ312097**	**DQ311842**	P	16
T. nigrescens Viv. ssp. *meneghinianum* (Clem.)ined.	NZ / MPN	AZ3296	**DQ312102**	**DQ311847**	A	?
T. nigrescens Viv. ssp. *nigrescens*	NZ / MPN	AZ2225	**DQ312101**	**DQ311846**	A	16
T. nigrescens Viv. ssp. *petrisavii* (Clem.)Holmboe	NZ / MPN	AZ125	**DQ312103**	**DQ311848**	A	?
T. occidentale Coombe	US / MPN	G 31525	**AF053168**	**DQ311852**	P	16
T. pallescens Schreber	NZ / MPN	AZ1895	**DQ312111**	**DQ311859**	P	16
T. parnassii Boiss. & Spruner	B	Willing 5135	**DQ312114**	**DQ311862**	P	16
T. repens L.	US	PI 376882	**DQ311962**	**DQ311961**	P	32
T. retusum L.	US	PI 120203	**DQ312145**	**DQ311895**	A	16
T. suffocatum L.	NZ / MPN	AZ2329	**DQ312173**	**DQ311928**	A	16
T. thalii Villars	NZ / MPN	AZ1792	**DQ312176**	**DQ311931**	P	16
T. uniflorum L.	US / MPN	PI 369138	**DQ312184**	**DQ311939**	P	32
Trifolium* sect. *Involucrarium						
T. albopurpureum Torrey & A.Gray	US / MPN	PI 593321	**AF053143**	**DQ311722**	A	16
T. amabile Kunth	NZ / MPN	AZ2424	**AF053144**	**DQ311958**	P	16
T. amoenum Greene	KY / MPN	S-249-1	**DQ311996**	**DQ311727**	A	?
T. andersonii A.Gray	KY	S-256-2	**DQ311997**	**DQ311728**	P	16
T. andinum Torrey & A.Gray	KY	S-158-2	**DQ311998**	**DQ311729**	P	16
T. argentinense Speg.	NZ	Brazil/Dall' Agnol	**DQ312002**	**DQ311735**	P	?
T. barbigerum Torrey	US / MPN	593311	**DQ312010**	**DQ311743**	A	16
T. beckwithii S.Watson	KY	S-202-3	**DQ312012**	**DQ311745**	P	48
T. bejariense Moric.	NZ / MPN	AZ2263	**AF053147**	**DQ311746**	A	16
T. bifidum A.Gray	US	PI 593297	**AF053156**	**DQ311748**	A	16
T. bolanderi A.Gray	KY / MPN	S-262-2	**DQ312018**	**DQ311753**	P	?

T. brandegei S.Watson	US	PI 611663	**DQ312019**	**DQ311754**	P	16
T. breweri S.Watson	OSC	McNeal 3783	**DQ312020**	**DQ311755**	P	16
T. buckwestiorum Isely	US / MPN	PI 593307	**AF053148**	**DQ311757**	A	?
T. calcaricum J.L.Collins & Wieboldt	KY	S-209-6	**DQ312023**	**DQ311760**	P	16
T. carolinianum Michaux	US	PI 516271	**DQ312027**	**DQ311765**	A	16
T. chilense Hook. & Arn.	NZ	AZ1490	**DQ312032**	**DQ311771**	A	16
T. ciliolatum Benth.	US / MPN	PI 593302	**AF053152**	**DQ311772**	A	16
T. cyathiferum Lindley	US	PI 516291	**DQ312037**	**DQ311776**	A	16
T. dasyphyllum Torrey & A.Gray	OSC	EPOB 4520	**DQ312039**	**DQ311778**	P	16,24
T. depauperatum Desv.	US / MPN	PI 516294	**DQ312042**	**DQ311780**	A	16
T. dichotomum Hook. & Arn.	US / MPN	PI 516298	**DQ312043**	**DQ311781**	A	32
T. douglasii House	KY	S-253-1	**DQ312046**	**DQ311784**	P	16
T. eriocephalum Torrey & A.Gray	KY	S-254-1	**DQ312049**	**DQ311787**	P	16
T. fucatum Lindley	US / MPN	PI 516311	**DQ312054**	**DQ311792**	A	16
T. gracilentum Torrey & A.Gray	US / MPN	PI 516319	**DQ312060**	**DQ311798**	A	16
T. gymnocarpon Torrey & A.Gray	KY	S-158-3	**DQ312063**	**DQ311801**	P	?
T. haydenii Porter	KY	S-204-2	**DQ312065**	**DQ311803**	P	16
T. howellii S.Watson	OSC	Fosback s.n.	**DQ312067**	**DQ311806**	P	?
T. jokerstii M.A.Vincent & R.Morgan	KY / MPN	S-270-1	**DQ312071**	**DQ311812**	A	?
T. kingii S.Watson var. *productum* (Greene) Jeps.	KY / MPN	S-251-2	**DQ312072**	**DQ311813**	P	16
T. latifolium (Hook.)Greene	KY	S-255-1	**DQ312074**	**DQ311815**	P	16,32
T. leibergii Nelson & Macbr.	US	W6 17564	**DQ312076**	**DQ311817**	P	?
T. lemmonii S.Watson	KY	S-261-1	**DQ312077**	**DQ311818**	P	?
T. longipes Torrey & A.Gray ssp. *elmeri* (Greene) J.M.Gillett	OSC	Chambers 5804	**DQ312221**	**DQ311823**	P	16
T. longipes Torrey & A.Gray ssp. *longipes*	US / MPN	W6 17547	**DQ312220**	**DQ311822**	P	16,32, 48
T. macilentum Greene	KY	S-99-3	**DQ312084**	**DQ311828**	P	16
T. macraei Hook. & Arn.	KY / MPN	S-192-2	**DQ312085**	**DQ311829**	A	16
T. macrocephalum (Pursh)Poiret	US	W6 17664	**DQ312086**	**DQ311830**	P	32,48
T. microcephalum Pursh	US	PI 593292	**DQ312092**	**DQ311837**	A	16
T. microdon Hook. & Arn.	US / MPN	PI 593288	**DQ312093**	**DQ311838**	A	16
T. monanthum A.Gray	KY	S-263-1	**DQ312095**	**DQ311840**	P	16
T. nanum Torrey	US	G 31370	**DQ312100**	**DQ311845**	P	16
T. obtusiflorum Hook.f.	US / MPN	PI 593296	**DQ312106**	**DQ311851**	A	16
T. oliganthum Steudel	US / MPN	PI 593289	**DQ312108**	**DQ311855**	A	16
T. owyheense Gilkey	KY	S-257-1	**DQ312109**	**DQ311857**	P	?
T. palmeri S.Watson	KY	S-135-8	**DQ312061**	**DQ311799**	A	?
T. parryi A.Gray	OSC	Holmgren 9998	**DQ312115**	**DQ311863**	P	16,32

T. peruvianum Vog.	NY	Sanchez 2382	**DQ312119**	**DQ311868**	P	?
T. physanthum Hook. & Arn.	NZ	AZ3200	**DQ312124**	**DQ311873**	A	?
T. pinetorum Greene	US / MPN	PI 516391	**DQ312130**	**DQ311879**	P	16
T. plumosum Hook.	KY	S-252-1	**DQ312132**	**DQ311881**	P	32
T. polymorphum Poiret	US / MPN	PI 233554	**DQ312133**	**DQ311882**	P	16,32
T. polyodon Greene	NZ / MPN	AZ4272	**DQ312134**	**DQ311884**	A	?
T. reflexum L.	KY / MPN	S-34-6	**DQ312143**	**DQ311893**	A	16
T. riograndense Burkart	US	PI 516425	**DQ312146**	**DQ311896**	P	16
T. rusbyi Greene ssp. *oreganum* (Howell) D.Heller & M.Zohary	OSC	Chambers 5805	**DQ312150**	**DQ311900**	P	?
T. rusbyi Greene ssp. *rusbyi*	NZ / MPN	AZ3221-1	**DQ312149**	**DQ311899**	P	16
T. stoloniferum Muhlenb.	US / MPN	PI 516440	**AF053176**	**DQ311920**	P	16
T. thompsonii Morton	NZ	AZ4367	**DQ312177**	**DQ311932**	P	16
T. triaristatum Colla	US	PI 516280	**DQ312179**	**DQ311934**	A	?
T. trichocalyx A.Heller	KY / MPN	S-246-1	**DQ312180**	**DQ311935**	A	?
T. variegatum Torrey & A.Gray	US / MPN	PI 516464	**DQ312185**	**DQ311941**	A	16
T. vernum Philippi	US	PI 516470	**DQ312189**	**DQ311945**	A	16
T. virginicum Small & Vail	US / MPN	PI 516471	**DQ312191**	**DQ311947**	P	16
T. wigginsii J.M.Gillett	KY / MPN	S-271-4	**DQ312193**	**DQ311949**	P	?
T. willdenovii Sprengel	NZ / MPN	AZ1290	**DQ312194**	**DQ311950**	A	16
T. wormskioldii Lehm.	US	PI 604683	**DQ312195**	**DQ311951**	P	16,32