Phylogeny and divergence times of the Coluteoid clade with special reference to Colutea (Fabaceae) inferred from nrDNA ITS and two cpDNAs, matK and rpl32-trnL(UAG) sequences data

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To cite this article: M. Moghaddam, S. Kazempour Oosaloo, H. Hosseiny & F. Azimi (2016): Phylogeny and divergence times of the Coluteoid clade with special reference to Colutea (Fabaceae) inferred from nrDNA ITS and two cpDNAs, matK and rpl32-trnL(UAG) sequences data, Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology, DOI: 10.1080/11263504.2016.1244120

To link to this article: http://dx.doi.org/10.1080/11263504.2016.1244120

Published online: 19 Oct 2016.
Phylogeny and divergence times of the Coluteoid clade with special reference to Colutea (Fabaceae) inferred from nrDNA ITS and two cpDNAs, matK and rpl32-trnL(UAG) sequences data

M. MOGHADDAM1, S. KAZEMPOUR OSALOO1, H. HOSSEINY1, & F. AZIMI2

1Department of Plant Biology, Faculty of Biological Sciences, Tarbiat Modares University, Iran and 2Natural Resource Research Center of Ardabil Province, Iran

Abstract

This study reconstructed the phylogeny of the Coluteoid clade using nrDNA ITS and plastid matK and rpl32-trnL(UAG) sequences data. The analyses resolve a well-supported Coluteoid clade, as sister to Astragalus s.str. + Oxytropis, nested within the larger, strongly supported Astragalean clade. The Coluteoid clade is now composed of 12 genera including Podlechiella, Swainsona, Carmichaelia, Clianthus, Montigena, Phyllolobium, Lessertia, Sutherlandia, Sphaerophysa, Smirnowia, Eremosparton and Colutea. Within this clade, Podlechiella is the first diverging lineage followed by successive subclades of Carmichaelia + Clianthus + Swainsona, Phyllolobium, Lessertia + Sutherlandia, Sphaerophysa + Smirnowia + Eremosparton, and Colutea. We assigned the formal tribal name to this clade and redefined the tribe Coluteae. A diagnostic key to the genera of the tribe is presented. Astragalus cysticalyx and A. sinicus have no relationship with the Coluteoid clade, instead, they are nested in Astragalus s. str. Resolution within Colutea is rather low, but several smaller subclades with low to high supports are found in the genus. None of the large sections in Colutea are monophyletic. Divergence time estimates revealed that the Coluteoid clade originated in the Early Miocene (20.4 Ma). Most of its members were diverged during the Late Miocene to Pliocene. Colutea and Podlechiella form the youngest lineages where the diversification occurred in the Pliocene-Pleistocene.

Keywords: Coluteae, divergence time, nrDNA ITS, phylogeny, plastid DNA, the Coluteoid clade

Introduction

The Coluteoid clade is a heterogeneous group of morphologically and ecologically distinct genera and species, which distributed throughout Africa to Australasia. Currently, it contains 12 genera, i.e. Colutea (including Oreophysa, see Kazempour Osaloo et al. 2006), Smirnowia, Eremosparton, Sphaerophysa, Lessertia, Sutherlandia, Carmichaelia, Clianthus, Montigena, Swainsona, Phyllolobium and Podlechiella as well as the two Astragalus species, A. sinicus L. and A. cysticalyx Ledeb. (Wojciechowski et al. 1999; Wojciechowski 2005; Lock & Schrire 2005). Colutea and its allied genera were previously treated in tribe Galegeae subtribe Coluteinae (Polhill 1981), while Carmichaelia and its allies formed subtribe Carmichaelinae (Heenan 1998a, 1998b; Wagstaff et al. 1999) or carmichaelioid group (Lock & Schrire 2005). Phyllolobium and Podlechiella have been recently segregated from Astragalus (Kazempour Osaloo et al. 2003; Zhang & Podlech 2006). Most members of the clade are characterized by non-interlocking wing and keel petals and a ciliate style (Lavin & Delgado 1990). All members of the clade except the annual Podlechiella are perennial herbs, subshrubs, or shrubs/small trees. Based on the phylogenetic analysis of the combined nrDNA ITS and matK sequences, Wojciechowski (2005) indicated that Podlechiella is the first diverging taxon as sister to the other representatives of the clade. Carmichaelia and allies formed own subclade, as for Lessertia and Sutherlandia. In this study, Phyllolobium chinense Fisch. (=A. complanatus Bunge) formed a common clade with A. sinicus, and Colutea, represented by C. arborescense L., was sister to A. cysticalyx. Furthermore, in the earlier study of Wojciechowski et al. (1999), Smirnowia and allies (including A. cysticalyx) formed a distinct subclade within the Coluteoid clade. Kazempour Osaloo et al. (2003) using molecular data (nrDNA ITS and plastid ndhf) clearly indicated that A. sinicus is more closely related to members of Astragalus s.str. than to the Coluteoid clade. However, the taxonomic composition of the Coluteoid clade and the exact phylogenetic status of A. cysticalyx are still unclear.
After *Swainsona* (about 80 spp.) and *Lessertia* (about 50 spp.), *Colutea* is the third largest genus of the clade with about 30 species. *Colutea* is distributed from Mediterranean region to China, Himalaya, and east–northeast Africa. In Iran, *Colutea* has nine species in 3–5 sections (Browicz 1984; Pooyan et al. 2014). Among the species-rich genera of the Coluteoid clade, *Carmichaelia* and *Swainsona* (Wagstaff et al. 1999), *Phyllolobium* (Kang et al. 2003; Zhang et al. 2012) and *Colutea* (Mirzaei et al. 2015) have been subjects of phylogenetic analyses using nrDNA ITS data.

By employing penalized likelihood estimates of ages and rates of nucleotide substitutions using *matK* sequences, Wojciechowski (2005) provided the estimated mean age of 13.7 (±1.70) Mya, the middle Miocene, and mean rate of 0.000894 (±0.000080) for the Coluteoid Clade.

Hitherto, no detailed phylogenetic treatment and divergence time estimates using multiple DNA sequence data have been conducted on the Coluteoid clade and *Colutea* in particular. In this study, the nuclear ribosomal DNA internal-transcribed spacer (nrDNA ITS) and two plastid fragments, *matK* gene and *rpl32-trnL* (UAG) intergenic spacer, were sequenced for phylogenetic reconstructions and estimation of ages and rates.

The main goals of present study are: (1) to evaluate the taxonomic status of the Coluteoid clade and to elucidate relationships within it, (2) to test the monophyly of *Colutea* and its major sections, (3) to assess the accurate taxonomic position of *Astragalus cysticalyx*, and (4) to estimate molecular divergence times and ages of the Coluteoid clade and its major subclades.

**Materials and methods**

**Taxon sampling**

Forty-three accessions representing 29 species belonging to Coluteoid clade and 12 species of the related clade (*Astragalus* + *Oxytropis*) were included in the analyses. One species of *Caragana* and one species of *Eversmania* were selected as outgroups. In addition to the newly generated nrDNA ITS (23), *matK* (21) and *rpl32-trnL* (UAG) (28) sequences, 20 nrDNA ITS, 16 *matK*, and 1 *rpl32-trnL* (UAG) sequences were retrieved from GenBank. All taxa together with their origin, voucher information, and GenBank accession numbers are listed in Table I.

**DNA isolation, PCR and sequencing**

Total genomic DNA was isolated from leaf materials using the modified CTAB method of Doyle and Doyle (1987). The nrDNA ITS region was amplified using the primers ITS5 m of Sang et al. (1995) and ITS4 of White et al. (1990) or AB101F and AB102R (Douvrey et al. 1999). The *matK* gene was amplified using the primer of *trnK685F* (Steele & Wojciechowski 2003) and *matK1320R* (designed in this study). The *rpl32-trnL* (UAG) region was amplified using primer *rpl32-P* and *trnL* (UAG) described in Shaw et al. (2007). The PCR amplification was carried out in the volume of 20 μl, containing 8.2-μl deionized water, 10 μl of the 2 × Taq DNA polymerase master mix Red (Amplicon, Cat. No. 180301, 150-mMTris-HCl Pd 8.5, 40-Mm (NH4)2SO4, 3.0-Mm MgCl2, 0.4-Mm dNTPs, 0.05 units μl −1Amplicon Taq DNA polymerase, iner red dye and a stabilizer) 0.5 μl of each primer (10 pmol/μl), and 1 μl of template DNA (20 ng/μl). PCR procedures for nrDNA ITS region were 2 min 30 s at 94°C for predenaturation followed by 30 cycles of 45 s at 94°C for denaturation, 40 s at 56°C for primer annealing and 40 s at 72°C for primer extension, followed by a final primer extension of 7 min at 72°C. for *matK* region, the PCR condition was 2 min 30 s at 94°C followed by 32–35 cycles of 40 s at 94°C, 40 s at 53°C, and 1 min 20 s at 72°C, and final extension of 7 min at 72°C was performed. For *rpl32-trnL* (UAG) region, the PCR condition was 5 min at 80°C followed by 28 cycles of 50 s at 95°C, 45 s at 54°C, and 50 s at 65°C, and the final extension of 5 min at 65°C was performed. The ensuring PCR fragments were separated by electrophoresis in 1% agarose gels in 1 × TAE (pH = 8) buffer, stained with ethidium bromide and were photographed with a UV gel documentation system (UVitec, Cambridge, UK). Purification of PCR products was performed using NucleoSpin Extract kits (MACHERY-NAGEL April 2004/Rev.01). Each region was directly sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction kit with the same primers in an ABI 3730xl DNA Analyzer (Applied Biosystems, USA) through Pishgam Inc.

**Sequence alignment**

Each of the individual data-sets was edited using BioEdit ver. 7.0.9.0 (Hall 1999) and aligned with web-based version of MUSCLE (Edgar 2004; at http://www.ebi.ac.uk/Tools/msa/muscle/) and adjusted manually. The alignment of the data-sets required for the introduction of numerous single- and multiple-base indels (insertions/deletions). The indels were treated as missing data for all data-sets.

**Phylogenetic analyses**

**Parsimony method.** Maximum parsimony (MP) analyses were conducted using PAUP” version
Table I. Taxa included in the nrDNA ITS, matK and rpl32-trnL(UAG) analyses.

<table>
<thead>
<tr>
<th>Species</th>
<th>DNA source (location, voucher)</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Colutea cilicica</em> Boiss. &amp; Bal.</td>
<td>Turkey: Ozbek 1569 (GAZI)</td>
<td>LC164570/LC164592/LC164609</td>
</tr>
<tr>
<td><em>C. gracilis</em> Freyn &amp; Sint.</td>
<td>Iran: Faghhiinia &amp; Zangui 18724 (FUMH)</td>
<td>LC164572/-/LC164611</td>
</tr>
<tr>
<td><em>C. porphyrrogenum</em> Rech.f.</td>
<td>Iran: Ghahreman et al. 22335 (TUH)</td>
<td>LC164577/LC164596/LC164616</td>
</tr>
<tr>
<td><em>C. persica</em> Boiss.</td>
<td>Iran: Manucher et al. 277 (TARI)</td>
<td>LC164576/LC164595/LC164615</td>
</tr>
<tr>
<td><em>C. glieana</em> Parsa</td>
<td>Iran: Memariani &amp; Zangui 39615 (FUMH)</td>
<td>LC164571/LC164593/LC164610</td>
</tr>
<tr>
<td><em>C. paulsensi</em> Freyn</td>
<td>Tajikistan: Pekofo 223 (TARI)</td>
<td>LC164575/LC164594/LC164614</td>
</tr>
<tr>
<td><em>C. armena</em> Boiss.&amp; Huet.</td>
<td>Turkey: Duzenli 1210 (ANK)</td>
<td>LC164568/LC164591/LC164607</td>
</tr>
<tr>
<td><em>C. melanocaulis</em> Boiss.&amp; Heldr.</td>
<td>Turkey: Pesemen &amp; Guner 1522 (ANK)</td>
<td>LC164574/LC164593/LC164613</td>
</tr>
<tr>
<td><em>C. homarvi</em> Tahkt.</td>
<td>Iran: Ghahremani &amp; Imani 7815 (TMUH)</td>
<td>LC164573/LC164582/LC164612</td>
</tr>
<tr>
<td><em>C. triphylla</em> Bunge ex Bioss.</td>
<td>Iran: Assadi &amp; Mozaffarian 33325 (TARI)</td>
<td>LC164578/LC164583/LC164617</td>
</tr>
<tr>
<td><em>C. arborescens</em> L.</td>
<td>GenBank</td>
<td>U56009, U56010b/AY386874b/-</td>
</tr>
<tr>
<td><em>C. uniflora</em> G. Beck</td>
<td>Iran: Charakhchian, 1687 (TMUH)</td>
<td>LC164579/-/LC164618</td>
</tr>
<tr>
<td><em>C. buhsei</em> (Boiss.) Shap.</td>
<td>Iran: Hosseiny 2008-5-8 (TMUH)</td>
<td>LC164585/LC164603/LC164621</td>
</tr>
<tr>
<td><em>Eremosparton flaccidum</em></td>
<td>Iran: Khakani &amp; Talaii 3687 (TMUH)</td>
<td>LC164588/LC164601/LC164628</td>
</tr>
<tr>
<td><em>P. v. vogelii</em> (Chiov)</td>
<td>Iran: Mozaffar et al. 39103 (TARI)</td>
<td>LC164586/LC164601/LC164628</td>
</tr>
<tr>
<td><em>ssp. fatimensis</em></td>
<td>Iran: Assadi &amp; Mozaffarian 33325 (TARI)</td>
<td>LC164578/LC164583/LC164617</td>
</tr>
<tr>
<td><em>ssp. steudelii</em></td>
<td>Iran: Assadi &amp; Mozaffarian 33325 (TARI)</td>
<td>LC164578/LC164583/LC164617</td>
</tr>
<tr>
<td><em>ssp. vogelii</em> Podlechiella vogelii (L.) R. Br.</td>
<td>South Africa: Mummenhoff 851 (TMUH)</td>
<td>LC164589/LC164604/LC164627</td>
</tr>
<tr>
<td>Algeria: Podlech 36700 (TARI)</td>
<td>LC164585/LC164603/LC164621</td>
<td></td>
</tr>
<tr>
<td><em>E. Mey</em></td>
<td>South Africa: Giessjun 55 (MSB)</td>
<td>LC164582/LC164588/LC164621</td>
</tr>
<tr>
<td><em>Lessertia capitata</em> E. Mey</td>
<td>South Africa: Giessjun 55 (MSB)</td>
<td>LC164582/LC164588/LC164621</td>
</tr>
<tr>
<td><em>L. benguellensis</em> Baler</td>
<td>South Africa: Giessjun 55 (MSB)</td>
<td>LC164582/LC164588/LC164621</td>
</tr>
<tr>
<td><em>L. annularis</em></td>
<td>South Africa: Giessjun &amp; Milliss 28236 (MSB)</td>
<td>LC164581/LC164585/LC164620</td>
</tr>
<tr>
<td><em>L. benguellensis</em> Baler</td>
<td>South Africa: Giessjun 55 (MSB)</td>
<td>LC164582/LC164588/LC164621</td>
</tr>
<tr>
<td><em>Sutherlandia frutescens</em></td>
<td>South Africa: Giessjun 55 (MSB)</td>
<td>LC164582/LC164588/LC164621</td>
</tr>
<tr>
<td><em>Smirnovia turkestana</em> Bunge</td>
<td>Morocco: Vogel et al. 38117 (TARI)</td>
<td>LC164578/LC164583/LC164622</td>
</tr>
<tr>
<td><em>Podlechiella vogelii</em> (Pall.) DC.</td>
<td>South Africa: Giessjun 55 (MSB)</td>
<td>LC164582/LC164588/LC164621</td>
</tr>
<tr>
<td><em>S. kotschyana</em> Boiss.</td>
<td>South Africa: Giessjun 55 (MSB)</td>
<td>LC164582/LC164588/LC164621</td>
</tr>
<tr>
<td>Turkey: Aytac et al. 6585 (GAZI)</td>
<td>South Africa: Giessjun 55 (MSB)</td>
<td>LC164582/LC164588/LC164621</td>
</tr>
<tr>
<td><em>Sphaerophysa salsula</em> (Pall.) DC.</td>
<td>South Africa: Giessjun 55 (MSB)</td>
<td>LC164582/LC164588/LC164621</td>
</tr>
<tr>
<td><em>S. kotschyana</em> Boiss.</td>
<td>South Africa: Giessjun 55 (MSB)</td>
<td>LC164582/LC164588/LC164621</td>
</tr>
<tr>
<td>Turkey: Assadi &amp; Mozaffarian 33325 (TARI)</td>
<td>South Africa: Giessjun 55 (MSB)</td>
<td>LC164582/LC164588/LC164621</td>
</tr>
<tr>
<td><em>C. buhsei</em> (Boiss.) Shap.</td>
<td>South Africa: Giessjun 55 (MSB)</td>
<td>LC164582/LC164588/LC164621</td>
</tr>
</tbody>
</table>

4.0b10 (Swofford 2002). The heuristic search option was employed for each of the data-sets, using tree bisection-reconnection (TBR) branch swapping, with 1000 replication of random addition sequence and an automatic increase in the maximum number of trees. Uninformative characters were excluded from the analyses. Branch support values were calculated using a full heuristic search with 1000 bootstrap replicates (Felsenstein 1985) each with simple addition sequence.

**Bayesian method.** Model of sequence evolution for the combined data-set was selected using the program MrModeltest version 2.3 (Nylander 2004) based on the Akaike information criterion (AIC) (Posada & Buckley 2004). The data-set was analyzed as a single partition with GTR + I + G model using the program MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). Posteriori on the model parameters were estimated from the data, using the default priors. The analysis was done with 5 million generation, using Markov chain Monte Carlo search. MrBayes performed two simultaneous analyses starting from different random trees (Nruns = 2) each with for Markov chain and trees sampled at every 100 generations. The first 25% of trees were
discarded as the burn-in. The remaining trees were then used to build a 50% majority rule consensus tree accompanied with posterior probability (PP) values. Tree visualization was carried out using Tree View version 1.6.6 (Page 2001).

**Likelihood method.** Maximum likelihood (ML) analyses were performed for the data-sets in the program raxmlGUI (Silvestro & Michalak 2012). The model of evolution employed for each data-set is the same as that of Bayesian analyses. Parametric bootstrap values for ML were calculated in raxmlGUI based on 1000 replicates with one search replicate per bootstrap replicate.

**Incongruent length difference test.** Combinability of nuclear and plastid data-sets was assessed using the partition homogeneity test [the incongruence length difference test (ILD)] of Farris et al. 1995 as implemented in PAUP* (Swofford 2002). The test was conducted with the exclusion of invariant characters (Cunningham 1997) using the heuristic search option involving simple addition sequence and TBR branch swapping with 1000 homogeneity replicates.

**Divergence time estimation.** Divergence times were estimated from the combined phylogeny using BEAST ver. 1.7 (Drummond et al. 2012). Since the reliable fossil records for the Coluteoid clade are rather poor, the clock was calibrated using the estimate of mean age 33.0 ± 2 Mya for a node, designated as node 76, encompassing from *Caragana arborescens* through *Astragalus americanus* based upon *matK* sequence data of Lavin et al. (2005). A likelihood-ratio test implemented in MEGA 6 (Tamura et al. 2013) was used to test if the different partitions included in dating analysis were evolving clock-like. This information was used to choose between the strict-clock and the relaxed uncorrelated lognormal clock priors implemented in BEAST. In this study, strict clock prior was selected. Analyses were run for $10 \times 10^6$ generations, with a burn-in of one million generations.

**Results**

**Phylogenetic analyses.** The visual inspection showed that all of the clades present in the nrDNA ITS tree were almost recovered in the plastid (*matK + rpl32-trnL(UAG)*) tree (Figure 1). Also, the partition homogeneity test suggested that the trees obtained from the nrDNA ITS and the plastid data-sets were congruent ($p = 0.4$); and thus, we combined these data-sets (Figure 2). Both nrDNA ITS and, in particular plastid (*matK + rpl32-trnL(UAG)*) topologies have,

![Figure 1. A comparison of A. nrDNA ITS and B. *matK + rpl32-trnL(UAG)* datasets for Coluteoid clade and *Astragalus s.str.* obtained from Bayesian inference.](image-url)
however, generally lower resolution and node support than the combined data-set. We described and interpreted the results mainly based on the combined nrDNA ITS and plastid data-set.

Maximum parsimony, maximum likelihood, and Bayesian analyses of the combined data-set gave very similar results. We here show only Bayesian tree along with posterior probabilities and ML and MP

Figure 2. Fifty percent majority rule consensus tree resulting from Bayesian analysis of the combined nrDNA ITS, matK and rpl32-trnL(GAU) dataset. Numbers above branches are posterior probability and likelihood as well as parsimony bootstrap values, respectively. Values <50 % were not shown.
bootstrap values (Figure 2). The length and composition of each DNA region sequenced, as well as the tree statistics from the nuclear and plastid as well as combined analyses are summarized in Table II. As shown in Figure 2, *Oxytropis* and *Astragalus* s.str. are sister taxa and, in turn, united with the Coluteoid clade. This clade was composed of six well-supported, successive sub-clades (indicated by “I” through “VI”). *Podlechiella* was the first diverging lineage (I) as sister to the remaining subclades. Three Australian/New Zealand taxa (*Carmichaelia*, *Chianthus*, and *Swainsona*), constituted the subclade “II,” followed by *Phyllolobium* (the subclade “III”). Next clade was the South African subclade (“IV”) comprising *Lessertia* and *Sutherlandia*, followed by the Central Asian subclade (“V”) comprising *Sphaerophysa* (represented by three accessions of two species), *Smirnowia* and *Eremosparton* and the subclade *Colutea* (“VI”). Within *Colutea* subclade, *C. ciliica* Boiss. & Bal. are sister to the remaining species.

**Divergence time estimates**

Our BEAST chronogram is largely consistent with those resulting from Bayesian analyses (Figure 2). The overall estimated mean ages and mean rates of evolution are presented in Table III. Our mean nodal Bayesian divergence time estimates indicated that stem–node (the root) is estimated to be Early Oligocene, ~33 Ma. Furthermore, the analysis suggested an Oligocene–Miocene origin of the Astragalean clade (Figure 3). The most recent common ancestor (MRCA) of the Coluteoid clade was dated to the Early Miocene (20.4 Ma), most genera of which originated in the Late Miocene through Pliocene to Pleistocene (Figure 3). When two molecular clock rate variation models (strict vs. relaxed clock) were tested, the mean rate of the individual clock models did not show any variation. The fastest and the slowest rate were found in the Astragalean clade (node 1) and the Coluteoid clade (node 3), respectively. The rate of evolution in node 1 was 0.0026 substitutions per site per million years (s/s/Ma) (95% HPD: 0.0002–0.0051) and in node 3, 0.0008 s/s/Ma (95% HPD: 0.0001–0.0026).

**Discussion**

**Taxonomic status of and relationships within the Coluteoid clade**

The present work in agreement with previous studies (Wojciechowski et al. 1999; Lock & Schrire 2005; Wojciechowski 2005) retrieved the Coluteoid clade as a well-supported lineage. According to the data presented here, the clade is now only composed of 12 genera including *Podlechiella*, *Swainsona*, *Carmichaelia*, *Chianthus*, *Montigena* (not analyzed herein), *Phyllolobium*, *Lessertia*, *Sutherlandia*, *Sphaerophysa*, *Smirnowia*, *Eremosparton*, and *Colutea* (including the monotypic *Oreophysa*, Kazempour Osaloo et al. 2006). The members of the clade are characterized by having pubescent styles and mainly unilocular pods. Unlike previous studies

### Table II. Dataset and tree statistics from separate and combined analyses of the nuclear and chloroplast regions.

<table>
<thead>
<tr>
<th></th>
<th>nrDNA ITS</th>
<th>plastid (ma+K + rpl32-trnL(UAG))</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sequences</td>
<td>43</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>Nucleotide sites</td>
<td>681</td>
<td>2711</td>
<td>3392</td>
</tr>
<tr>
<td>Informativ characters</td>
<td>146</td>
<td>243</td>
<td>389</td>
</tr>
<tr>
<td>Uninformativ characters</td>
<td>535</td>
<td>2468</td>
<td>3003</td>
</tr>
<tr>
<td>CI of MPTs</td>
<td>0.647</td>
<td>0.711</td>
<td>0.676</td>
</tr>
<tr>
<td>RI of MPTs</td>
<td>0.828</td>
<td>0.808</td>
<td>0.812</td>
</tr>
<tr>
<td>Number of MPTs</td>
<td>322</td>
<td>&gt;200000</td>
<td>136584</td>
</tr>
<tr>
<td>Length of MPTs (steps)</td>
<td>326</td>
<td>418</td>
<td>752</td>
</tr>
<tr>
<td>Evolutionary model selected (under AIC)</td>
<td>SYM+G</td>
<td>GTR+G</td>
<td>GTR+I+G</td>
</tr>
</tbody>
</table>

### Table III. The node mean ages and rates and range (95% HPD) of evolution with the posterior probability are shown.

<table>
<thead>
<tr>
<th>Node</th>
<th>Node definition</th>
<th>Mean age (Ma)</th>
<th>Min (Ma)</th>
<th>Max (Ma)</th>
<th>Mean rate (s/s/Ma)</th>
<th>95% HPD (Ma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td><em>Caragana grandiflora</em> - <em>Colutea uniflora</em></td>
<td>33</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td><em>Astragalus patagonicus</em> - <em>Colutea uniflora</em></td>
<td>24.5</td>
<td>15.9</td>
<td>34.1</td>
<td>0.0026</td>
<td>0.0002–0.0051</td>
</tr>
<tr>
<td>2</td>
<td><em>Oxytropis aicheri</em> – <em>Astragalus patagonicus</em></td>
<td>20.8</td>
<td>11.7</td>
<td>34.2</td>
<td>0.0017</td>
<td>0.0002–0.0049</td>
</tr>
<tr>
<td>3</td>
<td><em>Podlechiella vogelii</em> - <em>Colutea uniflora</em></td>
<td>20.4</td>
<td>11.8</td>
<td>32.2</td>
<td>0.0008</td>
<td>0.0001–0.0026</td>
</tr>
<tr>
<td>4</td>
<td><em>Chianthus punicus</em> – <em>Swainsonia pterostylis</em></td>
<td>8.5</td>
<td>3.2</td>
<td>16.3</td>
<td>0.0013</td>
<td>0.0004–0.0034</td>
</tr>
<tr>
<td>5</td>
<td><em>Phyllolobium balfourianum</em> – <em>P chinense</em></td>
<td>6.2</td>
<td>1.6</td>
<td>13.09</td>
<td>0.0015</td>
<td>0.0004–0.0035</td>
</tr>
<tr>
<td>6</td>
<td><em>Sphaerophysa saldana</em>– <em>Smirnowia turkestana</em></td>
<td>6.3</td>
<td>2.7</td>
<td>11.4</td>
<td>0.0022</td>
<td>0.0006–0.006</td>
</tr>
<tr>
<td>7</td>
<td><em>Colutea ciliica</em> – <em>C. uniflora</em></td>
<td>3.2</td>
<td>1.3</td>
<td>6.6</td>
<td>0.0013</td>
<td>0.0003–0.0023</td>
</tr>
<tr>
<td>8</td>
<td><em>Lessertia capitata</em> – <em>Sutherlandia frutescens</em></td>
<td>4.5</td>
<td>1.5</td>
<td>9.5</td>
<td>0.0013</td>
<td>0.0003–0.0023</td>
</tr>
<tr>
<td>9</td>
<td><em>Podlechiella vogelii</em> ssp. fatimensis – <em>P vogelii</em> ssp. vogelii</td>
<td>3.2</td>
<td>0.7</td>
<td>8.1</td>
<td>0.0016</td>
<td>0.0007–0.0028</td>
</tr>
</tbody>
</table>

Notes: Node numbers refer to Figure 3. Mean ages and mean rates and 95% highest posterior density (HPD) intervals of divergence times are in millions of years before the present. Ma = million years; s/s/Ma = substitutions per site per million years.
Phylogeny and divergence times of the Coluteoid clade

In contrast, as mentioned above, our molecular data clearly revealed that *Astragalus cysticalyx*, (the herbarium specimen used for this species preserved in MSB and confirmed by D. Podlech) has no relationship with the Coluteoid clade, instead, it is well united with *A. adsurgens* Pall. within *Astragalus* s.str. clade. The identical *rpoC*, *matK*, and *nrDNA ITS* sequences of *A. cysticalyx* with *S. salsula* indicates that the voucher specimen of *A. cysticalyx* used as a source of DNA in the previous studies definitely belongs to *S. salsula*. Therefore, the Coluteoid clade has no species of *Astragalus*. The present study suggests that the Coluteoid clade should be redefined to encompass all members of the tribe Coluteae mentioned above (see “Taxonomic treatment”).

Within the Coluteoid clade, *Podlechiella* (subclade “I”) formed the most basal branch. This conclusion was first reached by Wojciechowski (2005). It is a monotypic genus with two subspecies, *P. vogelii* (Webb) (Liston & Wheeler 1994; Sanderson & Liston 1995; Wojciechowski et al. 1999; Wojciechowski 2005), results of our study indicated that neither *Astragalus sinicus* L. nor *A. cysticalyx* Lede. is closely related to the Coluteoid clade, instead nested within *Astragalus* s.str. (Figures 1 and 2). Kazempour Osaloo et al. (2003, 2005) have already revealed the accurate position of *A. sinicus* as a member of *Astragalus* s.str. and well united with the Taiwanese endemic *A. nankotaizanensis* Sasaki. Liston and Wheeler (1994), based on parsimony analysis of restriction site data of plastid gene *rpoC*, firstly pointed out that *A. cysticalyx* was placed within the Coluteoid clade. They noted that: “although this species has an identical haplotype with *Sphaerophysa salsula* (Pall.) DC., no obvious morphological characters link these two taxa.” The subsequent studies using *nrDNA ITS* and *matK* from the same aliquot (Wojciechowski et al. 1999; Wojciechowski 2005), reached the same conclusion. In contrast, as mentioned above, our molecular data clearly revealed that *Astragalus cysticalyx*, (the herbarium specimen used for this species preserved in MSB and confirmed by D. Podlech) has no relationship with the Coluteoid clade, instead, it is well united with *A. adsurgens* Pall. within *Astragalus* s.str. clade. The identical *rpoC*, *matK*, and *nrDNA ITS* sequences of *A. cysticalyx* with *S. salsula* indicates that the voucher specimen of *A. cysticalyx* used as a source of DNA in the previous studies definitely belongs to *S. salsula*. Therefore, the Coluteoid clade has no species of *Astragalus*. The present study suggests that the Coluteoid clade should be redefined to encompass all members of the tribe Coluteae mentioned above (see “Taxonomic treatment”).

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Maassoumi & Kaz. Osaloo ssp. vogelii and P. vogelii ssp. fatimensis (Chiov.) Maassoumi & Kaz. Osaloo distributed in subtropical deserts of North Africa and southwest Asia (Kazempour Osaloo et al. 2003). Among the members of the Coluteoid clade, this is the sole genus having annual life cycle, medifixed hairs, very small flowers (c. 3 mm), and autogamy (Podlech 1984, 1999, personal observation). The current hypothesis of phylogenetic relationships suggests that these features are plesiomorphic in the Coluteoid clade. The findings of the previous studies (Wagstaff et al. 1999; Wojciechowski et al. 1999; Wojciechowski 2005) are in accordance with our results in placing the Australian-New Zealand lineage (subclade “II”) as the next subclade. Another subclade is Himalayan Phyllolobium (subclade “III”) as successive sister to the remaining taxa. All Phyllolobium species are perennial and occur mainly in the southwestern high mountains of China (Zhang 2003; Zhang & Podlech 2006). As next, Lessertia and Sutherlandia (subclade “IV”) form a monophyletic group restricted to South Africa. The well-supported subclade “V” is composed of Eremosparton flaccidum Litw. together with the monotypic Smirnova (S. turkestana Bunge) and two species of Sphaerophysa. In this subclade, Eremosparton and Smirnova are restricted to central Asian deserts, Iran and Afghanistan, and Sphaerophysa has wide distribution in Central Asia, Iran, Afghanistan, and Turkey (Rechinger 1984). The last subclade (“VI”) comprises only the species of Colutea. The monophyly of this genus was also confirmed by the recent nrDNA ITS study of Mirzaei et al. (2015).

**Taxon relationships within Colutea**

*Colutea* is a medium-sized genus of ca. 30 species with shrubby to sub-shrubby habit or small tree having inflated pods (Browicz 1963a, 1968, 1984; Mabberley 2008; Pooyan et al. 2014). The genus was first subdivided into two sections, *Colutea* (as Eucolutea) and *Oreophya* Bunge ex Boiss. by Boissier (1872). In a monographic work, Browicz (1963a) classified the genus into four sections, *Armata* Browicz, *Colutea*, *Multiflora* Browicz, and *Rostrata* Browicz. Simultaneously, Browicz (1963b) resurrected *Oreophya* (Bunge ex Boiss.) Bornm. (Bornmüller 1905) as a monotypic genus, restricted to northern Iran in Elburz Mountains. In a recent molecular phylogenetic study, Kazempour Osaloo et al. (2006), however, reduced it to the sectional level, as sect. *Oreophya* within *Colutea* (*C. triphylla* Bunge ex Boiss.). Plastid and the combined analyses show that *Colutea cilicica* Boiss. & Bal. forms a sister-group to the remaining species of *Colutea*. But, in the nrDNA ITS tree, *C. arborescens* L. is the first diverging branch of *Colutea*. This discrepancy between the trees regarding the placements of these taxa may be the result of hybridization/introgression event and subsequent chloroplast capture or lineage sorting. The interspecific relationships are not sufficiently resolved within the bulk of *Colutea*. Three smaller subclades (*G. persica* Boiss./*G. komarovii* Takht.; *G. giffana* Parsa, *C. uniflora* G. Beck/C. paulsenni Freyn and *C. arborescens* L./*G. melanolaxys* Boiss.& Heldr.) with low to high supports are, however, found in the combined tree. The present data suggest that the multi-specific sections *Colutea*, *Armata*, and *Rostrata* are not monophyletic and that their representatives analyzed here are intermixed. This contradicts with the conclusions of Mirzaei et al. (2015). Hence, the delimitation of the sections based upon gross morphological characters (Browicz 1963a, 1984; Pooyan et al. 2014) seems to be artificial.

The low phylogenetic resolution within *Colutea* reflects the low sequence divergence values across it, i.e. less than 0.024 (0.000–0.023) substitutions per site for ITS and less than 0.007 (0.000–0.006) for plastid data-set. The low nucleotide variability during speciation might be an indicator of rapid radiation in a lineage’s evolutionary history (Baldwin & Sanderson 1998; Richardson et al. 2001; Hughes & Eastwood 2006; Sun et al. 2012). The lower nucleotide substitution rate among *Colutea* species might also be explained by the generation time (GT); Graur & Li 2000; Gaut et al. 2011) and the rate of mitosis (ROM; Lanfear et al. 2013) hypotheses. There is now a consensus that evolutionary rate is negatively correlated with both GT and ROM. Both phenomena in *Colutea* have to be longer and slower, respectively, because of the woody (shrub/tree) habit. Shrubs/trees are consistently evolving more slowly than related herbaceous plants (Smith & Donoghue 2008; Gaut et al. 2011; Lanfear et al. 2013).

**Divergence time estimation**

Based on an evolutionary rates analysis of the *matK* phylogeny, Wojciechowski (2005) indicated that the divergence time of the Astragalean clade is in the Early Miocene (16.1 Mya). Our dating analysis of the combined data estimates, however, the age of this clade 24.5 Mya (Oligocene-Miocene boundary), with the split between *Oxytropis/Astragalus* s.str. and the Coluteoid clade to be 20.8 and 20.4 Mya, respectively. This finding is in agreement with Axelrod’s hypothesis (1992) which postulated that representatives of the Astragalean clade were widely distributed in temperate regions in Oligocene. Our estimated mean age indicates that the Coluteoid clade dates to the Early Miocene. This is more or less consistent with estimates from analysis of *matK* sequences of Wojciechowski (2005), who suggest-
ed the mean age of 13.7 Mya (Middle Miocene). Our dating analysis indicates that the diversification of some subclades of the Coluteoid clade might have occurred contemporaneously during the Late Miocene. Among these subclades, the youngest age ones (3.2 Mya) are *Podlechiella vogelii* and *Colutea* (Figure 3). In the Coluteoid clade, the first branch, *P. vogelii*, diverged from the rest of the Coluteoid clade 20.4 Mya, although it began to diversify to two subspecies (Kazempoor Osaloo et al. 2003) in the Pliocene (3.2 Mya). Next is Australian–New Zealand subspecies (Kazempoor Osaloo et al. 2003) in the Coluteoid clade. The second subclade is *Phyllolobium*, which diverged by the Late Miocene (6.2 Mya) according to our results. Zhang et al. (2012) based on nrDNA ITS, however, estimated 3.96 Mya (95% HPD: 1.84–6.59) for *Phyllolobium* and pointed out the genus has undergone rapid diversification in the Late Pliocene and the Early-to-Mid Pleistocene which matches with the beginning of the intense uplift of the Qinghai-Tibetan Plateau during the Late Pliocene. The South African subclade (*Sutherlandia* + *Lessertia*) diverged by the Pliocene (4.5 Mya). This is consistent with recent studies which suggesting a recent burst speciation might be occurred in the flora of Southern Africa triggered by the climatic changes near the Miocene–Pliocene boundary (Schnitzler et al. 2011). The South African endemics and Carmichaeloid group have independent northern hemisphere origin within the Coluteoid clade. This conclusion was first reached by Wagstaff et al. (1999). *Sphaerophyxa*, *Smirnovia*, and *Eremosparton*, which are restricted to dry sandy deserts in Central Asia, began to diversify in the Late Miocene (8.5 Mya). During the Middle–Late Miocene (17–5 Mya), the climate in Central Asia was drying to an increasing extent (Guo et al. 2008; Miao et al. 2012; Zhang et al. 2014). This harsh environmental condition accompanied by the special life form (subshrub) and restrictions provided by reproductive characteristics of these genera might explain the lower speciation in this subclade. It appears that *Colutea* was diverged from Central Asiatic relatives (Sphaerophyxa, Smirnovia, and Eremosparton) in the Middle Miocene period (10 Mya), but its diversification occurred during the Pliocene and Pleistocene. Unlike the Central Asian desertic relatives, *Colutea* has a shrubby/tree habit and is mostly distributed locally in drier mountainous regions. During the Pleistocene, the evolution of species in the mountains was more strongly enhanced. A correlation between the shift from herbaceous/subshrubby to shrub/tree habit and the shift from lowland to montane habitats is common among alpine species that might give rise to species radiations (Hughes & Atchison 2015; Schwy et al. 2015).

**Conclusions**

The present study using multiple sequence data demonstrated that the Coluteoid clade is a well-supported lineage and thus herein redefined to interpret its own tribe Coluteae, which comprises 12 genera. *Podlechiella vogelii* is retrieved as the basal most taxon of the tribe. *Colutea* is monophyletic as sister to the Central Asian desertic genera, although the resolution and relationships within it are not sufficiently resolved. Our data clearly demonstrate that *A. cysticalyx* and *A. sinicus* have no relationship with the Coluteoid clade, but they are belonging to Astragalus s. str. Based on this study, the tribe is originated in early Miocene and diversified during the late Miocene through the Pliocene–Pleistocene. The additional taxon sampling and DNA markers especially nuclear single copy genes are definitely necessary to be analyzed for getting a clear-cut picture of *Colutea* phylogeny. Moreover, it will be also merit to focus on the evolutionary history of *Swainsona* and *Lessertia*, as the largest genera of the tribe.

**Taxonomic treatment**


Shrubs, subshrubs, and perennial or rarely annual herbs, leaves imparipinnate, rarely 1-foliolate, rarely reduced to scales; leaflets entire; stipels absent; Indumentum of basifixed and rarely medifixed hairs, flowers in axillary sometimes pendulous racemes, flowers long rarely smaller (c. 3 mm); bracts and bracteoles small or absent; calyx-teeth subequal or upper two shorter; corolla papilionaceous, standard often reflexed, stamens free, anthers uniform 2 (1)-theoccus, ovules two-numerous, style barbate, rarely glabrous. Fruit often inflated or turgid, rarely not inflated, mostly uniocular, rarely semi-bilocular, usually indehiscent. Seeds reniform. The type genus: *Colutea* L.

A key to the genera of the tribe Coluteae adopted and modified from Hutchinson (1964) and Polhill (1981).

1. Annual herbs; North Africa, and southwest Asia..........................................................*Podlechiella*
   - Perennial herbs, shrubs, subshrubs, or small trees ........................................2
2. Pods unilocular or incompletely bilocular and not inflated; southwest China. Phyllolobium
   - Pods unilocular and inflated............................3

3. Anthers monothecous; New Zealand.................................Carmichaelia
   - Anthers bithecous, separate.................................4

4. Standard long-acuminate; leaves imparipinnate; New Zealand..................Chianthus
   - Standard emarginate to shortly acuminate, leaves pinnate.....................5

5. Style apically hooked with stigma on underside; South Europe, northeast and East Africa, West and
   Central Asia..................................................Colutea
   - Style not hooked at the tip......................................6

6. Style bearded with hairs on the upper or distal (adaxial) face..........................7
   - Style bearded only around the stig-

7. Keel erect, incurved, longer than the standard; South Africa..........................Sutherlandia
   - Keel obovate, triangular, apex obtuse, shorter
   than standard; New Zealand....................................8

8. Seed-bearing suture not intruded into the loculus; South Africa and South tropical Africa..................Lessertia
   - Seed-bearing suture intruded into the loculus................................................9

9. Standard reflexed from top of claw, erect, with contrasting-colored basal patch; Australia..................
   - Standard curved up less than a right-angle toward middle of the blade, and with sides also often folded back.....................................10

10. Leaves compound, at least on flowering shoots; calyx relatively short, the lobes subequil; Central
    Asia, Iran and Turkey..............................................Sphaerophysa
    - Leaves 1(-3)-foliolate or reduced; calyx at least 1/2
    as long as keel, with upper pair of lobes broader than lower ones......................11

11. Leaves 1(-3)-foliolate; many seeded; Central Asia and Iran............................Smirnowia
    - Leaves reduced to scales; 1–3-seeded; Central Asia..........................Eremosparton

Acknowledgments
We wish to thank M. F. Wojciechowski and S. Zarre for editing and improving linguistic of the text as well as for giving useful comments.

Funding
This work was supported by Tarbiat Modares University.

References


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