SYSTEMATIC POSITIONS OF MEDICAGO EDGEWORTHII AND M. ARCHIDUCIS-NICOLAI (LEGUMINOSAE) INFERRED FROM PLASTID trnK/matK, NUCLEAR GA30X1 AND ITS SEQUENCES

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Abstract

This paper characterizes the systematic positions of Medicago edgeworthii and M. archiducis-nicolai. The combined data set of chloroplast trnK/matK, nuclear GA3ox1 and ITS sequences provided a substantial amount of informative characters. The methods of Maximum parsimony, Bayesian inference, and Maximum likelihood were employed. The results showed that M. edgeworthii formed a monophyletic group with M. biflora and M. brachycarpa, both of which are members of section Lunatae; M. archiducis-nicolai is closely related to M. platycarpa and M. ruthenica. Our study supports the previous view that M. edgeworthii belongs to section Lunatae, and M. archiducis-nicolai belongs to section Platycarpae. In addition, the study suggests that M. lupulina is a member of a clade having M. tenoreana and M. minima, which indicates that M. lupulina and M. secundiflora should probably not be placed in the same section.

Introduction

Medicago L. (Leguminosae) is distributed from the Mediterranean to central Asia and consists of about 87 species including some important forage species such as M. sativa L., M. scutellata (L.) Mill. and M. lupulina L. (Small, 2011). Systematists had been progressively revising the genus Medicago and clarifying the systematic position of its species (Urban, 1873; Lesins & Lesins, 1979; Small, 1987a, b; Small & Jomphe, 1989a, b). Bena (2001) concluded that 23 Trigonella species previously known as medicagoids were better placed in Medicago rather than assigned to a new genus by using nrDNA ITS and ETS sequences. In addition, the phylogenetic researches of Steele et al., (2010) using 73 Medicago species and plastid trnK/matK and nuclear GA3ox1 supported certain currently recognized taxonomic groups, e.g., section Medicago (with M. sativa) and section Buceras. But some strongly supported clades, related to M. lupulina, M. murex, M. polymorpha, and M. truncatula, contradict the current classification. Small (2011) divided the genus into 14 sections based on both morphological and nucleotide sequences from molecular data, e.g. plastid gene (trnK/matK), mitochondrial region (rps14-cob), nuclear genes (GA3ox1, CNGC S, β-cop, ITS and ETS).

The systematic position of M. edgeworthii is controversial (Small & Jomphe, 1989b; Maureira-Butler et al., 2008; Small, 2011). Moreover, M. archiducis-nicolai, a valuable forage species was not included in previous studies. In the present study, we focus on the systematic positions of M. edgeworthii and M. archiducis-nicolai inferred from the combined sequences of plastid trnK/matK and nuclear GA3ox1 and ITS, based on Bena (2001) and Steele et al., (2010).

This paper firstly explores the systematic positions of M. edgeworthii and M. archiducis-nicolai, using the combined dataset of chloroplast trnK/matK, nuclear GA3ox1 and ITS sequences. In addition, this is the first study that uses molecular data to examine the systematic position of M. archiducis-nicolai.

Materials and Methods

Plant samples: The species used for the present study are listed in Table 1. They were all collected in the field in China. Healthy, clean leaves were fast-dried using silica gel. The voucher specimens have been deposited at the Herbarium of Wuhan Botanical Garden, Chinese Academy of Science (HIB).

DNA sequencing and alignment: Total genomic DNA was isolated using the modified CTAB method (Doyle & Doyle, 1987). The polymerase chain reaction (PCR) was used for double stranded DNA amplification. Each 25 μL reaction contained 0.25 μL of Ex Taq (2.5 μL), 2.5 μL of 10× Ex Taq buffer (Mg2+ concentration of 25 mM), 2.0 μL of dNTP mix (at 2.5 mM concentration for each dNTP), 1 μL of each, forward and reverse primers at 5 μmol/μL. The following molecular markers primers were used: plastid trnK/matK (Hu et al., 2000; Steele and Wojciechowski, 2003; Wojciechowski et al., 2004; Bruneau et al., 2008), nuclear GA3ox1 (Steele et al., 1999) and ITS (Bena, 2001) sequences. For PCR amplifications, pre-denaturation was first conducted at 94°C for 5min, followed by 30 cycles of (1) denaturation at 94°C for 30 s, (2) annealing at 50°C-58°C for 30 s, and (3) extension at 72°C for 1 min. At the end of the cycles, a final extension was used at 72°C for 10 min. The PCR products were purified and sequenced by Sangon Biotech (Shanghai) Co., Ltd.

Clustal X (Thompson et al., 1997) was used to produce an aligned matrix, which was corrected manually using the BioEdit program (Hall, 1999). All gaps were treated as missing characters. Finally, the sequences of trnK/matK, GA30x1 and ITS were combined for phylogenetic analyses.
Phylogenetic analyses: The phylogenetic analyses (Maximum Parsimony, Bayesian Inference and Maximum Likelihood) of combined datasets of trnK/matK, GA3oxI and ITS sequences were conducted using PAUP* 4.0b10 (Swofford, 2002), MrBayes 3.1 (Huelsenbeck & Ronquist, 2001) and Phylm 3.1 (Guindon & Gascuel, 2003), separately. Maximum parsimony searches were performed using heuristic search methods: tree-bisection-reconnection (TBR), branches collapsed (creating polytomies) if the maximum branch length was zero, and all characters weighed equally. The analyses were repeated 100 times with a random order of sequence addition in an attempt to weigh the results equally. The analyses were repeated 100 times maximum branch length was zero, and all characters (TBR), branches collapsed (creating polytomies) if the maximum branch length was zero, and all characters weighed equally. The analyses were repeated 100 times with a random order of sequence addition in an attempt to sample multiple islands of the most parsimonious trees. Bootstrap analyses (Felsenstein, 1985) under MP analyses were performed to assess the relative support of the branches. Heuristic search settings identical to those above were used to estimate bootstrap values (BS) with 10,000 replicates. Bayesian analyses were conducted using MrBayes, version 3.1.1 (Ronquist & Huelsenbeck, 2003). Four chains were run (Markov Chain Monte Carlo), beginning with a random tree and saving a tree every 100 generations, for one million generations. For searching the likelihood tree, we used Phylm 3.1. Support rates are calculated by 1000 repeat. For ML analyses, Modeltest 3.7 (Posada & Crandall, 1998) was used to select the best model (GTR+G+I) for the combined dataset based on the Akaike information criterion (Akaikie, 1974).

The incongruence length difference (ILD) test (Farris et al., 1994; 1995) for the combined dataset of three genes was implemented in PAUP*.

Results

Aligned DNA sequences: DNA site variation and tree statistics from maximum parsimony analyses for combined dataset are shown in Table 2. The three-gene dataset was not significantly incongruent based on the ILD tests ($P = 0.134$).

Phylogenetic analyses: The MP analysis of combined data constructed one most parsimonious tree of 3344 steps (Fig. 1, CI=0.60, RI=0.76, RC=0.45). The numbers of MP, PP and ML stand for bootstrap percentages of MP, Bayesian posterior probabilities and ML bootstrap support values found in parsimony, Bayesian and ML trees, separately. M. edgeworthii is related to M. biflora and M. brachycarpa with higher support value (MP/PP/ML = 73/1.0/95), M. biflora and M. brachycarpa are members of section Lunatae. Section Buceras is sister of section Lunatae (MP/PP/ML = 72/0.92/89). The clade formed by M. platycarpus, M. archiducis-nicolai and M. ruthenica is strongly supported (MP/PP/ML = 100/1.0/100). M. lupulina (section Luparia), M. tenoreana (section Spirocarpos, subsection Leptospireae) and their sister species, M. minima (also subsection Leptospireae) form a well-supported clade (MP/PP/ML = 100/1.0/100). M. secundiflora, the only species in section Luparia, is not included in this group.

Table 1. Information of taxa used for the present study.

<table>
<thead>
<tr>
<th>Species</th>
<th>GenBank #</th>
<th>Locality information</th>
<th>Voucher</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. archiducis-nicolai</td>
<td>KC333393, KC333397</td>
<td>Dawu Xian, Ganzi, Sichuan, China</td>
<td>J.Q. Li 972 (HIB)</td>
</tr>
<tr>
<td>M. edgeworthii Sirj. ex Hand.-Mazz</td>
<td>KC333394, KC333398</td>
<td>Dafou Xian, Ganzi, Sichuan, China</td>
<td>J.Q. Li 973 (HIB)</td>
</tr>
<tr>
<td>M. ruthenica (L.) Ledebour.</td>
<td>KC333396, KC333400</td>
<td>Bei’an Shi, Heihe, Heilongjiang, China</td>
<td>J.Q. Li 961 (HIB)</td>
</tr>
<tr>
<td>M. lupulina L.</td>
<td>KC333395, KC333399</td>
<td>Balikun Xian, Hami, Xinjiang, China</td>
<td>J.Q. Li 938 (HIB)</td>
</tr>
</tbody>
</table>

Table 2. DNA site variation and tree statistic from separate maximum parsimony analyses for combined dataset.

<table>
<thead>
<tr>
<th>Result</th>
<th>trnK/matK+GA3oxI+ITS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of species</td>
<td>66</td>
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<tr>
<td>Number of sequences</td>
<td>70</td>
</tr>
<tr>
<td>Number of characters</td>
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<tr>
<td>Number of variable sites</td>
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<td>Number of informative sites</td>
<td>885</td>
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<td>No. trees</td>
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</tr>
<tr>
<td>Tree length</td>
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</tr>
<tr>
<td>CI</td>
<td>0.60</td>
</tr>
<tr>
<td>RI</td>
<td>0.76</td>
</tr>
<tr>
<td>RC</td>
<td>0.45</td>
</tr>
</tbody>
</table>

CI, consistency index; RI, retention index; RC, rescaled consistency index

Discussion

Based on the research of Bena (2001) and Steele (2010), four species M. edgeworthii, M. archiducis-nicolai, M. ruthenica and M. lupulina were used in this study. Of these, M. ruthenica and M. lupulina had been previously collected by Steele (2010), but we collected our own specimens of these species in China and included these in our study (Fig. 1). M. edgeworthii and M. archiducis-nicolai were not involved in the data matrix of Bena (2001) and Steele (2010). Overall, the topology of our phylogenetic tree is consistent with that of previous research.

Systematic position of M. edgeworthii: The genus Medicago belongs to the Subtribe Trigonellinae of Tribe Trifolieae together with genera Trigonella and Melilotus. Within Medicago, the species in section Buceras and section Lunatae all have pulvinate cotyledons, distinct from other species of Medicago (Small & Brookes, 1984; Small, 1987a).

Section Lunatae, is known to consist of M. biflora, M. brachycarpa, M. huberi and M. rostrata. In addition M. edgeworthii was previously attributed to section Platycarpae (a group without pulvini) (Small, 1989b), but Maureira-Butler et al., (2008) found M. edgeworthii was in a strongly supported group with two of the species from section Lunatae: M. brachycarpa and M. huberi.
Interestingly, Small (2011) indicated that contrary to previous reports (e.g., Small & Jomphe, 1989b), *M. edgeworthii*, does have pulvini. Referring to molecular evidences of Maureira-Butler et al. (2008), Small (2011) transferred *M. edgeworthii* from section *Platycarpae* to section *Lunatae*. In this study, by using plastid *trnK/matK* and nuclear *GA3ox1* and ITS sequences, the systematic position of *M. edgeworthii* is obvious. *M. edgeworthii* is shown to have close relationship with *M. biflora* and *M. brachycarpa* (Fig. 1). This coincides with the classification system of Small (2011). Our results also support the results of Maureira-Butler et al., (2008). Therefore, combined molecular and morphological data confirm that *M. edgeworthii* should be placed in section *Lunatae*.

Systematic position of *M. archiducis-nicolai*: *M. archiducis-nicolai* is endemic in China, and usually grows on upland slopes, valleys or grasslands at 3000-4000 m. Based on its distinct morphological characteristics, it was placed in section *Platycarpae* (Small, 1989b and 2011). It has epulvinate cotyledons and uncoiled, very flat pods.
Acknowledgements

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References


Bena, G. 2001. Molecular phylogeny supports the morphologically based taxonomic transfer of the “medicagoid” *Trigonella* species to the genus *Medicago*. *Lupularia*, subsection *Leptospireae* and their sister species, *minima* (subsection *Leptospireae*) (Fig. 1) constituted a well-supported clade. *Medicago* is more appropriate to consider as sister species. Based on the present study it seems that it is more appropriate to consider *M. lupulina*, *M. tenoreana* and *M. minima* as sister species.

and these characteristics also found in *M. playtcarpos* and *M. ruthenica*. Molecular evidence about the systematic position of *M. archiducis-nicolai* has not been previously reported. The species in section *Playtcarpeae* are *M. playtcarpos*, *M. ruthenica*, *M. hybrida* and *M. archiducis-nicolai* (Small, 1989b). The topology of our tree gives a similar result, with high support (MP/PP/ML = 100/1.0/100) (Fig. 1). Considering the strong relationship between *M. archiducis-nicolai* and *M. playtcarpos*, *M. archiducis-nicolai* can be placed in section *Playtcarpeae*.

*Medicago lupulina* and *M. secundiflora* are currently placed in section *Lupularia* (Small & Jomphe, 1989b). However, in our analyses (Fig. 1), *M. lupulina* and *M. secundiflora* were found to belong to separate clades. *M. lupulina* (section *Lupularia*), *M. tenoreana* (section *Spirocarpos*, subsection *Leptospireae*) and their sister species, *minima* (subsection *Leptospireae*) (Fig. 1) constituted a well-supported clade. *Medicago* is more appropriate to consider as sister species. Based on the present study it seems that it is more appropriate to consider *M. lupulina*, *M. tenoreana* and *M. minima* as sister species.


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