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Full Length Research Paper

Analysis of chloroplast ribosomal subunit S16 (*rpS16*) intron sequences in *Morus* (Urticales: Moraceae)

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In this study, the chloroplast *rps*16 sequence variation of *Morus* was examined. Sequence data were obtained from 18 mulberry individuals belonging to 13 species and three varieties, and two accessions of *Broussonetia papyrifera* and *Ficus carica* of the related Moraceae, designed as outgroup were analyzed. The nucleotide diversity (0.016±0.006) covered 113 polymorphic sites of which 22 were parsimony informative. A total of 20 haplotypes were identified, producing a high overall haplotypic diversity (1.00±0.02). Inferred phylogenetic relationship using the neighbor-joining method indicated genus *Morus* was a monophyletic and phylogenetic relationship among 18 mulberry materials was further determined. The result from cluster analysis indicates that they are basically consistent with the morphological classification.

Keywords: Mulberry, Phylogeny, *rps* 16.

INTRODUCTION

Mulberry (*Morus* L.; Family Moraceae) is a perennial and economically important plant in the sericulture industry and has traditionally been used for feeding the monophagous silkworm, *Bombyx mori* L. Furthermore, being a perennial tree crop with a crop cycle of over 50 years, mulberry offers additional benefits such as conservation of soil and water, enhancement of biodiversity by providing shelter to shade loving plants, and food to birds and small animals. Mulberry has a long cultivation history and is widely distributed in China, India, Bangladesh, Pakistan, and several other Asian countries. Mulberry (*Morus*) is believed to have originated in the northern hemisphere, particularly in the Himalayan foothills, and spread to the tropics of southern hemisphere (Benavides et al., 1994; Hou, 1994). While reviewing the centers of origin of crop plants, Vavilov (1951) placed *Morus* L. in China–Japan center of plant origin. Most of the contemporary molecular studies also revealed an early diversification of Moraceae in Eurasia and subsequent migration into the southern hemisphere (Zerega et al., 2005).

Taxonomy of the genus *Morus* was started by Linneaus (1753) by recognizing seven species based on morphological characteristics; considerable differences exist among systematists as to the number of species that exist in this genus (Koidzumi, 1917, 1923; Hotta, 1958; Katsumata, 1972; Airy, 1973). So far, more than 150 species of mulberry have been cited in the Index Kewensis, but a majority of them have been treated either as synonyms or as varieties rather than species, and some have been transferred to allied genera. It is remarkable that *Morus* is the only genus of the Moraceae that has not been revised yet (Berg, 2001). Nonetheless, species of mulberry have now been widely recognized and majority of them are found in Asia, especially in China, Japan, Korea, and India (Datta, 2000). However, it is important to note that most of these species undergo natural cross hybridization and produce fertile hybrids (Dwivedi et al., 1989; Tikader and Dandin, 2001). Moreover, most of the putative mulberry species are dioecious and can cross-pollinate among

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Abbreviations: *rps*16, ribosomal subunit **S16; RAPD,** random amplified polymorphic **DNA.**

Table 1. Information of 20 accessions used in this study

Number	Species	Accession	Origin	GeneBank accession number
1	*M.atropurea* Roxb.	Lunjiao 40	Shunde city, Guangdong province, China	EF687665
2	*M.cathayana* Hemsl.	Baojing 5	Baoqing city, Hunan province, China	EF687666
3	*M.alba* var. *venose* Delile.	Wenqisang	Zhouzhi city, Shanxi province, China	EF687668
4	*M.mizuho* Hotta.	Housang	Yuhang city, Zhejiang province, China	EF687669
5	*M.alba* Linn.	Niuersang	Yangcheng city, Shanxi province, China	EF687670
6	*M.atropurea* Roxb.	Qiner	Shunde city, Guangdong province, China	EF687671
7	*M.wittiorum* Hand-Mazz.	Qianesang 1	Dejiang city, Guizhou province, China	EF687673
8	*M.nigra* Linn.	Yaosang	Xingjiang autonomous region, China	EF687674
9	*M.mongolica* Schneid.	Jimengsang	Jilin province, China	EF687675
10	*M.rotundiloba* Koidz.	T12	Thailand	EF687676
11	*M.mongolica* var.*diabolica* Koidz.	Youmaoyansang	Guizhou province, China	EF687677
12	*M.Laevigata* Wall.	Dejiang 10	Dejiang city, Guizhou province, China	EF687678
13	*M.alba* var. *macrophlla* Loud.	Gongxianheiyou	Gongxian city, Sichuan province, China	EF687679
14	*M.bombycis* Koidz	Changnongshan	Shandong province, China	EF687680
15	*M.Australis* Poir.	Chasang	Sichuan province, China	EF687681
16	*M.alba* Linn.	Sanglian	Fujian province, China	EF687682
17	*M.alba* var. *pendula* Dipp.	Chuizhisang	Korea	EF687667
18	*M. multicaulis* Pree.	Husang32	Zhenjiang city, Jiangsu province, China	EF687683
Outgroup				
19	*Broussonetia* papyrifera L.	Goushu	Zhenjiang city, Jiangsu province, China	EF687672
20	*Ficus carica* Linn.	Wuhuaguo	Zhenjiang city, Jiangsu province, China	EF687684

All mulberry materials sampled were from National mulberry Genbank in the Sericultural Research Institute, CAAS, Zhenjiang, Jiangsu Province, China.

themselves to produce fertile hybrids, suggesting that they have relatively close genetic relationships. Such a high degree of cross-species repro-ductive success is not encountered often in nature, and thus, their “species” status needs to be investigated further (Wang and Tanksley, 1989). Another reason for this paucity of information on the general taxonomy of Morus is that most of the recent investigations were confined only to the sericulturally important species. Therefore, information on other species and their relationships with these species remains very scanty (Dandin, 1998; Zhao et al., 2006).

Molecular methods, including deoxyribonucleic acid (DNA) sequence analysis, have wide application in solution of controversial phylogenetic and taxonomic problems. Plant molecular systematics studies often refer to polymorphisms in organelles DNA sequence and in the first place chloroplast DNA (cpDNA) (Sang et al., 1997; Gielly and Taberlet, 1994; Hanilton et al., 2003). The ribosomal subunit S16 (*rps*16) intron was chosen because the marker has proven useful for inferring phylo-genetic relationships at generic or higher levels (Andersson and Rova, 1999; Bremer and Manen, 2000; Nie et al., 2005; Abbasi et al., 2010). For the first time, the gene for the small *rps16*, containing a group II intron was used by Oxelman et al. (1997) and then frequently has been applied to molecular phylogeny studies of various plant taxa.

Earlier, the nucleotide variability including ITS, cpDNA *trn*L-F and *trn* L intron in *Morus* was determined (Zhao et al., 2004, 2005; Wang et al., 2008). However, because of ambiguous results, additional studies are still needed including intraspecies diversity analyses. The aim of this work was to evaluate *rps*16 intron variability for clarifi-cation of *Morus* phylogenetic relationship at different taxonomic levels.

MATERIALS AND METHODS

Materials

18 accessions representing 11 species and three varieties of genus *Morus* and two accessions of *Broussonetia papyrifera* and *Ficus carica* Linn. of the related Moraceae, designated as outgroup species were included in this study. All mulberry specimens were deposited in the National Mulberry Genbank of the Sericultural Research Institute, Chinese Academy of Agricultural Sciences (CAAS), Zhenjiang, Jiangsu province, China (Table 1).

DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

Total genomic DNA was extracted from 2 g of fresh leaves of

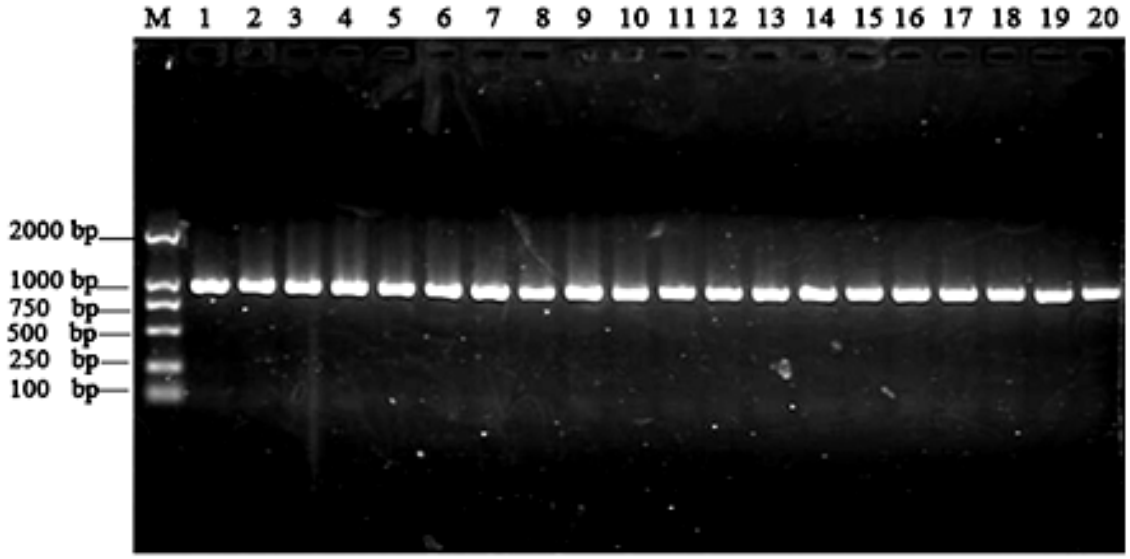

Figure 1. Electrophoretic pattern amplified in 20 materials. Lane 1-20 represents accessions, as shown in Table 1. M is a DNA marker DL 2,000 (MBI).

mulberry using the CTAB method (Zhao and Pan 2004). PCR amplification were performed with universal primers for *rps*16 (5'-AAACGATGTGGTAGAAAGCAAC-3') and "f" (5'-AACATCAATT-GCAACGATTCGAT A-3') (Oxelman et al., 1997). PCR amplification was conducted in a 25 µl volume containing 2.5 mM $MgCl_2$, 200 µM of each dNTP, 5 pM of each primer, 1.5 U of Taq DNA polymerase (Takara Bio Inc.), 10 x PCR Buffer (100mM Tris-HCl pH 8.3, 500mM KCl, 0.01% gelatin), and approximately 25 ng DNA template. Amplification reaction was carried out with following thermal cycles profiles: 1 cycle for 5 min at 95℃ then 25 cycles of 30 s at 95℃, 30 s at 58℃, 1 min at 72℃ followed by a final extension of 7 min at 72℃. The fragment amplified was purified, ligated into the clone vector and transformed into the *E. coli* competent cells. Finally, the recombinant fragment was sequenced by Sangon (Shanghai, China).

Data analysis

Sequence alignments were conducted using the Clustal X, version 1.81 (Thompson et al., 1997) and finally adjusted manually where necessary. The insertion/deletion mutations (indels) of unambiguous alignment were recoded as separate characters appended in the matrix. The data matrices are available upon request from the authors. The aligned sequences were analyzed for diversity using DnaSP version 5.0 (Librado, P. and Rozas, 2009). Two estimates of diversity, π and θ, were calculated. π is the average number of nucleotide differences per site between two sequences and θ per site is derived from the total number of mutations (Eta) and corrected for sample size (Rozas et al., 2003). The phylogenetic tree was constructed by MEGA4.0 using the neighbor-joining method (Tamura et al., 2007).

RESULTS AND DISCUSSION

PCR amplification with primers proposed by Oxelman et al. (1997) yielded fragments of predicted length (about 1kb) in all samples (Figure 1). Sequences of *rps* 16 were deposited in the GenBank database under the accession numbers EF687665–EF687684. The *rpS16* intron amplicons vary in length from 938 bp in *F. carica* to 973 bp in *Morus*. The average nucleotide composition of all sequences was 36.1% A, 31.3%T, 14.2% G and 18.4% C and the average nucleotide content of A+T (67.4%) was obviously higher than that of G+C (40.44%), indicating that they are AT-rich. These data are in agreement with nucleotide compositions of the *rpS16* intron in other plant taxa (Andersson and Rova, 1999; Lee and Hymowitz, 2001).

The results obtained by using DNAsp 5.0 (Librado, P. and Rozas, 2009) revealed a low mean nucleotide diversity (π) (0.016±0.006) covering 113 polymorphic sites (11.3% of the whole alignment) of which 22 were parsimony informative. A total of 20 haplotypes (excluding sites with gaps and missing data) were identified, producing high overall haplotypic diversity (1.00 ± 0.02). Theta (θ) (per site) from Eta was 0.03604, the total number of mutations was 117, while the total number of InDel sites was 85.

Sequence alignment revealed that all the nucleotide sequences above appear to be rather conserved. The identity between these sequences varies in a range from 93.0 to100 %, while the average identity between all mulberry accessions sequence was above 99%, with a range from 95.1 to 100%. In addition, the identity between mulberry accessions and genus *F. carica* sequence was below 94.0% while the identity between mulberry accessions and genus *B. papyrifera* sequence was below 95.0%. The result shows that mulberry accessions had higher genetic similarity than genus *B. papyrifera* and *F. carica.*

Molecular phylogenetic analysis was done using *rpS16*

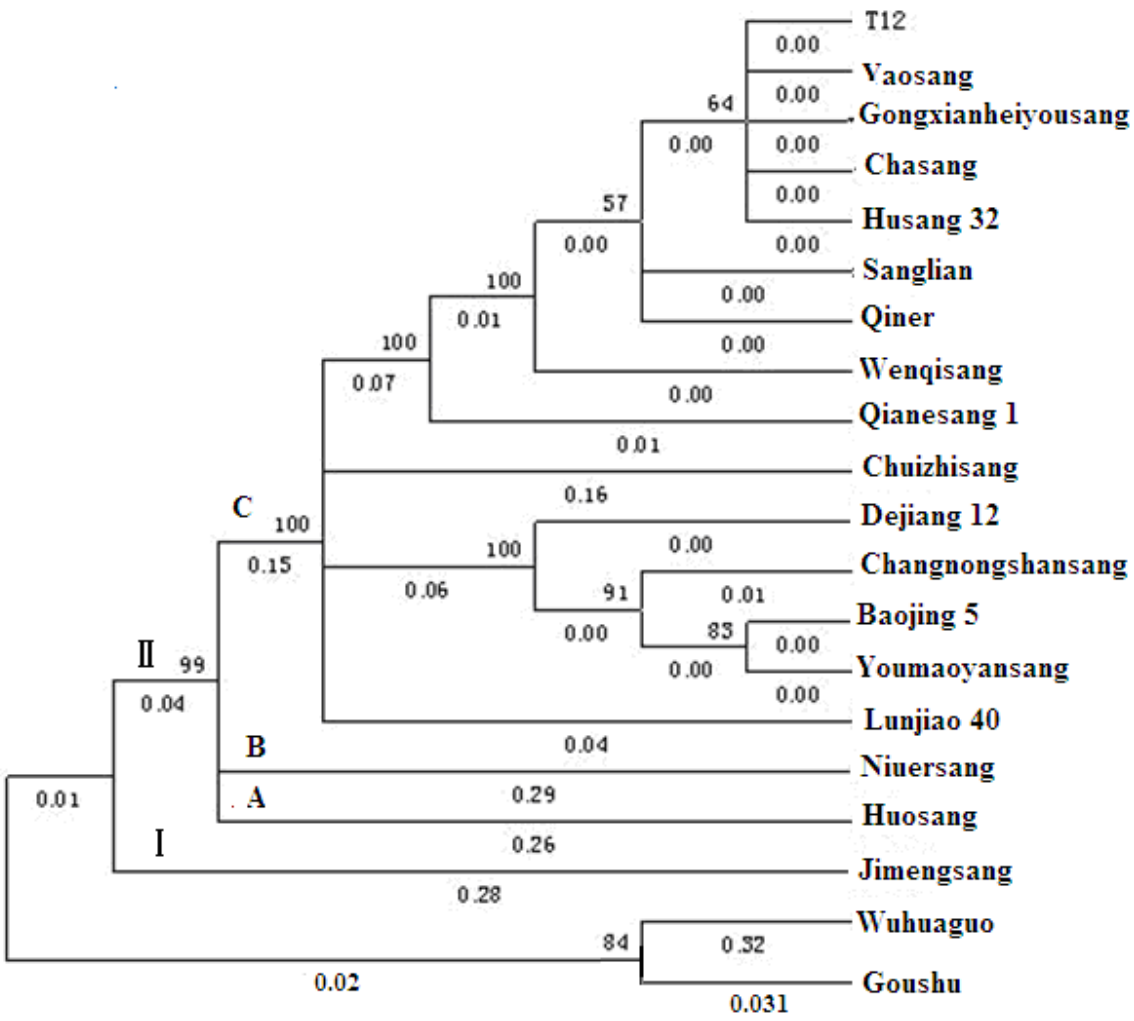

Figure 2. Phylogenetic trees obtained from the analysis of *rps* 16 data through the neighbor-joining method.

sequence data through the neighbor-joining method (Figure 2). The clustering result show that all species of genus *Morus* were grouped together, which indicated that the genus *Morus* was a monophyletic. The above result obtained from DNA sequences was in consistent with previous research based on the study on ITS, *trn*L-F and *trn*L intron sequences, isozymes and random amplified polymorphic DNA (RAPD) marker (Hirano, 1982; Zhao et al., 2004; Zhao and Pan, 2004; Zhao et al., 2005; Wang et al., 2008). In the genus *Morus* clade, Jimengsang was generally the most distant varieties in comparison with other mulberry varieties clustered into cluster I group; other mulberry cultivars were clustered into cluster II group. The above result is consistent with previous research based on the study on ITS and *trn*L intron sequences (Zhao et al., 2004; Wang et al., 2008). The cluster II was divided into three subgroups: A, B, and C cluster groups. Niuersang and Huosang formed independently the A and B subgroups, respectively. In C subgroup, Dejiang 12, Changnongshansang, Baojing 5 and Youmaoyansang were grouped together, indicating their closer relationship. T12, Yaosang, Gongxian-heiyousang, Chasang and Husang 32 were grouped together with above 99.0% sequence similarity, indicating a closer relationship. The results from cluster analysis were basically in agreement with the existing morphologic classification. This study also indicate that phylogenetic analysis using cpDNA *trn*L-F intergenic spacer region sequence data offer a reliable and an efficient method of assessing phylogenetic relationship but more definitive resolution of the problems existing in mulberry classification may require utilization of results from additional studies using nuclear along with extra-nuclear informative data in future.

Conclusion

The phylogeny of genus *Morus* inferred from *rps* 16 sequences is congruent with our current understanding of

the group. These DNA regions offer a reliable and an efficient method of assessing phylogenetic relationship at the interspecific and intergeneric levels in mulberry. It is also helpful for the conservation and identification of mulberry collections, and in mulberry breeding.

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