

MOLECULAR AUTHENTICATION OF THYMUS PERSICUS BASED ON nrDNA ITS SEQUENCES DATA

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Thymus persicus is an endemic species of the genus *Thymus* (Lamiaceae) which grows in Zanzan and West Azerbaijan (Takab) provinces of Iran. Among the Iranian *Thymus* species, *T. persicus* is well differentiated by having the smallest leaf width among *Thymus* species with long non-glandular and short glandular hairs. In order to evaluate the phylogenetic relationships of the *T. persicus* and *T. marandensis* Jamzad, the recently and assumed related species to *T. brachychilus* Jalas and *T. leucotrichus* Hal., a molecular analysis based on nrDNA ITS sequences of 25 accessions belonging to 19 species of *Thymus* together with representatives of genera *Origanum*, *Thymbra*, *Satureja*, *Micromeria*, *Gontscharovia*, *Ziziphora*, *Saccocalyx* and *Zataria* was performed. The results of phylogenetic analysis showed that the genus *Thymus* is paraphyletic when the monotypic genus *Saccocalyx* is included and its sister relationship with *Origanum* was confirmed. While the phylogenetic position of *T. persicus* among the other taxa of *Thymus* is appeared unresolved. *T. marandensis* turned out to be the sister to a group of *Thymus* species including *T. carmanicus*, *T. migricus*, *T. pubescens*, *T. trautvetteri* and *T. daenensis*.

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شناسایی و تایید مولکولی آویشن ایرانی (*Thymus persicus*) بر اساس توالی‌های nrDNA ITS

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آویشن ایرانی (*Thymus persicus*) یکی از گونه‌های انحصاری جنس *Thymus* است که در استانهای زنجان و آذربایجان غربی (تکاب و حومه) ایران رویش دارد. این گونه با داشتن برگ‌های باریک خطی شکل با کرک‌های غیرغده‌ای بلند به همراه کرکهای غده‌ای تک سلولی کوتاه در بین سایر گونه‌های جنس آویشن به خوبی متمایز است. برای تعیین جایگاه تبارزایی گونه آویشن ایرانی و روابط خویشاوندی آن با سایر گونه‌ها و تایید مولکولی این گونه، 25 نمونه متعلق به 19 گونه از جنس *Thymus* به همراه گونه‌هایی از جنس‌های خویشاوند *Origanum*, *Satureja*, *Thymbra*, *Micromeria*, *Ziziphora*, *Gontscharovia*, *Saccocalyx* و *Zataria* انتخاب و آنالیز تبارزایی بر اساس توالی‌های nrDNA ITS انجام شد. کلاودوگرام حاصل از آنالیز بی‌زین نشان داد که جنس آویشن با در نظر گرفتن جنس *Saccocalyx* پیرا تبار است و رابطه خویشاوندی خوهری آن با جنس *Origanum* تایید شد. گونه *T. persicus* با وجود صفات متمایز کننده خاص به همراه سایر گونه‌های این جنس در کلاذ تک تبار *Thymus* بصورت پلی‌تومی و حل نشده ظاهر شد. گونه *T. marandensis* به عنوان یک گونه خوهری با برخی از گونه‌های این جنس از جمله *T. daenensis*, *T. migricus*, *T. pubescens* و *T. trautvetteri* قرار گرفت.

INTRODUCTION

The genus *Thymus* L. belongs to the *Nepetoideae* subfamily of *Lamiaceae* family and comprising about 220 species that are widely distributed all over the world. The Mediterranean region is considered being the center of diversification of the genus. *Thymus* species have been used for many centuries in traditional medicine for their antiseptic, carminative, antimicrobial, antiviral and antioxidant properties (Morales 2002). The genus *Thymus* is represented in flora of Iran by 18 species of which four are endemics (Jamzad, 2012). *Thymus persicus* (Ronniger ex Rech. f.) Jalas is an endemic species which is distributed in restricted regions of the northwest of Iran including Zanzan and West Azerbaijan provinces (Rechinger, 1982; Jamzad, 2009). Bacterial susceptibility and chemical composition of the essential oil of *T. persicus* have already been reported (Rasooli & Mirmostafa, 2003). The main chemical constituents of the essential oil of *T. persicus* have been reported to be thymol and carvacrol in different quantities (Sefidkon & al., 2002). Morphologically, *T. persicus* is well differentiated in the genus *Thymus* by the smallest leaves width among *Thymus* species with long non-glandular and short-stalked glandular hairs (Fig. 1). *Thymus marandensis* Jamzad has been described from northwest of Iran, East Azerbaijan, Marand and assumed to be closely related to *T. brachychilus* Jalas. and *T. leucotrichus* Halácsy (Jamzad, 2009).

Recently, there have been an increasing number of molecular studies in *Nepetoideae* with focus on the selected genera, e.g. *Nepeta* L. (Jamzad & al., 2003), *Mentha* L. (Bunsawat & al., 2004), *Micromeria* L. (Bräuchler & al., 2005) and *Salvia* L. (Walker & al., 2004; Walker & Sytsma, 2007) and some investigation at the subtribal level such as *Menthinae* (Bräuchler & al., 2010) with emphasize on the phylogenetic reconstruction. Therefore, in the present study nrDNA ITS sequences were used for authentication of *T. persicus* and its phylogenetic position and relationships with other *Thymus* species especially *T. marandensis*.

Materials and methods

Taxon sampling

The present dataset consists of 36 taxa (42 accessions), of which 25 accessions belonging to the genus *Thymus* and some representatives of the genera *Origanum* L. (4), *Satureja* L. (2), *Thymbra* L. (4), *Micromeria* (2), *Zataria* L. (1), and *Gontscharovia* Tzvelev (1). Four taxa of *Thymus* and altogether five accessions are newly generated in the present study. Two species of the genus *Ziziphora* L. were chosen as outgroup (Bräuchler & al., 2010). Information concerning voucher specimens or already published sequences is

given in Table 1.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted either from fresh leaves of cultivated specimens in the field or herbarium specimens following a manufacturer's protocols of the Bioflux plant DNA extraction kit (China). Polymerase chain reaction (PCR) amplifications of the nrDNA ITS region were performed using the primers ITS4 and ITS5A (White & al., 1990) PCR amplifications were conducted according to the following protocol described in Sonboli & al. (2010). The PCR products were purified and used for sequencing reaction. Forward and reverse sequencing of the nrDNA ITS fragments was performed in an ABI sequencer. The new sequences of nrDNA ITS were submitted to the DDBJ sequence data bank (see Table 1 for accession numbers).

Sequence alignment and phylogenetic analyses

The DNA sequences obtained were carefully checked for the presence/absence of polymorphic sites using Chromas and Sequencher ver. 4.9. The IUPAC (International Union of Pure and Applied Chemistry) ambiguity code was used to indicate nucleotide polymorphisms. Sequences were aligned using Clustal W (Thompson & al., 1994) as implemented in BioEdit version 7.05.2 (Hall, 1999) and the alignment was optimized manually. Gaps were treated as missing data. The maximum parsimony (MP) analysis of the nrDNA ITS dataset was performed using the heuristic search algorithm of PAUP* 4.0 version beta 10 (Swofford, 2002), with ACCTRAN, MULPARS, tree bisection–reconnection (TBR) branch swapping, and random addition sequence replicates. Character states were treated as unordered and unweighted. To test the robustness of individual branches, bootstrap (BS) values (Felsenstein, 1985) were calculated using heuristic searches with 1,000 bootstrap replicates, each with a simple addition sequence, ACCTRAN, TBR branch swapping, MULPARS, and saving 10 trees per replicate.

The Bayesian inference (BI) phylogenetic analysis of the nrDNA ITS dataset was carried out with MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). A SYM+G model was selected based on the results of the program MrModeltest 2.3 (Nylander 2004) as implemented in MrMTgui (Nuin, 2005) and using the Akaike information criterion for model comparisons (Posada & Buckley, 2004). The analysis was conducted using two parallel searches with four MCMC chains each and was run for a total of 3,000,000 generations with trees sampled at every 100th generation. To ensure approximation to stationary, we checked that the



Fig.1. *Thymus persicus* in its natural habitat (Takab: Baderlu). Scale bar=1 cm.

average standard deviation of split frequencies was approaching a value of 0.01 as suggested by Ronquist & al. (2005). After discarding the first 25% of trees as burn-in, a 50%-majority-rule consensus tree of the remaining trees was computed accompanied with posterior probability (PP) values for individual clades. Tree visualization was carried out using Tree View version 1.6.6 (Page, 2001).

Results AND Discussion

The aligned nrDNA ITS dataset consisted of 634 characters, of which 139 nucleotide sites were parsimony informative. Of the remaining characters, 432 and 63 were constant and variable, respectively. The MP analysis resulted in 180 shortest trees of 366 steps length, a consistency index (CI) of 0.719, and retention index (RI) of 0.858. The strict consensus tree of the MP analysis (tree not shown), is topologically similar to that of the Bayesian analysis (Fig. 2). In the present study, the paraphyly of the genus *Thymus* is indicated with respect to the other taxa, when the monotypic genus *Saccocalyx* along with two species of the genus *Thymus* (*T. saturejoides* and *T. sespitiatus*) are included. This is in agreement with the results of Bräuchler et al., 2010.

Fifty percent majority rule consensus tree obtained from nrDNA ITS sequences data using Bayesian method in *Thymus* and relative genera revealed two main sister clades, one clade encompassed the genus *Saccocalyx* and *T. saturejoides* and *T. caespititius* with 0.91 posterior probability support and the other clade included all *Thymus* samples (PP=1.0; BS=78) (Fig. 2). Within the core-group of *Thymus*

included in our present analysis some species groups such as *T. vulgaris*, and *T. marandensis* and its relatives received high statistical support (PP=1.0; BS=93 and BS=84, respectively), while the overall phylogenetic relationships between the most species remained largely unresolved.

The sister-group relationship position of the genus *Origanum* with *Thymus* suggested by Bräuchler & al., (2010) is in agreement with our results. The position of the recently described endemic *T. marandensis* Jamzad in the reconstructed molecular phylogeny could not be effectively inferred because of lack of sampling of Turkish *Thymus* taxa (*T. brachyichilus* and *T. leucotrichus*) which were assumed to be closely related to it. They belong to *Thymus* section *Hyphodromi* (A. Kerner) Halácsy subsection *Subbracteati* (Klokov) Jalas. The species belonging to this section and its subsequent subsection are characterized by more or less revolute or convolute leaves, holotrichous stems and inflorescence frequently capituliform with bracts different from the leaves. From the species belonging to this subsection, *Thymus marandensis* has morphological similarities with Turkish taxa mentioned above, i. e. leaf shape and indumentum, habit and stem indumentum.

However, based on examined taxa its phylogenetic position was found to be closely related to a species group including: *T. migricus*, *T. pubescens*, *T. daenensis*, *T. carmanicus* and *T. trautvetteri*. It should be admitted that more sampling of the Iranian and Turkish *Thymus* species may provide a better resolved tree and define the phylogenetic relationships of species. Meanwhile, representative species from

Table 1. List of selected taxa used in the current phylogenetic analysis and GenBank accession numbers of ITS. The specimens from which DNA was extracted for the present study and newly generated sequences are indicated with an asterisk.

Taxa	Geographic source	Reference	GenBank accession number
<i>Gontscharovia popovii</i> (B. Fedtsch. & Gontsch.) Boriss.	Tajikistan	Brauchler et al., 2010	GU381439
<i>Micromeria benthami</i> Webb & Berthel.	Spain	Brauchler et al., 2010	GU381446
<i>M. hyssopifolia</i> Webb & Berthel.	Spain	Brauchler et al., 2010	GU381448
<i>Origanum elongatum</i> (Bonnet) Emb. & Maire	Morocco	Brauchler et al., 2010	GU381465
<i>O. microphyllum</i> (Benth.) Vogel	Greece	Brauchler et al., 2010	GU381467
<i>O. rotundifolium</i> Boiss.	Armenia (cult.)	Brauchler et al., 2010	GU381463
<i>O. vulgare</i> L.	Germany	Brauchler et al., 2010	GU381469
<i>Saccocalyx saturejoides</i> Coss. and Dur.	Algeria	Brauchler et al., 2010	GU381462
<i>Satureja linearifolia</i> (Brullo & Furnari) Greuter	Libya	Brauchler et al., 2010	GU381455
<i>S. thymbra</i> L.	Greece	Brauchler et al., 2010	GU381441
<i>Thymbra calostachya</i> (Rech.f.) Rech.f.	Greece	Brauchler et al., 2010	GU381452
<i>T. capitata</i> L.	Spain (cult.)	Brauchler et al., 2010	GU381453
<i>T. sintenisii</i> Bornm. & Azn.	Turkey	Brauchler et al., 2010	GU381451
<i>T. spicata</i> L.	Israel	Brauchler et al., 2010	GU381456
<i>Thymus amurensis</i> Klovov	China	Quan et al., 2008	EU556515
<i>T. broussonetii</i> Boiss.	Morocco	Brauchler et al., 2010	GU381458
<i>T. caespititius</i> Brot.	Madeira	Brauchler et al., 2010	GU381457
<i>T. carmanicus</i> Jalas	Iran: Kerman	This study	submitted*
<i>T. daenensis</i> Celak.	Iran: East Azerbaijan	Abdolahinia et al., 2011	FJ236467
<i>T. dahuricus</i> Serg.	China	Quan et al., 2008	EU556511
<i>T. magnus</i> (Nakai) Nakai	Korea	Kim et al., 2003	AY443446
<i>T. magnus</i> (Nakai) Nakai	Korea	Kim et al., 2003	AY443448
<i>T. mandschuricus</i> Ronniger	China	Quan et al., 2008	EU556512
<i>T. marandensis</i> Jamzad	Iran: Marand	This study	submitted*
<i>T. mastichina</i> (L.) L.	-	Prather et al., 2002	AY029168
<i>T. mongolicus</i> (Ronniger) Ronniger	China	Quan et al., 2008	EU556509
<i>T. mongolicus</i> (Ronniger) Ronniger	China	Quan et al., 2008	EU556510
<i>T. persicus</i> (Ronniger ex Rech. f.) Jalas	Iran: Ardabil	Abdolahinia et al., 2011	EU735058
<i>T. persicus</i>	Iran: Zanjan	This study	submitted*
<i>T. persicus</i>	Iran: Takab	This study	submitted*
<i>T. pubescens</i> Boiss. & Kotschy ex Celak.	Iran: Ardabil	Abdolahinia et al., 2011	EU374715
<i>T. pulegioides</i> L.	-	Drew & Sytsma 2012	JQ669138
<i>T. quinquecostatus</i> Celak.	Korea	Kim et al., 2003	AY443436
<i>T. quinquecostatus</i> Celak.	Korea	Kim et al., 2003	AY443440
<i>T. saturejoides</i> Coss.	Morocco	Brauchler et al., 2010	GU381460
<i>T. serpyllum</i> L.	-	Kersten & Knoess 2008	EU796890
<i>T. trautvetteri</i> Klovov & Des.-Sosht.	Iran: East Azerbaijan	Abdolahinia et al., 2011	EU735059
<i>T. vulgaris</i> L. (MPH)	Iran: Tehran (cult.)	This study	submitted*
<i>T. vulgaris</i> L.	France (cult.)	Trusty et al., 2003	AY329369
<i>Zataria multiflora</i> Boiss.	Afghanistan	Brauchler et al., 2010	GU381450
<i>Ziziphora clinopodioides</i> Lam.	Turkey	Brauchler et al., 2010	GU381386
<i>Z. hispanica</i> L.	Morocco	Brauchler et al., 2010	AF369162

different sections of *Thymus* may provide different topology which will give us better result to infer the relationship of species.

While two accessions of *T. persicus* (Takab and Zanjan) analyzed in the present investigation occupy an unresolved position in the *Thymus* clade,

another specimen of *T. persicus* collected from Ardabil province which has been analyzed in a previous work (Abdolahinia & al., 2011) and its sequence was retrieved from GenBank (EU735058) is nested in a well supported clade composed of *T. migricus* and other relatives (Fig. 2). These species belong to sect.

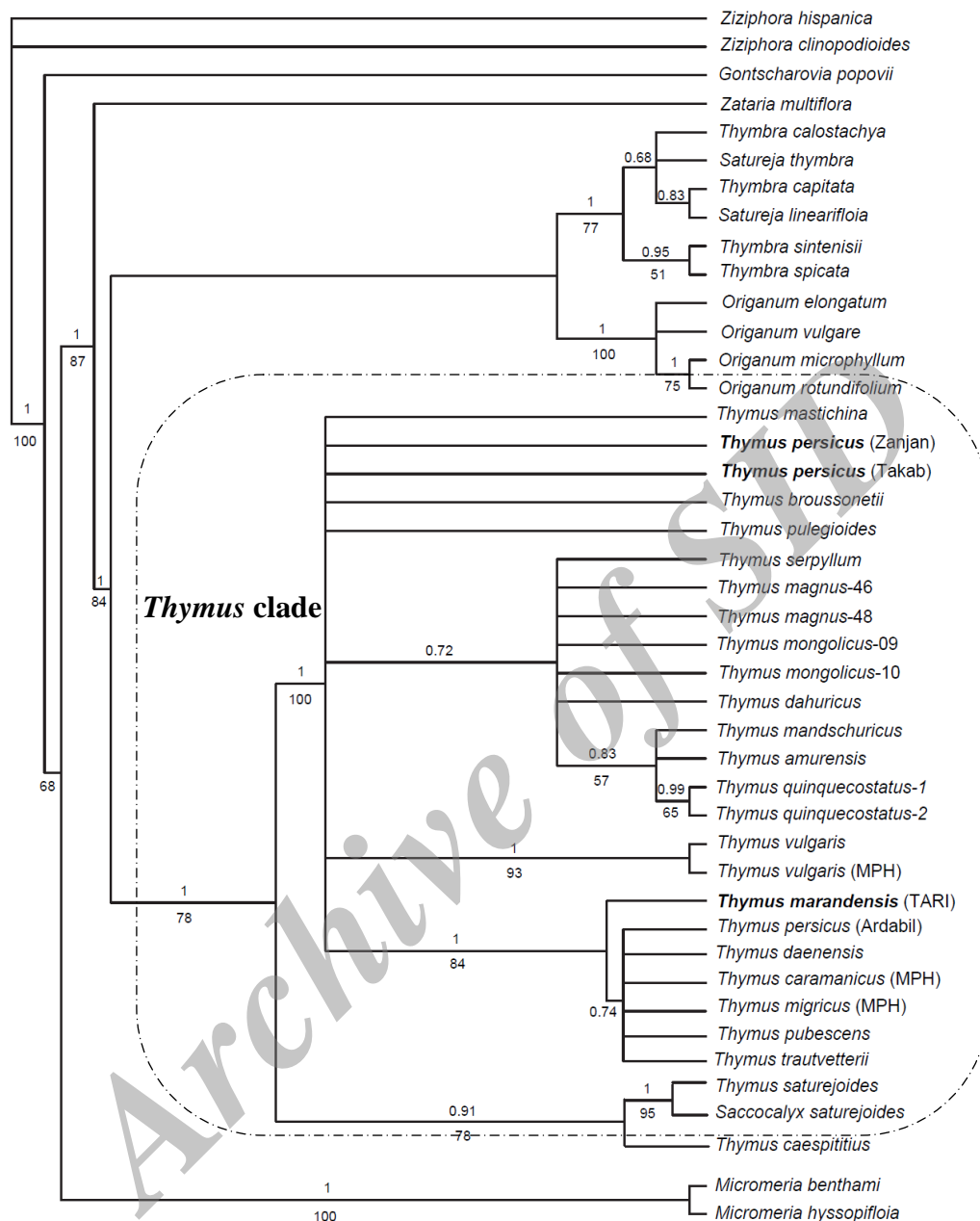


Fig. 2. Fifty percent majority rule consensus tree obtained from nrDNA ITS sequences data using Bayesian method in *Thymus* and relative genera. Posterior probabilities are presented on the branches. Bootstrap values are indicated under the branches.

Serpyllum (Miller) Benth. subsect. *Kotschyani* (Klokove) Jlas. They are characterized by having a woody habit or woody only at base, with holotrichous, goniotrichous or alelotrichous stems, flat leaves, usually ciliate at the base, with distinct lateral veins; inflorescence spike like or more or less globose. According to the phylogenetic position of *T. persicus*

(Ardabil) it could be considered as misidentification of plant species as it can also be observed from the image provided by the authors (Abdolahinia & al., 2011, Fig. 1-B), the specimens does not belong to *T. persicus*, the leaf shape (ovate, flat) is quite different and resembles more to the species belonging to sect. *Serpyllum* subsect. *Kotschyani*. This is more justified when we

consider the *T. persicus* geographical distribution which is far from Ardabil and restricted to West Azerbaijan and Zanjan provinces. Regarding to misidentification of the plant specimen (Abdolahinia & al., 2011), the subsequent sequencing does not reflect the actual case for the *T. persicus*. Sequence alignments indicated the existence of three nucleotide changes in Zanjan and Takab accessions of *T. persicus* in the ITS1 region (C40, C41 and C110) vs. Ardabil accession (A40, A41 and T110 with reference to sample EU735058). These differences along with morphological features can be used for differentiating the correct identified *T. persicus* from its adulterants. It should be admitted that in DNA barcoding projects and submitting DNA sequences to GenBank an authentication of identification of plant specimen should be considered, otherwise we will have a conflicting data source which will be problematic for our future works.

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