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MOLECULAR PHYLOGENY AND SYSTEMATIC STATUS OF SOME *TANACETUM* L. (ASTERACEAE) TAXA FROM TURKEY

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Abstract

Tanacetum is an important member of the Asteraceae family and considered the most problematic genus and the phylogenetic position of some taxa is of great interest because of the high morphologic diversity and taxonomical complexity. Molecular phylogeny and systematic status of some *Tanacetum* taxa from Turkey has been carried out. *T. heterotomum* (endemic), *T. cadmeum* subsp. *orientale* (endemic), *T. cappadocicum*, and *T. eginense* have not been included in any molecular phylogenetic analysis yet. To determine the phylogenetic relationships and taxonomic status of *Tanacetum* L. taxa we analysed internal transcribed spacer (ITS) from nuclear ribosomal DNA (nrDNA) and *trnL* (UAA) intron, intergenic spacer between the *trnL* (UAA) 3` exon and *trnF* (GAA) from chloroplast DNA (cpDNA). According to the phylogenetic trees two main clades were formed. First clade included *T. eginense*, *T. cadmeum* subsp. *orientale* and *T. cappadocicum*, the other clade included *T. heterotomum* according to the data based upon the nrDNA. On the other hand, according to the cpDNA data, all four taxa were located in the same branch.

Key words: *Tanacetum*, nrDNA, cpDNA, Phylogeny.

Introduction

The genus *Tanacetum* L. is an important member of the Asteraceae (Compositae) family and widespread mainly in Europe and western Asia through the northern temperate regions. Anthemideae is a medium-sized tribe in the family (Valles *et al.,* 2005) comprising of 111 genera and about of 1,800 species worldwide (Oberprieler *et al.,* 2007). *Tanacetum* consists of about 150-200 species and it is the third largest genus of the Anthemideae tribe after Artemisia (522 ssp.) and Anthemis (175 spp.) (Heywood 1976; Soreng & Cope, 1991). The Asteraceae family is the second largest family according to the Flora of Turkey with regard to number of genera (Ozhatay *et al.,* 2011). In Flora of Turkey 143 genera and 1484 species are recorded that belong to Asteraceae family, among them 474 species are endemic and endemism ratio is approximately 38% (Davis 1982; Ozhatay *et al.,* 2011). *Tanacetum* contains several annual and perennial taxa and it is represented in Turkey by 46 species, 18 of which are endemic and endemism rate is 40% (Güner *et al.,* 2012).

Tanacetum has not been divided in sections but placed in three groups in Flora of Turkey (A, B and C) based largely on capitula and flower characters (Davis 1982). According to Flora of Turkey discrimination of this genus is done as follows:

1. Capitula heterogamous; marginal female flowers present, ligulate but sometimes inconspicuous and scarcely longer than disc flowers;
2. Female flowers white, pale sulphur yellow (but not bright yellow) or pinkish red, always with conspicuous ligules (Group A)
2. Female flowers bright or deep yellow, ligules sometimes inconspicuous (Group B)
1. Capitula homogamous, discoid; female flowers completely absent (Group C)

Until today many molecular-phylogenetic studies have been done to resolve generic delimitation and infrageneric classifications of many groups of Anthemideae (Watson *et al.,* 2000; Masuda *et al.,* 2009; Zhao *et al.,* 2010; Sonboli *et al.,* 2011; Sonboli *et al.,* 2012). *Tanacetum* is considered to be one of the most problematic genera and the phylogenetic position of some taxa is of great interest because of the high morphologic diversity and taxonomical complexity. *Tanacetum* which is a polymorphic genus is described to have important variation in flowers, inflorescence morphology, and achenes (Sonboli *et al.,* 2012). Among the published studies, Sonboli *et al.,* (2012) was studied the phylogenetic position of 80 *Tanacetum* taxa within the tribe.

The use of the internal transcribed spacer of the nuclear ribosomal repeat (nrDNA ITS) region in plant molecular systematics has been reviewed by Baldwin *et al.,* (1995). The entire ITS region is now a widely used data source in molecular systematic studies of plants at lower taxonomic levels for three principal reasons. First, the high copy number allows easy amplification of the region from total DNA. Second, the spacer sequences evolve rapidly and can therefore resolve lower level relationships better than slowly evolving genes, such as 18S and *rbc*L (Baldwin, 1992; Baldwin *et al.,* 1995; Baker *et al.,* 1999). Third, the availability of several sets of universal (or near so) PCR primers working with a large diversity of taxonomic groups is easy (White *et al.,* 1990; Gardes & Bruns, 1993). In addition to nrDNA sequences, noncoding chloroplast sequences as the *trnL* (UAA) intron and the intergenic spacer between the *trnL* (UAA) 3' exon and the *trnF* (GAA) gene also have phylogenetic potential (Taberlet *et al.,* 1991).

The aim of this study is to provide first report on the systematic position of *T. heterotomum*, *T. cadmeum* subsp. *orientale*, *T. cappadocicum*, and *T. eginense* which have been not included in any molecular phylogenetic analysis yet. Among these *T. heterotomum* and *T. cadmeum* subsp. *orientale* are endemic taxa to Turkey (Davis, 1982). In this study, we used molecular data from entire nrDNA ITS region and we further included sequence information from the cpDNA non-coding regions *trnL* (UAA) intron, intergenic spacer between the *trnL* (UAA) 3' exon and *trnF* (GAA) to provide a more comprehensive taxonomic and phylogenetic results and a more stable classification.

Material and Methods

Plant material: Plant material was obtained from silica-gel dried leaved of collected specimens in the wild. *T. abrotanifolium* was collected from natural habitats in Sancak (Bingol), 2008, *T. argenteum* subsp. *argenteum* was collected from (Harput) Elazig, 2008, *T. balsamita* subsp. *balsamita* was collected from Metan village (Bingol), 2014, *T. cappadocicum* was collected from Munzur valley (Tunceli), 2008, *T. cadmeum* subsp. *orientale* was collected from Kayalık Village-Palu (Elazig), 2013, *T. cilicicum* was collected from Saban village (Bingol), 2014, *T. chiliophyllum* var. *chiliophyllum* was collected from Sancak (Bingol), 2013, *T. chiliophyllum* var. *chiliophyllum* was collected from Sivrice (Elazig), 2008, *T. densum* subsp. *amani* was collected from Tecer mountain (Sivas), 2008, *T. eginense* was collected from Darende (Malatya), 2008, *T. heterotomum* was collected from Kangal (Sivas), 2008, *T. kotschyi* was collected from Şaban village (Bingol), 2014, *T. mucroniferum* was collected from Munzur valley (Tunceli), 2008, *T. nitens* was collected from Baskil (Elazig), 2008, *T. parthenium* was collected from Pınaraltı village (Bingol), 2013, *T. parthenifolium* was collected from Saban village (Bingol), 2014, *T. vulgare* was collected from Munzur mountain (Tunceli), 2008, *T. zahlbruckneri* was collected from Saban village (Bingol), 2014. The plant materials were identified by Dr. A. Kocak. Voucher specimens were deposited at the Molecular Biology and Genetics Laboratory of Bingol University and Plant Products and Biotechnology Research Laboratory of Firat University.

DNA extraction, amplification, and sequencing: Total genomic DNA was extracted by modified protocol of the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). Polymerase chain reaction (PCR) of the whole region of nrDNA ITS were performed using the ITS AB101 and ITS AB102 primers (Douzery *et al.,* 1999) and ITS4 and ITS5 primers (White *et al.*, 1990) in some cases. PCR amplifications were conducted according to the protocols described in Sonboli *et al.*, (2010). Amplification of intergenic spacer *trnL* (UAA) intron (B49317 and A49855 primers) and intergenic spacer between the *trnL* (UAA) 3' exon and *trnF* (GAA) (B49873 and A50272 primers) were performed according to the protocols of Taberlet *et al.,* (1991). Sequencing reactions were performed using ABI 3730 XL (Applied Biosystems).

Alignment and phylogenetic analyses: Phylogenetic analysis were undertaken using three data sets of samples and each included the sequences from the GenBank database of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) were aligned using ClustalW (Thompson *et al.,* 1994) software and subsequently checked visually. Indels were not treated in final datasets. Ultimately, evaluation carried out by grouping the data into three sets as nrDNA and two regions from cpDNA. The first dataset was comprised of both studied taxa ITS (ITS 1, 5.8S and ITS 2) sequences and the ITS sequences from closely related taxa retrieved from the NCBI database (Table 1). The second dataset was composed of the sequences of the species of the genera of *trnL* from current study, the third and the last dataset included both *trnL-F* and the GeneBank sequences.

Variable sites, number of parsimony-informative sites, transition, transversion, genetic distance, nucleotide diversity, and divergence within species were computed as molecular diversity statistics for each dataset using Molecular Evolutionary Genetics Analysis software (MEGA 6.0; Tamura *et al.*, 2013). Ultimately, pylogenetic tree was constructed by Maximum Likelihood Method with 1000 bootstrap replicates.

Table 1. Accession number from the NCBI database (out group).

Specimens	Internal transcribed spacer (ITS)	*trnL*	*trnL-F*
T. millefolium	AY603263	-	-
T. vulgare	EF577323	-	EF577378
T. vulgare	AY603264	-	-
T. cinerariifolium	EF577319	-	EF577374
T. macrophyllum	AY603262	-	-
T. ptarmiciflorum	EF577322	-	EF577377
T. parthenium	EF577320	-	EF577375
Achillea biebersteinii	AY603218	-	-
Achillea millefolium subsp. *sudetica*	AY603187	-	-
Achillea millefolium	AY603186	-	-
Anthemis arvensis	EU179214	-	-
Anthemis melampodia	KJ004380	-	-
Anthemis cotula	EU179216	-	-

Table 2. Numeric information of ITS, *trnL* and *trnL-F*.

	ITS	*trnL*	*trnL-F*
Length of the aligned sequence (including all taxa with out group)	826	555	514
GC% content (including all taxa with out group)	50.4	34.1	36.8
Parsimony informative sites (including all taxa with out group)	118	17	20

Results

The characteristics of sequences: The aligned data set of entire ITS, *trnL* and *trnL-trnF* included a total of 37 (10), 26 and 29 (4) taxa. The number in the parenthesis indicates the taxa taken from GenBank. ITS, *trnL* and *trnL-trnF* sequences length varied from 826, 555, and 514 respectively. The aligned data comprised of 593bp from nrDNA, 418 and 337bp from cpDNA without gaps and missing data including the taxa taken from GenBank and all data sets comprised different numbers of taxa from five different genera (*Tanacetum*, *Anthemis*, *Achillea*, *Bellis* and *Paeonia*) including outgroup taxa. The parsimony informative sites were 118 for nuclear DNA gene region, 17 for trn*L* and 20 for *trnL-trnF* of cpDNA (Table 2). Considering *Tanacetum* taxa without other taxa parsimony informative sites were 43, 8 and 5 respectively.

The evolutionary characteristics: According to the three phylogenetic trees, constructed by using both chloroplast and nuclear DNA data showed that all taxa of *Tanacetum* supported as a monophyletic group, but basically two main clades were observed in the *Tanacetum* taxa (Figs. 1, 2 and 3). One clade, including most specimens of *Tanacetum* especially the taxa *T. eginense*, *T. cadmeum* subsp. *orientale* and *T. cappadocicum*, the other clade comprised of three subclades with *T. heterotomum* according to the data based upon the nuclear DNA (Fig. 1). On the other hand, based upon the chloroplast DNA data, all four taxa were located in the same clade (Fig. 2). In Flora of Turkey, members of *Tanacetum* fall into three main clades (Group A, B and C) according to the gender status of the capitula and colour of the flowers. It is clearly seen on phylogenetic tree obtained from ITS data that Clade 1 composed of mainly Group B members, on the other hand, Clade 2 encompassed Group A *Tanacetum* members.

Discussion

In this study identification of molecular systematic position of *T. heterotomum, T. cappadocicum, T. eginense* and *T. cadmeum* subsp. *orientale* taxa was done by using nrDNA and cpDNA sequence analysis. Whereas these taxa were simply classified by their capitulum sexual status, ligular and tubular flower structure and leaf fragmentation characters. According to Flora of Turkey *T. heterotomum* is in the group A, *T. cappadocicum, T. eginense* and *T. cadmeum* subsp. *orientale* are in the group B (Davis, 1982).

The results of nuclear DNA analysis correspond to the group separation in Flora of Turkey. Phylogenetic trees which are based upon the nuclear DNA show that basically two main clades are formed among taxa of *Tanacetum* and they were collected together and separated from outgroup taxa completely. While first clade includes three species; *T. eginense, T. cadmeum* subsp. *orientale* and *T. cappadocicum*, the other clade includes *T. heterotomum* (Fig. 1). These results show conformity to the groups separation based on morphological charters in Flora of Turkey (Davis, 1982). However, according to the one type of chloroplast marker (cpDNA *trnL*), all four species were located in the same branch and this result was not compatible with the group recognized in Flora of Turkey (Fig. 2). These analysis results on the other hand don't comply with the groups recognized in Flora of Turkey based on morphological charters (Davis, 1982).

On the other hand with the other chloroplast marker (cpDNA *trnL-F*) analysis shows that *T. cappadocicum, T. eginense* and *T. cadmeum* subsp. *orientale* are in the same clade but *T. heterotomum* could not be evaluated because PCR studies with primers for *trnL-F* region didn't work (Fig. 3).

T. balsamita subps. *balsamita* which is defined in both group A and group C according to the Flora of Turkey, located among the taxa with heterogamous capitulum (group A). Also it is obvious that the genus *Tanacetum* is closely related to the genera *Achillea*, and *Anthemis*.

Sonboli *et al.*, (2012) studied molecular phylogeny of *Tanacetum* genus and based on the nrDNA (ITS), cpDNA (*trn*H-*pbs*A) sequence variation they introduced the infrageneric taxonomy and Bayesian tree of *Tanacetum*. In addition to Sonboli *et al.*, (2012), with this study the position of *T. heterotomum* (endemic), *T. cadmeum* subsp. *orientale* (endemic), *T. cappadocicum*, and *T. eginense* in the phylogenetic tree of *Tanacetum* was determined for the first time.

In conclusion, dendrograms drawn by the results of both nrDNA (ITS) and cpDNA (*trnL* and *trnL-F*) sequence analysis put forth immense diversity. In future detail evaluation with other variable markers between all members of the *Tanacetum* genus and with closely related genera will make this study more meaningful.

Acknowledgement

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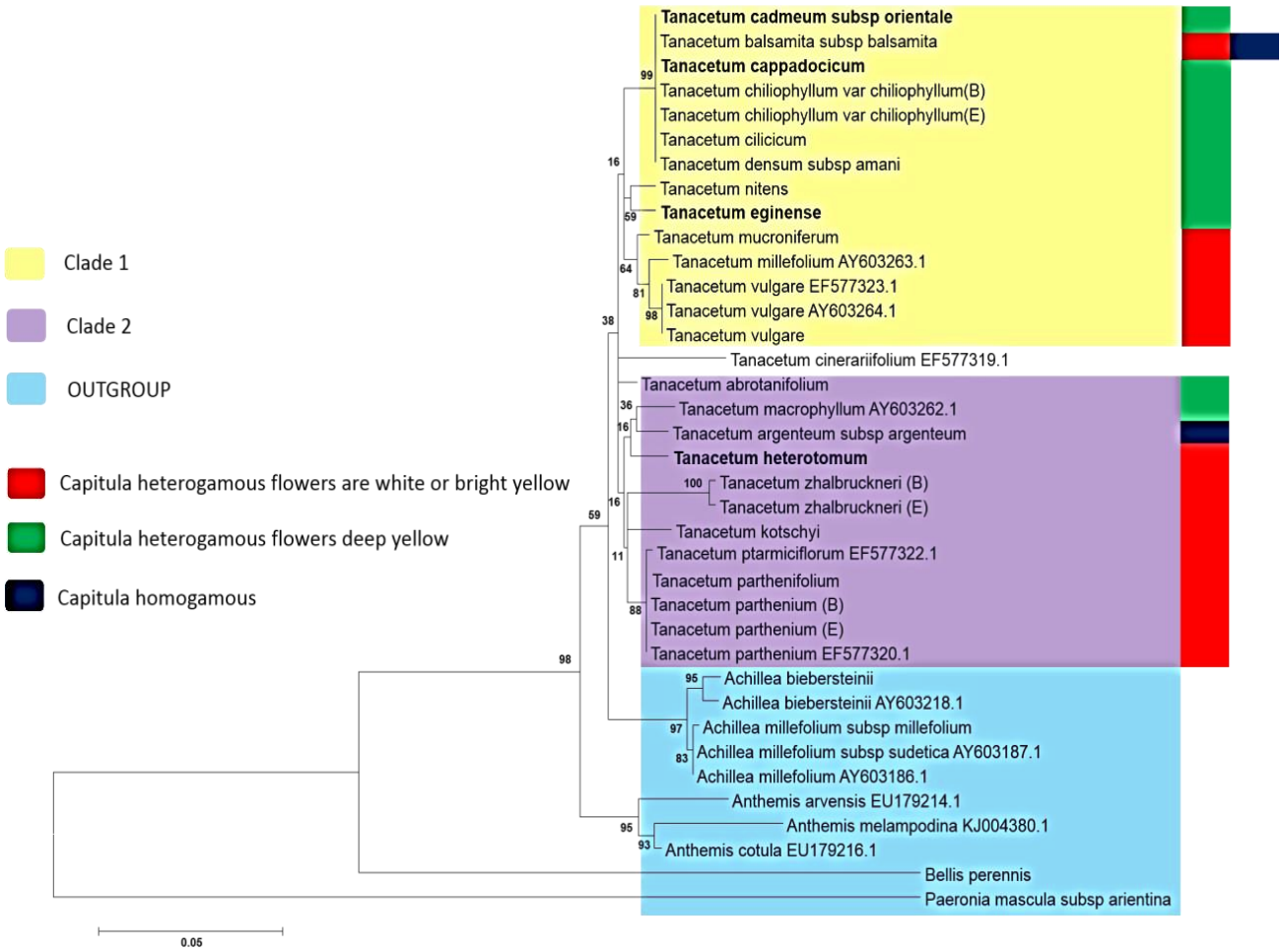

Fig. 1. Maximum Likelihood tree based upon the Tamura-Nei model of nrDNA ITS region with 1000 bootstrap replicates. Bootstrap values are represented next to the each node of the branches. Important clades and flower information are indicated in different colours on the tree.

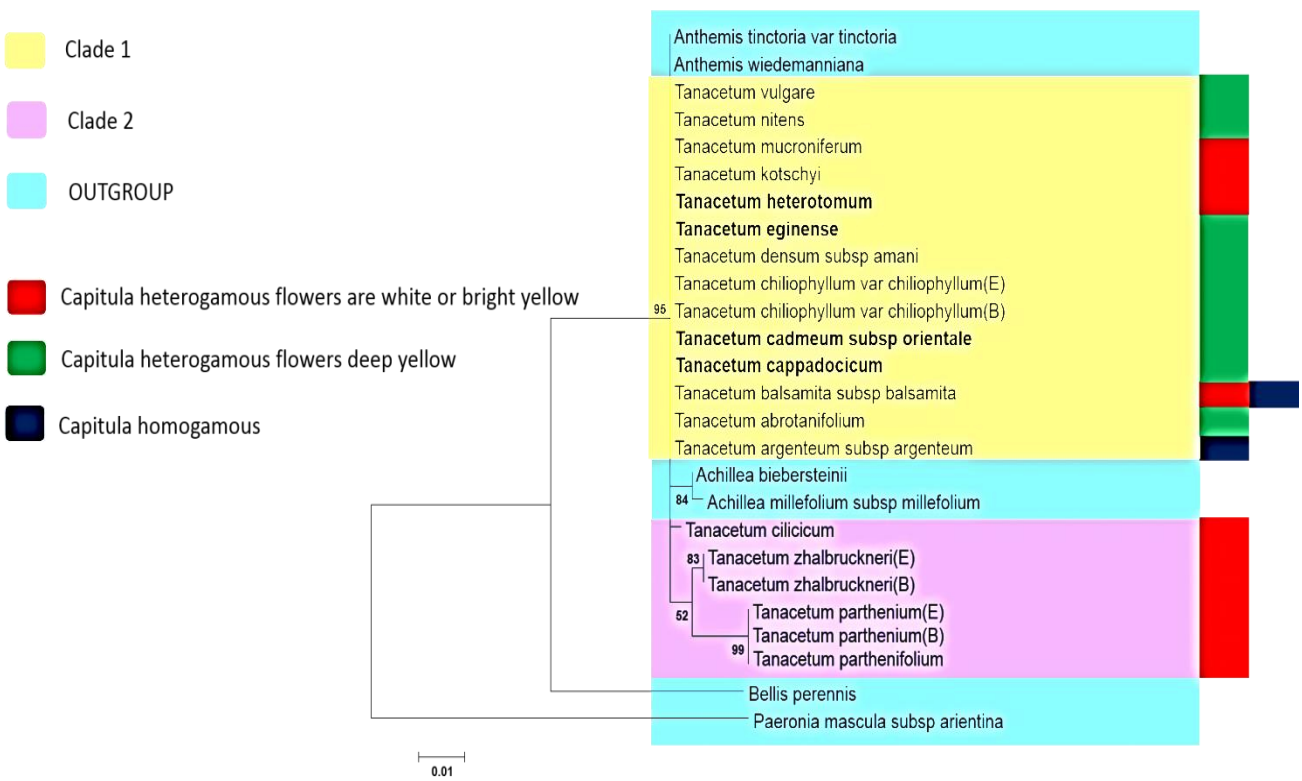

Fig. 2. One of the cpDNA, based Maximum Likelihood method tree specifically the sequence data of *trnL* region.

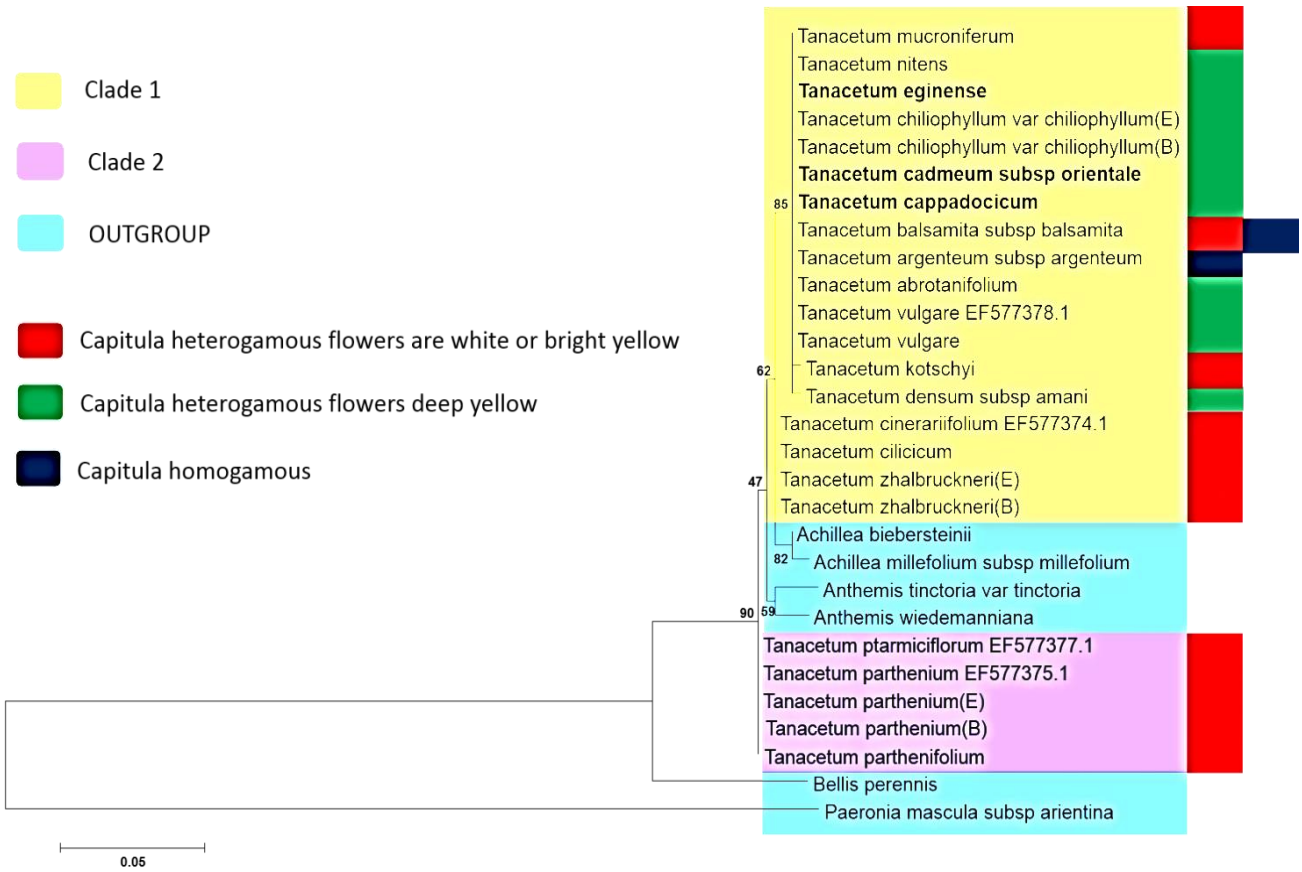

Fig. 3. Maximum Likelihood cpDNA tree based on the data obtained from the sequences of the *trnL-trnF*.

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