



## RESEARCH PAPER

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## A phylogenetic analysis of *Hyoscyamus* L. (Solanaceae) species from Iran based on ITS and *trnL*-F sequence data

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### Abstract

A phylogeny of *Hyoscyamus* L. (Solanaceae) that represents two different subgenera (i.e. *Hyoscyamus* subgen. *Dendrotrichon* SchÖnbeck-Temesy and *Hyoscyamus* subgen. *Hyoscyamus*) based on ITS and *trnL*-F sequence data were studied. In this study five species of *Hyoscyamus* were Iran endemic. ITS and *trnL*-F sequence data for 18 species of this genus from Iran were studied. In addition we used 8 ITS sequence data and 13 *trnL*-F sequence data from genbank for another taxa in Solanaceae family. For phylogenetic analysis were used Bayesian and Maximum parsimony methods. Parsimony analyses of the ITS and *trnL*-F regions formed a dendrogram with strong bootstrap (ca. ≥70%) that showed *Hyoscyamus* is a monophyletic group. In this study four synonymous species (*H.insanus* Stocks, *H.rosularis* SchÖnbeck-Temesy, *H.orthocarpus* SchÖnbeck-Temesy, *H.nutans* SchÖnbeck-Temesy) were stand in one clade and two similisar species *H.bornmulleri* Khatamsaz and *H.tenuicaulis* SchÖnbeck-Temesy constituted one clade. Also two species *H.senecionis* Willd and *H.pusillus* L. were formed one clade in *trnL*-F and combined analyses

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## Introduction

The Solanaceae are one of the most important family of flowering plants economically, floristically, entomobotanically and scientifically. *Hyoscyamus* belongs to Solanaceae that has 94 genera and 2950 species, which are mostly distributed in tropical lands of America (Mabberley, 1987; Willis, 1973). This genus belongs to subfamily solanoideae, tribe Hyoscyameae and order Solanales (Olmstead *et al.*, 2008; TU *et al.*, 2010; APGII, 2003). In traditional classification Solanaceae included two subfamily such as Solanoideae and Cestroideae (D'Arcy, 1979, 1991; Hunziker, 1979, 2001; Olmstead and Palmer, 1992). Some species of this genus such as *H.niger* L. is cosmopolitan (Kouhsari *et al.*, 2006). *Hyoscyamus* is distributed in Turkey with six species, the former USSR with eight and Pakistan with four species (Baytop, 1978; Pojarkovae, 1955; Nasir, 1985) and has 18 species and two subgenus in Flora Iranica (SchÖnbeck-Temesy, 1972). *H.bornmulleri* was introduced as a new species from Shiraz (Khatamsaz, 1377). Most of the species of this genus belong to Iranian-Turanian area and some belong to European-Siberian and Sahara-Sandy. Distribution of various species of this genus in most provinces of Iran indicated that this genus is adaptable to different climates. Six species of this genus are Iran endemic and two species are plateau Iran endemic. (SchÖnbeck-Temesy, 1972). In Flora of Iran *H.insanus* from south, south east and center of Iran is synonymous with *H.orthocarpus*, *H.rosularis* and *H.nutans* but in Flora Iranica *H.orthocarpus* from west and south, *H.nutans* from south, *H.rosularis* from center and *H.insanus* from south of Iran are reported as separate species and three species *H.orthocarpus*, *H.rosularis* and *H.nutans* are Iran endemic but *H.insanus* is plateau Iran endemic (Khatamsaz, 1377; SchÖnbeck-Temesy, 1972). We use phylogenetic analysis for survey relationship of Iranian species of *Hyoscyamus* in this study.

## Material and methods

### Taxon sampling

Samples were collected in the field and dried in silica

gel or taken from the herbarium material deposited at TARI (Table1). Also the list of some taxa of Solanaceae for ITS and *trnL-F* sequence data with genbank accession number are given in table 2.

### DNA extraction, amplification and sequencing

Total DNA was extracted using the DNeasy plant Mini kit (zoofa gene). Amplification of the ITS region used forward primer ITSleu1(5'-GTCCACTGAACCTTATCATTAG-3') and reverse primer ITS4(5'-TCCTCCGCTTATTGATATGC-3') in 25 µL reactions containing the following: 12.5 µL Master mix contain of (Taq DNA polymerase, dNTP, MgCl<sub>2</sub>) 10.5 µL water, 1 µL DMSO, 0.5 µL forward primer, 0.5 µL reverse primer plus 1 µL DNA for each reaction. The PCR program used for ITS amplification was 97 C for 2 min followed by 30 cycles of 97 C for 1min, 50 C for 1min, 72 C for 45 sec, with a 3 sec extension per cycle and a single cycle of 72 C for 7 min (Bohs and Olmstead, 2001). Amplification of the *trnL-F* region used universal primers c (5'-CGAAATCGGTAGACGCTACG-3') and f (5'-ATTTGAACTGGTGACACGAG-3').

The PCR program used for *trnL-F* amplification was 94 C for 1min followed by 35 cycles 50 - 55 C for 1min, 72 C for 2 min and a single cycle of 72 C for 5 min (Taberlet *et al.*, 1991). Purification and sequencing of PCR products were performed in South Korea.

### Sequence editing, alignment and data analysis

We edited the PCR products after sequencing with Sequencher and Bioedit and MEGA 5.1(Tamura *et al.*, 2011). Then for aligning the sequence data we used the Mesquite (version2:75) and Mac clade (Maddison, DR and Maddison, 2000; Maddison, WP and Maddison, 2010). The matrix data were analyzed with two methods (Maximum parsimony and Bayesian) with PAUP\*4.0b10 (Swofford, 2002) and MrBayes 3 (Ronquist and Huelsenbeck, 2003). In parsimony analysis all characters had equal weight that implement tree- bisection - reconnection (TBR) branch swapping with 10 heuristic random addition

sequence replicates. Gaps were treated as missing data. Phylogenetic analyses of 18 species of *Hyoscyamus* in two subgenus from Iran were studied. In this study we used the ITS sequence data of two species of *Anisodus*, one species of *Atropa*, one species of *Atropanthe*, three species of *Scopolia* and *trnL-F* sequence data of five species of *Hyoscyamus*, one species of *Anisodus*, one species of *Atropa*, two species of *Physochlaina*, one species of *Przewalskia*

and two species of *Scopolia* from genbank.

### Results and discussion

In ITS and *trnL-F* the best modeltest of sequence evolution was modeltest 3.4 (GTR+I+G) with the following setting: nst=6, rates=gamma, base=est, rmat=est, pinv=est (Huelsenbeck and Crandall, 1997).

**Table 1.** List of Iranian *Hyoscyamus* species with collector name and herbarium code All collected species are saving in herbarium of research institute of forests and rangeland (TARI).

Taxon	Collector name and herbarium code
<i>H.nutans</i> SchÖnbeck–Temesy	Poormirzayi & Shakeri 4968(TARI)
<i>H.senecionis</i> Willd	Shams & Feizy 12828(TARI)
<i>H.tenuicaulis</i> SchÖnbeck–Temesy	Assadi & Aboohamzeh 38538(TARI)
<i>H.orthocarpus</i> SchÖnbeck–Temesy	Khatamsaz & Ghafari 72954(TARI)
<i>Hyoscyamus kurdicus</i> Bornm	Mozaffarian 45931(TARI)
<i>Hyoscyamus pojarkovae</i> SchÖnbeck–Temesy	Bazargan & Arazm 21735(TARI)
<i>Hyoscyamus squarrosus</i> Griff	Assadi & Mozaffarian 35824(TARI)
<i>Hyoscyamus reticulatus</i> L.	Khatamsaz & Farzaneh 73146(TARI)
<i>Hyoscyamus bornmulleri</i> Khatamsaz	Mozaffarin 71291(TARI)
<i>Hyoscyamus rosularis</i> SchÖnbeck–Temesy	Wendelbow & Foroughi 15292(TARI)
<i>Hyoscyamus malekianus</i> Parsa	Mozaffarian 53092(TARI)
<i>Hyoscyamus turcomanicus</i> Pojark	Mozaffarian 5758(TARI)
<i>Hyoscyamus leptocalyx</i> Stapf	Wendelbow & Assadi 16736(TARI)
<i>Hyoscyamus niger</i> L.	Massoumi & Hajrasouliha 97869(TARI)
<i>Hyoscyamus leucanthera</i> Bornm & Gauba	Reshinger 50924(TARI)
<i>Hyoscyamus pusillus</i> L.	Foroughi 8546(TARI)
<i>Hyoscyamus arachnoideus</i> Pojark	Khatamsaz & Farzaneh 73089(TARI)
<i>Hyoscyamus insanus</i> Stocks	Assadi 22750(TARI)

#### ITS sequence data

The ITS data included 503 characters that 247 characters constant and 256 characters were variable. In variable characters 101 characters parsimony uninformative and 155 characters were parsimony informative.

Strict consensus tree in ITS with 520 steps was included consistency index(CI)= 0.679 and the retention index (RI) = 0.766.

All the species that we studied belong to tribe

Hyoscyameae. Within the Hyoscyameae five major clades were identified (*Atropa*, *Atropanthe*, *Anisodus*, *Scopolia* and *Hyoscyamus*). Clade *Atropa* included two samples of *Atropa belladonna* with two different genbank accession numbers (PP=1, BS=100%). *Atropanthe sinensis* constituted clade *Atropanthe* (PP=0.92, BS=100%). *Anisodus loridus* constituted *Anisodus* clade (PP=0.96, BS=100%) and *Anisodus tanguticus* was outgroup. Three species of *Scopolia* such as *Scopolia lutescens*, *Scopolia parviflora* and *Scopolia carniolica* were formed a monophyletic group (PP=1, BS=100%). And all

species of *Hyoscyamus* were formed a monophyletic group (18 species; PP=1, BS=100%). *Hyoscyamus* clade included six major clades (A-F): Clade A included four species *H.insanus*, *H.rosularis*, *H.orthocarpus* and *H.nutans* from subgenus *Dendrotichon* (PP=1, BS=100%). This clade has two subclade, one subclade included three species *H.insanus*, *H.rosularis*, *H.orthocarpus* (PP=0.98, BS=80%). *H.untans* consitituted another subclade. Clade C included two species *H.bornmulleri* and *H.tenuicaulis* from subgenus *Dendrotichon* (PP=1, BS=100%). Clade F included nine species *H.senecionis*, *H.kurdicus* Bornm, *H.leucanthera*

Bornm & Gauba, *H.pojarkovae* Schönbeck-Temesy, *H.reticulatus* L., *H.squarrosus* Griff, *H.arachnoideus* Pojark, *H.pusillus*, *H.turcomanicus* Pojark from subgenus *Hyoscyamus* (PP=0.71). This clade has two subclade (FI, FII). In subclade FII six species *H.kurdicus*, *H.leucanthera*, *H.pojarkovae*, *H.reticulatus*, *H.squarrosus* and *H.arachnoideus* were formed a polytomy group (PP=0.9). Also two species *H.pusillus* and *H.turcomanicus* were formed another subclade (PP=0.9). Finally another species of *Hyoscyamus* clade such as *H.malekianus* Parsa were stand in clade B, *H.leptocalyx* Stapf in clade D, *H.niger* in clade E and *H.senecionis* in subclade FI.

**Table 2.** List of some taxa in Solanaceae with genbank accession number for ITS and trnL-F sequence data.

Taxon	GenBank accession number	
	ITS	trnLF
<i>Anisodus luridus</i>	AY478400.1_AY478414.1	
<i>Anisodus tanguticus</i>	AY478402.1_AY478416.1	
<i>Atropa belladonna</i>	AB019288_AB019948.1	
<i>Atropa belladonna</i>	AY028129_AY028147	
<i>Atropanthe sinensis</i>	AY478403.1_AY478417.1	
<i>Scopolia carniolica</i>	AY478397.1_AY478411.1	
<i>Scopolia lutescens</i>	AY478395.1_AY478409.1	
<i>Scopolia parviflora</i>	AY478390.1_AY478404.1	
<i>Hyoscyamus muticus</i>		EU58099.1
<i>Hyoscyamus albus</i>		EU580994.1
<i>Hyoscyamus boveanus</i>		EU580995.1
<i>Hyoscyamus desertorus</i>		EU580996.1
<i>Hyoscyamus pusillus</i>		EU580998.1
<i>Anisodus tanguticus</i>		EU580956.1
<i>Atropa belladonna</i>		EU580958.1
<i>Atropa belladonna</i>		EU580959.1
<i>Physochlaina infundibularis</i>		EU581046.1
<i>Physochlaina orientalis</i>		EU581047.1
<i>Przewalskia tangutica</i>		EU581050.1
<i>Scopolia carniolica</i>		EU581060.1
<i>Scopolia japonica</i>		EU681061.1

*TrnL-F sequence data*

The *trnL-F* data included 930 characters that 854 characters constant and 76 characters were variable. In variable characters 39 characters parsimony uninformative and 37 characters were parsimony informative. Strict consensus tree in *trnL-F* with 84

steps was included (CI)= 0.940 and (RI) = 0.970.

We studied 31 samples of Solanaceae from six different genus (*Atropa*, *Physochlaina*, *Przewalskia*, *Scopolia*, *Hyoscyamus*, *Anisodus*) in *trnL-F* analysis. All species of *Hyoscyamus* were formed a monophyletic group (21species, PP=1, BS=100%).

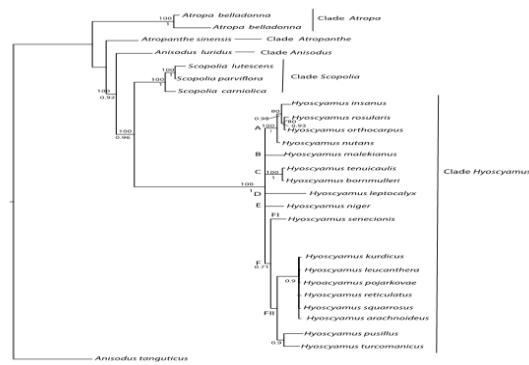
Within this group two major clade were identified (A - B). *H.muticus* constituted clade A. Clade B divided to five subclade (B1-B2-B3-B4 and B5). *H.desertorus* constituted clade B1. Clade B2 included eight species *H.boveanus*, *H.malekianus*, *H.bornmulleri*, *H.tenuicaulis*, *H.insanus*, *H.orthocarpus*, *H.rosularis* and *H.nutans* (PP=0.97). In this clade two species *H.bornmulleri* and *H.tenuicaulis* were formed one subclade (PP=0.99, BS=70%) and four species *H.insanus*, *H.orthocarpus*, *H.rosularis* and *H.nutans* were formed another subclade (PP=0.99, BS=100%). *H.leptocalyx* constituted clade B3. *H.turcomanicus* constituted clade B4. Clade B5 included ten species (PP=0.79, BS=73%) from subgenus *Hyoscyamus* and divided to two subclade (B5a - B5b). Eight species *H.albus*, *H.leucanthera*, *H.niger*, *H.pojarkovae*, *H.reticulatus*, *H.arachnoideus*, *H.squarrosus* and *H.kurdicus* were formed polytomy group (subclade B5a, PP=1, BS=90%). And two species *H.senecionis* and *H.pusillus* were formed subclade B5b (PP=1, BS=100%). Among another taxa from Solanaceae we studied two samples of *Atropa belladonna* with two different genbank accession numbers that constituted one clade (PP=1, BS=100%). Five species such as *Physochlaina orientalis*, *Physochlaina infundibularis*, *Przewalskia tangutica*, *Scopolia japonica* and *Scopolia carniolica* were formed one clade (PP=0.95). This clade divided to three subclade. Two species *Physochlaina orientalis* and *Physochlaina infundibularis* were formed monophyletic group (PP=0.85, BS=75%). Two species *Przewalskia tangutica* and *Scopolia japonica* were formed one subclade (PP=0.83, BS=70%) and *Scopolia carniolica* constituted another subclade and *Anisodus tanguticus* was outgroup.

**Combined sequence data**

In combined analysis the best fit model was (HKY+I+G) that selected by hLRT in model test 3.7 (Posada and Crandall, 1998). The base frequencies were freq A= 0.2867, freq C= 0.2328, freq G= 0.2452 and freq T= 0.2353.

The combined data included 1391 characters that 1117 characters constant and 274 characters were variable.

In variable characters 139 characters parsimony uninformative and 135 characters were parsimony informative.

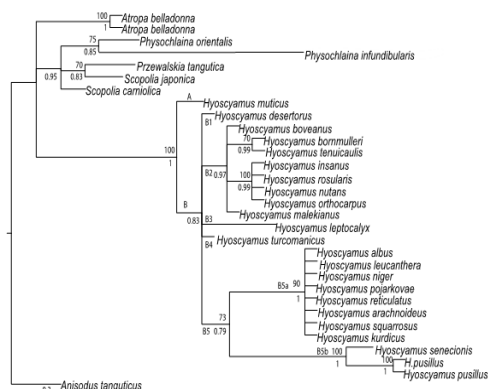


**Fig. 1.** The 50% majority rule consensus tree from Bayesian analysis of NrDNA ITS sequence data. Numbers above branches represent Boot strap support (BS) and numbers below branches represent posterior probabilities(PP).

Strict consensus tree in combined with 456 steps was included CI= 0.764 and RI= 0.724. homogeneity test (HI) = 0.1100.

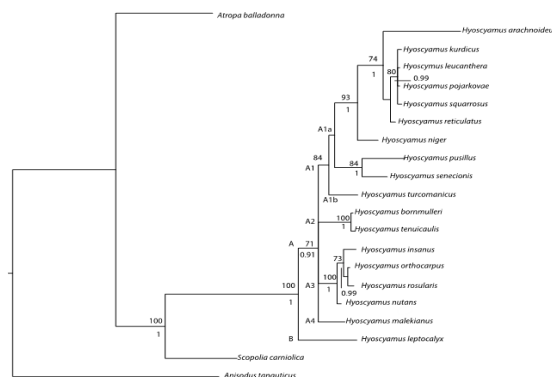
In combined analysis all species of *Hyoscyamus* were formed a monophyletic group (18 species, PP=1, BS=100%). Within this group two major clade were identified (A-B): *H.leptocalyx* constituted clade B. Clade A divided to four subclade (PP=0.91, BS=71%) (A1-A2-A3-A4): Clade A1 included two subclade A1a, A1b (BS=84%). *H.turcomanicus* constituted A1b. In clade A1a two species *H.pusillus* and *H.senecionis* were formed one clade (PP=1, BS=84%). Seven species *H.arachnoideus*, *H.kurdicus*, *H.leucanthera*, *H.pojarkovae*, *H.squarrosus*, *H.reticulatus* and *H.niger* were formed another clade (PP=1, BS=93%) In this clade *H.niger* constituted one subclade and another six species were formed one subclade (PP=1, BS=74%) that in this subclade four species *H.kurdicus*, *H.leucanthera*, *H.pojarkovae*, *H.squarrosus* were formed a polytomy group (PP=0.99, BS=80%). Clade A2 included *H.tenuicaulis* and *H.bornmulleri* (PP=1, BS=100). Clade A3 included *H.insanus*, *H.orthocarpus*, *H.rosularis* and *H.nutans* (PP=1, BS=100%). In this clade three species *H.insanus*, *H.orthocarpus* and *H.rosularis*

were formed one subclade (PP=0.99, BS=73%) and *H.malekianus* constituted clade A4. *H.insanus*, *H.orthocarpus*, *H.rosularis* and *H.nutans* reported as a synonymous in Flora of Iran and were located in subgenus *Dendrotrichon*. All phylogenetic trees resulted from MP and Bayesian analyses based on ITS, *trnL-F* and combined sequences data showed that these four species were formed one clade and MP supported this result with strong bootstrap 100%. Then the result of present study were confirmed by Flora of Iran (Khatamsaz, 1377). Also two species *H.bornmulleri* and *H.tenuicaulis* that have morphological similarity were located in one clade and belong to subgenus *Dendrotrichon* that placed at the closer distance from another taxa of this subgenus in *trnL-F* and combined analysis. *H.pusillus* and *H.senecionis* in two analyses *trnL-F* (BS=100%) and combined (BS=84%) were formed one clade.



**Fig. 2.** The 50% majority rule consensus tree from Bayesian analysis of *trnL-F* sequence data. Numbers above branches represent Boot strap support (BS) and numbers below branches represent posterior probabilities(PP).

Olmstead *et al* (2008) studied on 89 genera and 190 species from Solanaceae by using *ndhF* and *trnL-F* markers and reported that *Hyoscyamus* was a monophyletic group. Also two species *Physoclaina orientalis* and *Physoclaina infundibularis* were formed a monophyletic group and two species *Scopolia japonica* and *Przewalskia tangutica* were formed a one clade that agreement with our result.



**Fig. 3.** The 50% majority rule consensus tree from Bayesian analysis of combined sequence data. Numbers above branches represent Boot strap support (BS) and numbers below branches represent posterior probabilities(PP).

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