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SYSTEMATIC POSITIONS OF *MEDICAGO EDGEWORTHII* AND *M. ARCHIDUCIS-NICOLAI* (LEGUMINOSAE) INFERRED FROM PLASTID *TRN*K/*MAT*K, 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 *trn*K/*mat*K, 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 *trn*K/*mat*K 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 (*trn*K/*mat*K), mitochondrial region (*rpS14-cob*), nuclear genes (*GA3ox1*, *CNGC* 5, *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 *trn*K/*mat*K 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 *trn*K/*mat*K, 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 u/μL), 2.5 μL of 10× Ex Taq buffer (Mg^{2+} 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 *trn*K/*mat*K (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, predenaturation 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 *trn*K/*mat*K, *GA30x1* and ITS were combined for phylogenetic analyses.

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

Species	GenBank #			Locality information	Voucher
	***trn* K/*mat*K**	***GA3ox1***	**ITS**		
M. archiducis-nicolai Sirj.	KC333393	KC333397	KC333389	Dawu Xian, Ganzi, Sichuan, China	J.Q. Li 972 (HIB)
M. edgeworthii Sirj. ex Hand.-Mazz	KC333394	KC333398	KC333390	Daofu Xian, Ganzi, Sichuan, China	J.Q.Li 973 (HIB)
M. ruthenica (L.) Ledebour.	KC333396	KC333400	KC333392	Bei'an Shi, Heihe, Heilongjiang, China	J.Q. Li 961 (HIB)
M. lupulina L.	KC333395	KC333399	KC333391	Balikun Xian, Hami, Xinjiang, China	J.Q. Li 938 (HIB)

Phylogenetic analyses: The phylogenetic analyses (Maximum Parsimony, Bayesian Inference and Maximum Likelihood) of combined datasets of *trn*K/*mat*K, *GA3ox1* and ITS sequences were conducted using PAUP* 4.0b10 (Swofford, 2002), MrBayes 3.1 (Huelsenbeck & Ronquist, 2001) and Phyml 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 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 Phyml 3.1, Support rate are calculated by1000 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 (Akaike, 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. platycarpos*, *M. archiducis-nicolai* and *M. ruthenica* is strongly supported (MP/PP/ML = 100/1.0/100). *M. lupulina* (section *Lupularia*), *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 *Lupularia*, is not included in this group.

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

Result	***trn*K/*mat*K+*GA30x1*+ITS**
Number of species	66
Number of sequences	70
Number of characters	5200
Number of variable sites	1596
Number of informative sites	885
No. trees	1
Tree length	3344
CI	0.60
RI	0.76
RC	0.45

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*.

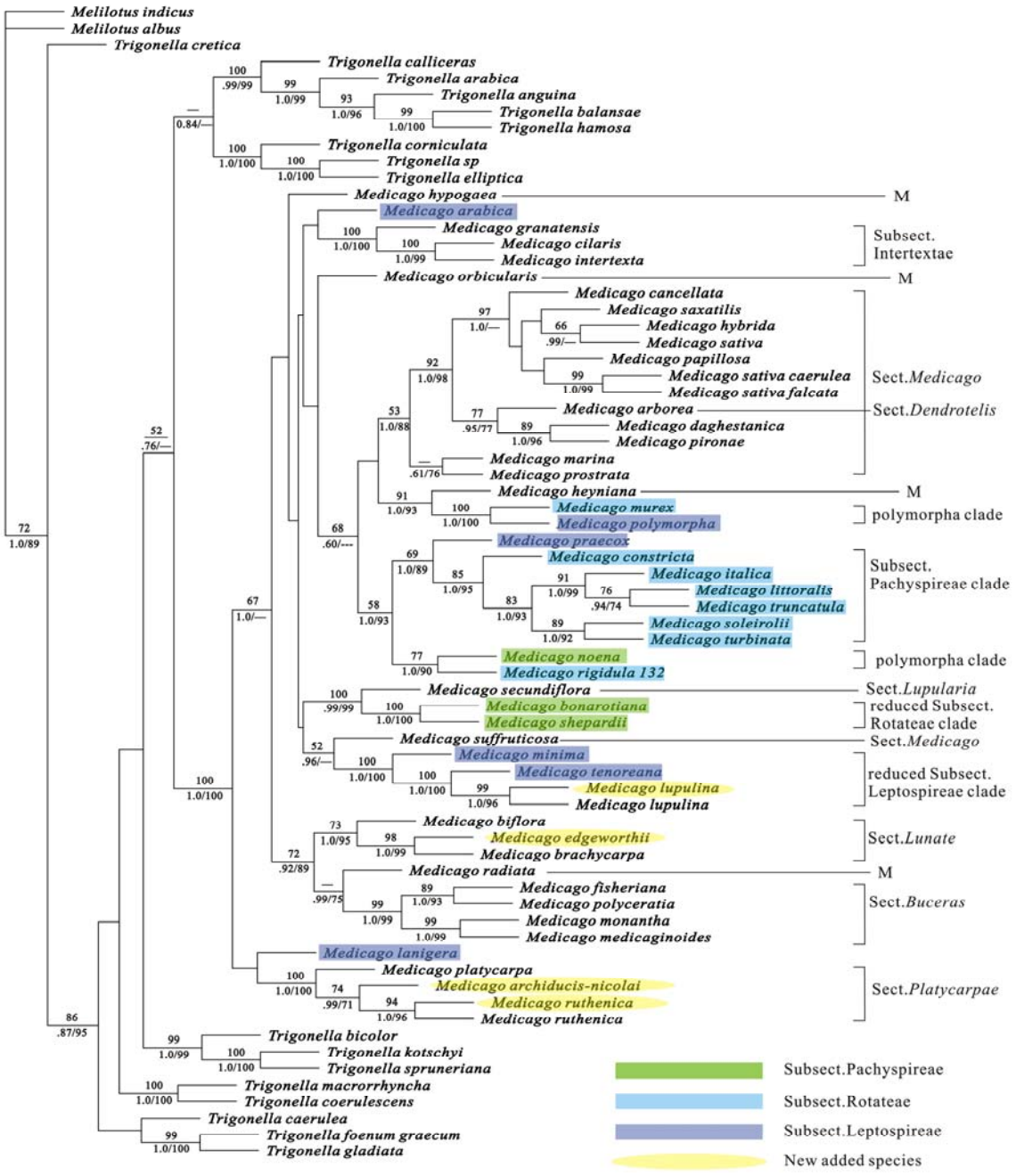

Fig. 1. Phylogenetic relationships of *Medicago* based on MP analyses of *trn*K/*mat*K+*GA30x1*+ITS combined sequences. Numbers along branches indicate bootstrap percentages above 50%. Numbers below branches are Bayesian posterior probabilities and ML bootstrap support values, indicated for those clades found in parsimony, Bayesian and ML trees. "M" indicates monotypic sections.

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 *trn*K/*mat*K 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,

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 *Platycarpae* 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 *Platycarpae*.

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, *M. minima* (subsection *Leptospireae*) (Fig. 1) constituted a well-supported clade. *M. secundiflora,* the only other species in section *Lupularia*, cannot be accommodated within the current classification scheme (Small & Jomphe, 1989b), though our molecular analysis corresponds well with previous molecular studies (Downie *et al*., 1998; Bena, 2001; Maureira-Butler *et al*., 2008; Steele *et al*., 2010). Considering the differences in morphology, karyotypes and restriction endonuclease fragment patterns of the chloroplast genome between the two species, we agree with Steele *et al*., (2010) that section *Lupularia*, that currently consists of the two species *M. lupulina* and *M. secundiflora*, should no longer be recognized. 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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