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Phylogeny analysis of *Colutea* L. (Fabaceae) from Iran based on ITS sequence data

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Abstract. *Mirzaei L, Mehregan I, Nejadsatari T, Assadi M. 2015. Phylogeny analysis of* Colutea *L. (Fabaceae) from Iran based on ITS sequence data. Biodiversitas 16: 168-172.* This study was carried out on the species of *Colutea* L. that growing in Iran. Some of these species are native to Iran. Internal transcribed spacer (ITS) sequences were obtained from 12 samples representing seven species of *Colutea* recognized by recent taxonomic treatments from Iran and we used 20 ITS sequences from GenBank to test the phylogeny of *Colutea*. Phylogenetic analyses were conducted using Bayesian inference and maximum likelihood methods. Our results of cladistic analysis of phylogenetic relationships among *Coluteae* tribes showed its monophyletic origin and *Astragaleae* was its sister group. In addition, *C. cilicica* was a sister group to *C. gifana* whose separated of other *Colutea* genus from Iran. Bayesian inference and maximum parsimony analyses confirmed the monophyly of three sections of *Colutea* in Iran. Our results showed that further investigation including application of larger number of markers and involving all the Iranian *Colutea* species will be more effective in estimation the relationships of the genus.

Keywords: *Colutea*, Fabaceae, Iran, ITS, phylogeny

INTRODUCTION

Fabaceae is the third largest family of angiosperms with 730 genera and more than 19,000 species that is distributed mainly in the temperate and subtropical parts of the world. Legumes are second grasses in their agricultural and economical value, and include many important species grown for food, fodder, wood, ornamentals, and raw materials for industry. In addition, they play ecologically important role in biological nitrogen fixation. This family of angiosperm without counting the genus *Astragalus* contains 429 species is distributed in Iran; among them 173 are rare and 117 species are endemics. About half of the observations in Iran were from the provinces Fars, Hormozgan, Tehran, Bushehr and Mazandaran (Yousefi 2006; Lewis et al. 2005; Mousavi and Khosravi 2010).

Colutea L. (Fabaceae L.) is a small genus included nearly 30 species of shrubs and small trees with inflated fruits and is distributed throughout the Mediterranean region, China, Himalaya, Eastern and North-Eastern Africa, mostly in dry mountains (Mabberley 1997). *Colutea* genus includes 13 species from three sections in the Iranian plateau. Seven species of this genus are growing in Iran, that five of them are endemic (Browizc 1959). In current scenario, the DNA markers become the marker of choice for the study of crop genetic diversity to revolutionize the plant biotechnology. The internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) is one of the most extensive sequenced molecular markers (Alvarez and Wendel 2003). The region is part of the rDNA cistron, which consists of 18S, ITS1, 5.8S, ITS2, 26S and present in several hundred copies in most eukaryotes.

The internal transcribed spacer (ITS) contains the signals need to process the rRNA transcript and often used for inferring phylogeny at intra and inter generic levels in many plant families (Baldwin 1992; Baldwin et al. 1995; Wojciechowski et al. 1999; Kazempourosaloo et al. 2005; Ahangarian et al. 2007). The length of the ITS region of flowering seed plants is highly uniform (Baldwin et al. 1995). By contrast, that of non-flowering seed plants shows much variation especially the length of the ITS1 region, which ranges from 630 to 3125 bp, is strikingly more labile in flowering plants (Liston 1995; Maggini et al. 2000). ITS1 and ITS2 are not equivalent structures, even though they are sometimes convergent in length as well as substitutional patterns; ITS1 evolved from an intergenic spacer and ITS2 from an expansion segment in the rDNA large subunit.

The previous phylogenetic studies of Fabaceae by using analyses of the nrDNA ITS were focused exclusively on *Hedysareae* tribe (Ahangarian et al. 2007; Lewke et al. 2013), *Vicieae* (*Fabeae*) tribe (Foladi et al. 2013), *Pogonophaca* subgenus (Kang et al. 2003), *Phyllolobium* genus (Zhang et al. 2012), *Genista* genus (Hakki et al. 2010), and *Astragalus* genus (Zarre and Azani 2012) and presented the monophyly of majority of this clades. Thereby, ITS sequencing provides valuable phylogenetic data for resolving relationships among species and genus of Fabaceae family by molecular markers. Major objective of this paper is to ascertain the taxonomic and phylogenetic relationships within species of *Colutea* genus in Iran.

MATERIALS AND METHODS

Taxon sampling

Accessions in the amount of 12 plant sample belonging to seven species of *Colutea* L. were collected by authors from wild populations in different regions of Iran (see Table 1). All the specimens examined (or its duplicates) are deposited in the Islamic Azad University Avicennia Herbarium (IAUH, without voucher number/in registration process). In this study we used 20 ITS sequence of 17 species obtained from the GenBank: three species of *Colutea*, three species of *Astragalus*, three species of *Swainsona*, *Chesneya kotschyi*, *Lessertia herbacea*, *Sutherlandia frutescens*, *Carmichaelia williamsii*, *Clianthus puniceus*, *Eremosparton flaccidum*, *Erophaca baetica* subsp. *Orientalis* and *Smirnowia turkestana*. The list of non-Iranian taxa used in our analysis with GenBank accession numbers are showed in Table 2.

Table 2. List of non-Iranian taxa with GenBank accession number used in our analysis.

Species	ITS GenBank accession number
Astragalus vogelii	U50499.1
A. complanatus	EU591995.1
A. cysticalyx	AF121682.1
Carmichaelia williamsii	AF113854.1
Colutea. abyssinica	GQ246039.1
C. arborescens	U56010.1
C. arborescens	U56009.1
C. atlantica	GQ246040.1
Chesneya kotschyi	GQ246104.1
Clianthus puniceus	L10801.1
C. puniceus	L10800.1
Eremosparton flaccidum	GQ246035.1
Erophaca baetica subsp. *orientalis*	EU070920.1
Lessertia herbacea	AF121752.1
Smirnowia turkestana	GQ246037.1
Sutherlandia frutescens	GQ246033.1
Swainsona canescens	GQ246042.1
S. formosa	U56008.1
S. pterostylis	U56007.1
S. pterostylis	GQ246032.1

DNA extraction and ITS sequencing

The DNA extraction was performed from leaves dried with silica gel by using the NucleoSpin Plant Kit (Macherey-Nagel GmbH & Co. KG, Du ren, Germany) after the manufacturer's protocol. Concentration and quality of extracted DNAs were checked by 1% agarose gel electrophoresis. We amplified the ITS region (ITS1-5.8S-ITS2) of the nuclear ribosomal DNA by using primer combinations AB101 and AB102 primers: a forward primer AB101annealing, 5'-ACG AAT TCA TGG TCC GGT GAA GTG TTC G-3', and a reverse primer (AB102) annealing, 5'-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3' (Kang et al. 2003).The PCR amplifications were performed in 25µL reaction volumes containing 1µL template DNA, 10.5µL ddH_2O, 10× buffer + $MgCl_2$ +Taq polymerase+Tween 20 + dNTP=12.5µLand 0.5µL each of the both primers. Thermal cycles were performed with 2min denaturation step at 95° C, followed by 35 cycles at 95 °C for 1 min, 51.5 °C for 1 min, and 72 °C for 1.5 min, followed by 7-10 min final extension at 72° C for completion of primer extension. PCR products were resolved by electrophoresis in 1% agarose gel and then visualized under UV light.

Phylogenetic analysis

Forward and reverse sequences were visually compared and edited, and then initially aligned using Sequencher 4 software (Gene Codes Corporation, Ann Arbor, MI USA). All ITS sequences were assembled and aligned using Mac Clade 4 (Maddison and Maddison 2010).

Maximum parsimony analyses (MP)

Parsimony analyses were implemented by employing PAUP ver. 4.0 (Swofford 2002) using following criteria: 100 heuristic search replicates, random stepwise addition of taxa, and tree-bisection reconnection (TBR) branch swapping. These parsimonious trees were used to calculate the consensus tree. Bootstrap analyses (BS) were applied to determine the clade support. BS for clades was calculated using PAUP with 100 replicates of heuristic searches, and randomly stepwise addition of taxa. Clades with a bootstrap value of 70% or more were considered as robustly supported nodes.

Table 1. List of *Colutea* species investigated and voucher specimen information (TARI = Herbarium of Research Institute of Forests and Rangelands, IAUH = Islamic Azad University Avicennia Herbarium).

Species	Origin, Voucher
Colutea buhsei (Boiss.) Shapar.	Iran, Prov. N. Gorgan, 1400 m, (30871 TARI).
C. buhsei (Boiss.) Shapar.	Iran, Prov. E. Khorasan, 1550 m, Foroghi, (50312 TARI).
C. buhsei (Boiss.) Shapar.	Iran, Prov. S. Ardebil, Khalkhal to chuli, 1000m (1), Ferguson, (000013619 IAUH).
C. buhsei (Boiss.) Shapar.	Iran, Prov. Tehran, 1800 m.Trott, (000013617 IAUH).
C. buhsei (Boiss.) Shapar.	Iran, Prov. Gorgan, Aliabad, 600 m, Gauba (88858 TARI).
C. gracilis Fryen & Sin.f.	Iran, Prov. N. Gorgan, 20800 m (000013611 IAUH).
C. persica Boiss.	Iran, Prov. Kerman, 2300m, Mussavi and Tehrani (16256 TARI).
C. persica Boiss.	Iran, Prov. Fars, Dahte arzhan, 2200 m,Foroghi,(45755 TARI).
C. porphyrogramma Rech.f.	Iran, Prov. Khorasam, Bojnord, 1350 m,Resh,(000013614 IAUH).
C. uniflora G .Beck. ex Stap f.	Iran, Prov. Khorasan, Gazvin, 1600 m (000013621 IAUH).
C. cilicica Boiss. & Balansa.	Iran, Prov. Azerbaijan, Kaleibar,vinag, 1000 m, Assadi & Wdb (000013620 IAUH).
C. gifana Parsa	Iran, Prov. E Khorasan, Gifan, 1300 m, Parsa (000013623 IAUH).

Bayesian analysis (BA)

The BA analyses of the ITS datasets were performed using MrBayes ver. 3 (Huelsenbeck and Ronquist 2001). In order to find the appropriate model of DNA substitution, the Maximum Likelihood criteria for datasets were determined by the Akaike Information Criterion (AIC; Akaike 1974) as implemented in the software ModelTest v 3.7 (Posada et al. 1998).

Modeltest

For the Maximum Likelihood (ML) and MrBayes analyses (MB), the best fit of DNA substitution model should be found. The Akaike information criterion (AIC) and hierarchical likelihood ratio test (hLRT) were calculated based on the log likelihood scores of 56 models using Modeltest 3.7 (Posada et al. 1998). In general, AIC was chosen (Posada 2008). For ITS spacer dataset of this paper, Likelihood settings from best-fit model (TVM+G) were selected by AIC in Modeltest 3.7 with the nucleotide frequencies A = 0.2072, C = 0.2576, G = 0.2751, T = 0.2601, a gamma shape parameter of 0.5955 and an assumed proportion of invariable sites of 0.0.

Maximum likelihood

Maximum search was performed on the basis of the result of Modeltest in PAUP. The parameters of best model, such as the base frequency, the mean relative substitution rates, proportion of invariable sites, Gamma distribution shape, were all employed. The heuristic search and bootstrap were implemented as in parsimony analysis in PAUP above mentioned.

Bayesian inference

Bayesian inference of phylogenetic trees was analyzed by some parameters from the Modeltest, and included in the analysis. The option was set up using 1,000,000 generations of Markov Chain Monte Carlo (MCMC) searches and a sample frequency of 1000. Saturation was reached after a burn-in of 1000 generations. The clade support was assessed using Bayesian posterior probabilities employing MrBayes version 3.0 (Huelsenbeck and Ronquist 2001).

RESULTS AND DISCUSSION

The data set of the ITS region included 432 characters with 270 including variable positions within the ingroup; 82 were parsimony informative. The Bayesian 50% majority- rule consensus tree for ITS contained 11 internal nodes with a posterior probability (PP) of 1.0 (Fig. 2). Strict consensus phylogeny trees, with 256 steps was included consistency index (CI) = 0.791, retention index (RI) = 0.799. Using the data of Figure 1, *Clianthus puniceus* and *Carmichaelia williamsii* taken as an outgroup form a separate clade, and *Swainsona canescens* species form a group which is sister to other *Swainsona* species. The ingroup consists of two main clades that labeled as A and B. In clade A, *Swainsona formosa* and *S. pterostylis* form a separate group. The support was occurred in clade A (PP=1; BS 96%). Clade B comprises two clades including BI and BII. Clade BI comprises two subclades including BI1 (*Astragalus complanatus, Chesneya kotschyi* and *Erophaca baetica* forming a separate group) and BI2 clades included a single species *Astragalus vogelii*. Clade BII, recognized in three subclades, BII1, BII2 and BII3, respectively. Within BII1 subclade *Astragalus vogelii* separated of *Eremosparton flaccidum* + *Smirnovia turkestana* subclade. Clade BII2 involves BII2a and BII2b that consisting of *Colutea* species. In clade BII2a *Colutea cilicica* and *C. gifana* grouped together separately from the other Iranian species of *Colutea*. The species of *Colutea arborescens* and *C. atlantica* recognize in BII2b subclades. In BII3 subclade, *Lessertia herbacea* and *Sutherlandia frutescens* are grouped together. The support was occurred in clade B for two sister-group BI and BII (PP=1; BS 98%). Best support relationship between three sister-group clades BII (PP =1; BS = 93%) and relationship between two sister group clade BII2a and BII1 was supported by (PP=1; BS= 99%). Clade BII2b was contain the *Colutea* species genus supported with pp=1, BS=74%. Clade BII2b divided into two sister groups BII2a and BII2b. Within the *Colutea* species three major clades were identified and named as D, E and F clade (12 species; PP = 0.90; BS = 56%). Clade D comprising 10 accessions of *C. buhsei*, *C. gracilis*, *C. uniflora* and *C. porphyrogramma* (PP = 0.97; BS = 74%) constitutes the unresolved group and shows polytomy. *C. gifana* in clade F together with its sister *C. cilicica* in clade E separated of other species of *Colutea* from Iran was sister group to *C. gifana* in clade E. Actually sampled species in three clade D, E, F included to Sect. Rostrata (five accessions of *C. buhsei*), two species of Sect. Armata (*C. uniflora, C. porphyrogramma*) and four species of Sect. *Colutea* (*C. gracilis, C. persica, C. cilicica*) from Iran that little supported relationships among main clades were existed. On the other side, relationships among D clades remained indefinite in our results. So our results show that *Astragalus cysticalyx* from *Astragaleae* tribe is close to *Colutea* tribe (Ahangarian et al. 2007).

According to the other studies, all species examined in our study nested within coluteoid clade in large astragalean clade; *Carmichaelia williamsii* and *Clianthus puniceus* species were basal group in sub tribe *Coluteinae*, and among members of the sub tribe closely relation existed (Polhill 1981; Lavin et al. 1990; Sanderson and Liston 1995; Wojciechowski 2005; Zhang et al. 2012). Wojciechowski et al. (2000) represented *Colutea istria* which is sister to the *Lessertia*+*Sutherlandia* complex plus *Eremosparton* + *Smirnowia.* As can be concluded from the phylogenetic study performed by Lock and Schrire (2005), *Swainsona* formed the monophyletic group sister to *Astragalus cysticalyx*, *Colutea istria* and *Colutea arborescens.* The results of the present work are consistent with those of Wagstaff et al. (1999) showing the genera *Carmichaelia* and *Clianthus* nested within the *Swainsona*, and thus the monophyly of *Carmichaelia* was reported by these authors. Therefore, our study supported these results. In the same study based on a *mat*K, *trn*L and ITS molecular markers *Astragalus epiglottis* was shown as the sister group

for *Colutea persica* (Liston 1995; Amirahmadi et al. 2014). Our results of cladistic analysis the phylogenetic relationships among *Coluteae* tribes showed its monophyletic origin and *Astragaleae* was its sister group. In addition, *C. cilicica* was a sister group to *C. gifana* whose separated of other *Colutea* genus from Iran. However, ITS marker could not sufficiently informative to provide adequate resolution for *Colutea* genus and the relationships between some of the major groups remained unresolved. According to our results, we can assume that further investigation insufficiently resolved nodes within the *Colutea* genus will provide important insight into the relationships between the species and, consequently, the genera of *Coluteae* tribe. The combined approach including application of different markers would increase the resolutions and supports of the clades in *Colutea.*

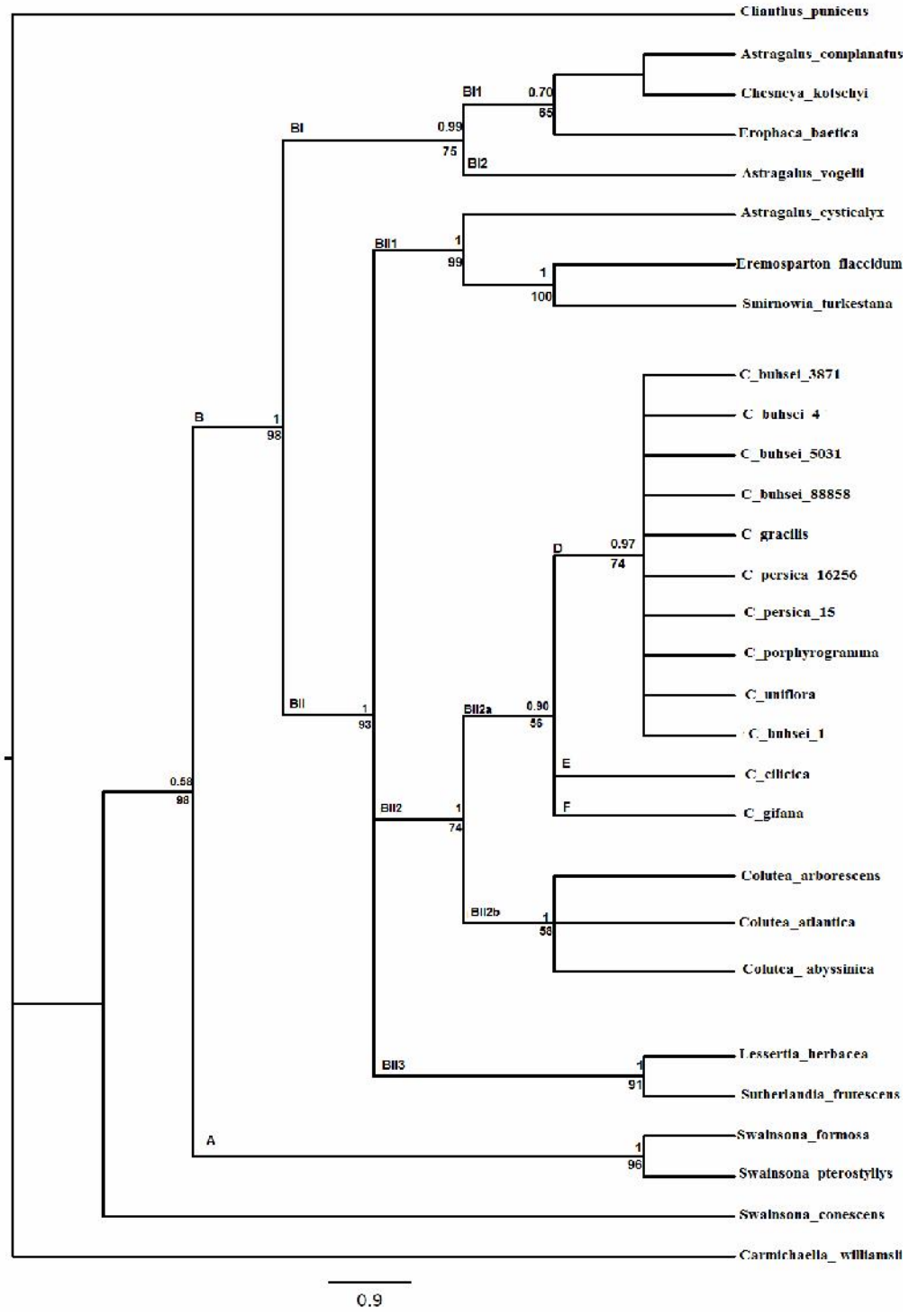

Figure 2. Bayesian consensus phylogram for combined data (Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum likelihood percentage bootstrap values).

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