African Journal of Biotechnology Vol. 4 (6), pp. 563-569, June 2005
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2005 Academic Journals

Full Length Research Paper

Phylogeny of the genus *Morus* (Urticales: Moraceae) inferred from ITS and *trn*L-F sequences

Zhao Weiguo[1,2], Pan Yile[1]*, Zhang Zhifang Jia Shihai[2], Miao Xuexia[2], Huang Yongping[2]

[1]Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang City, Jiangsu Province 212018, China.
[2]Shanghai Institute of Plant Physiology and Ecology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, China.

Accepted 20 April, 2005

Both nuclear ribosomal ITS and chloroplast *trn*L-F sequences were acquired from 13 mulberry genotypes belonging to nine species and three varieties, and one paper mulberry. The later belongs to genus *B. papyrifera,* designed as outgroup, and were analyzed. Within the genus *Morus*, the sequence diversity of ITS was much higher than that of *trn*L-F. The results of phylogenetic analyses based on these data (separately or combined) show that the genus *Morus* is monophyletic group. Strict consensus tree obtained through the Neighbor-joining method can be divided into five major clades in the genus *Morus*, according to combined sequence data. *M. bombycis*, *M. alba* var. *venose* formed clades A and B, respectively. Clade C comprises of 5 species; *M. rotundiloba, M. atropurpurea, M. mongolica, M. australi,* and *M. mongolica* var. *diabolica*. Clade D comprises of 3 species; *M. wittiorum, M. laevigata,* and *M. alba*. Clade E comprises of 2 species; *M. multicaulis,* and *M.alba* var. *macrophylla*. The results from cluster analysis were basically in agreement with the existing morphologic classification.

Key words: *Morus*, phylogeny, ITS, *trn*L-F.

INTRODUCTION

Mulberry (genus: *Morus*), grown as a perennial tree or shrub is an economically important plant used for sericulture, as it is the sole food plant for the domesticated silkworm, *Bombyx mori*. *Morus*, which is widely distributed in Asia, Europe, North and South America, and Africa, is cultivated extensively in East, Central and South Asia for silk production. A few species of mulberry are also valued for their edible fruit (*M. alba* and *M. laevigata*),and timber (*M. laevigata* and *M. serrata*). Whereas it has been widely believed that mulberry species originated on the low slopes of the Himalayas bordering China and India, the study of Hou suggests a multicentered origin (Hou, 1994).

Mulberry belongs to the order Urticales and the family Moraceae. Since the classification of the genus *Morus* was firstly established by Linnaeus in 1753 mainly 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 Shaw, 1973). Major contribution to the systematic of *Morus* was made by Koidzumi (1917, 1923). He classified the species under two sections, the Dolichostylae (long style) and the Macromorus (short style), under which recognized the group Papillosae and Pubescentae based on the nature of stigmatic hairs. For further classification, he used morphological characters of leaf, inflorescence and sorosis. So far, more than 150 species of mulberry have

*Corresponding Author. E-mail: wgzsri@sina.com.

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. Because of environmental influence, phenotypic traits in many cases fail to serve as unambiguous markers for systematics and diversity analysis (Wang, 1989). Moreover, most of the putative mulberry species are dioecious and can cross-pollinate among themselves to produce fertile hybrids, suggesting that they have relatively close genetic relationships. Such a high degree of cross-species reproductive success is not encountered often in nature, and has thus created considerable doubt with regard to the species status of mulberry. In addition, taxonomic studies have been confined only to the sericulturally important species; and for other species, enough information is not available. At present the classification of the *Morus* is rather highly debated and arbitrary one (Dandin, 1998).

Recent advance in molecular biology provides a convenient and rapid assessment of the differences in the genetic composition of the related individuals using DNA sequence data. In China, there are about 3,000 mulberry germplasm resources, which comprise 15 species and 4 varieties, its types and quantities ranks No.1 in the world (Pan, 2000). Taking advantage of numerous mulberry resources in China, we here assess the relationships of *Morus* and related genus with sequences from the chloroplast *trn* L(UAA)-*trn*F(GAA) region (*trn*L-F), and the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. Both of these regions have proven phylogenetically informative for inferring relationships in various plant groups (Carine Brouat et al., 2001; Akiko et al., 2004; Gang Hao et al., 2004), particularly at the intergeneric or interspecific levels (e.g., Chassot et al., 2001; Mast et al., 2001). The main objectives of this study are: (1) to evaluate molecular phylogenies of *Morus*, and (2) to assess whether they corroborate existing classifications of the genus.

MATERIALS AND METHODS

Materials

Fourteen individuals representing 9 species and 3 varieties of *Morus* and one individual of paper mulberry belonging to genus *B. papyrifera*, designated as outgroup, were included in this study. All mulberry genotypes are deposited in the National Mulberry Gene Bank of the Sericultural Research Institute, Chinese Academy of Agricultural Sciences (CAAS), Zhenjiang, Jiangsu province, China (Table 1).

DNA extraction

Total genomic DNA was extracted from fresh young leaves following the method described by Doyle and Doyle (1987) and modified as follows: 1.5 g young leaves were ground in liquid nitrogen to a fine powder and extracted with hot cetyltrimethylammonium bromide (CTAB) extraction buffer [50 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, 2% PVP(w/v) and 1% (v/v) β-mercaptoethanol]. The mixture was incubated at 60℃ for 30 min, followed by two extractions with chloroform/isoamyl alcohol (24:1). Isopropanol was used to precipitate nucleic acids and the pellet was dissolved in Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0). RNA was removed by digestion with deoxyribonuclease-free ribonuclease and remaining impurities were extracted with chloroform. Total DNA was precipitated using cold ethanol, the precipitate was washed twice with 75% ethanol, and the pellet was dissolved in TE buffer. The purified total DNA was quantified by gel electrophoresis and its quality verified by spectrophotometry. DNA samples were stored at 4℃.

PCR amplification

PCR amplification was performed in a 50 µl volume containing 2.5 mM $MgCl_2$, 200 µM of each dNTP, 5 pM of each primer, and 1.5 U of Taq DNA polymerase (Takara Bio Inc.), 10x PCR Buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 0.01% gelatin), and approximately 25 ng DNA template. The *trnL-trnF* intergenic spacer was amplified with primers “e” and “f”(Taberlet et al., 1991). The primers "ITS5" and "ITS4" (White et al., 1990) were used to amplify the ITS fragment comprising ITS1, the 5.8S gene, and ITS2. Amplification reaction was carried out with following thermal cycles profiles: 1 cycle for 10 min at 95℃, then 35 cycles of 30 s at 95℃, 30 s at 55℃, 90 s at 72℃, followed by a final extension of 7min at 72℃.

PCR product purification and sequencing

The PCR product was run through a 1% agarose gel in 1x TBE buffer at 70-100 V. The PCR amplification band was excised from the agarose gel with a sterile razor blade. The excised band was incubated with 500-600 µl of sodium iodide (NaI) solution (6 M) at 55℃ in a waterbath to melt the gel. Approximately 10 µl of GLASSMILK suspension was added and incubated at room temperature for 5 min. The solution was mixed with GLASSMILK by inversion every 1-2 min to ensure that it stays suspended. After 5 min, the GLASSMILK was spun down in a microcentrifuge for a few seconds. The NaI supernatant solution was removed and the GLASSMILK pellet was washed three times with 500-600 µl of NEW WASH solution (10 mM Tris-HCl, 0.5 mM EDTA, 50 mM NaCl, 50% ethanol). After washing, the pellet was dried for 5 min. The pellet was resuspended with 20 µl of sterile ddH_2O and incubated at 55℃ in a waterbath for 3 min. Then the pellet was spun down in a microcentrifuge for a few seconds and the supernatant containing eluted DNA fragment was transferred into a new microfuge tube. The purified PCR product was sequenced on an ABI 373 automated DNA sequencer using the Dye Terminator Cycle Sequencing Reaction Kit (PE Applied Bio-systems). Each fragment was sequenced for both strands.

Data analysis

Sequences of ITS and *trn*L-F were aligned with Clustal X, version 1.81, applying the default parameters (Thomson et al., 1997) and then manually adjusted for insertion/deletion mutations (indels) where necessary. The *trn*L-F sequences required only minor manual adjustment for alignment. ITS sequences required a higher level of manual adjustment for alignment. The regions of alignment ambiguity were excluded from phylogenetic analyses. The indels of unambiguous alignment were recoded as separate characters appended in the matrix. The data matrices are available upon request from the authors. The phylogenetic tree was constructed by the Neighbor-joining (NJ) method, version 3.6b of PHYLIP software. The phylogenetic tree was evaluated with bootstrap test on 1000 resamplings (Felsenstein, 1985).

Table 1. The source of materials and GenBank Accession No.

Taxon	Accessions	Ploidy and chromosome number	Locality	Morphological characters	GenBank Accession No. (ITS; *trnL-F*)
M. multicaulis Perr.	Naxisang	2n=2x=28	Naxi city, Sichuan province, China	Style absent or indistinct, stigma papillose with protuberances inside. Leaf cordate generally unlobed, surface has wrinkled appearance, margin serrate, apex sharp obtuse, base cordate.	AY345153; AY271293
M. alba Linn.	Niuersang	2n=3x=42	Yangcheng city, shanxi province, China	Style absent or indistinct, stigma papillose. Leaf ovate or wide ovate, lobed and unlobed, surface smooth without wrinkles, apex acute or acuminate, margin usually blunt serrate or dentate, few serrulate or denticulate, base indented or shallowly cordate.	AY345157; AY271279
M.. alba var *macrophylla* Loud.	Gongxianheiyousang	2n=2x=28	Gongxian city, Sichuan province, China	Style absent or indistinct, stigma papillose. Leaf lager, surface smooth, apex acuminate, base shallowly cordate or trunate.	AY345148; AY271289
M. alba var *venose* Delile.	Wenqisang	2n=2x=28	Zhouzhi city, shanxi province, China	Style absent or indistinct, stigma papillose. Leaf ovate with white vein, apex acuminate or argutidentate, margin serrate.	AY345149; AY271275
M. atropurpurea Roxb.	Lunjiao 40	2n=3x=42	Sunde city, Guangdong province, China	Style absent or indistinct, stigma pubescentes. Leaf smaller generally unlobed, surface smooth or little wrinkled appearance, glossy, apex long acuminate or caudate, base shallowly cordate or trunate.	AY345145; AY271270
M. bombycis Koidz	Jianchi	2n=2x=28	Japan	Female flower with distinct long style, stigma papillose or pilose. Leaf mostly dentato-serrate, crenato-dentate rarely argutidentato-serrate margin. Bud scales glabrous	AY345151; AY271290
M. laevigata Wall.	Dejiang 10	2n=3x=42	Dejiang city, gueizhou province,China	Style very short, stigma papillose inside. Flower in spikes of 4-16 cm length. Leaf ovate or wide ovate, comparatively larger, no hairs on ventral part, margin serrulate, apex long cuspidate, base shallow cordate to truncate.	AY345147; AY271288
M. mongolica Schneid.	Jimengsang	2n=2x=28	Jilin province,China	Style long, stigma papillosae. Leaf ovate or ovato-elliptical, smooth without hair, apex long caudate or long acuminate, margin with triangle serrate, base cordate or trunate.	AY345158; AY271285
M. mongolica var. *diabolica* Koidz	Youmaoyansang	2n=2x=28	Guizhou province, China	Style long, stigma papillosae. Leaf ovate-rotundate or cordate ,usually lobed and hairy, apex caudate , margin with triangle serrate.	AY345146; AY271287
M. rotundiloba Koidz.	T11	2n=2x=28	Thailand	Style long, stigma lancecolate, minutely papillose.Leaf margin dentato-serrate and denticels are nearly equal. Leaf is usually trilobate and apex of the lateral lobes is rotundate	AY345150; AY271286
M. wittiorum Hand-Mazz.	Qingesang 1	2n=2x=28	Dejiang city, gueizhou province,China	Style very short, stigma papillosae. Leaf long elliptical or elliptical generally unlobed, surface smooth , no hairs on ventral part, apex caudate, margin shallowly serrate or rotundate, base rotundate.	AY345154; AY271283
	Gui 14	2n=4x=56	Biejie city, gueizhou province,China		AY345155; AY271284
M. australis Poir.	Chasang	2n=2x=28	Sichuan province,China	Style long, stigma pilose, shoot errect. Under surface of leaf light green and slightly hairy, leaf ovate, ovato-oblong or ovato-elliptical, apex caudate, margin serrate with short prickles.	AY345152; AY271292
outgroups	*B. papyrifera*	_	Nanjing Forestry University,China		AY345156; AY271281

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

Table 2. Length (bp) and G+C contents of ITS1, 5.8s rDNA and ITS2 of genus Morus.

Materials	ITS1		5.8S rDNA		ITS2	
	Length (bp)	G+C %	Length (bp)	G+C %	Length (bp)	G+C %
Naxisang	189	59.3	152	55.3	217	60.8
Niuersang	181	59.1	152	54.6	217	62.2
Gongxianheiyousang	189	59.3	152	55.3	209	62.7
Wenqisang	190	60.0	152	55.3	219	62.1
Lunjiao 40	189	59.3	152	55.3	208	62.5
Jianchi	188	58.5	152	42.1	210	57.8
Dejiang 10	189	59.8	152	55.3	208	63.0
Jimengsang	197	58.9	154	55.2	210	62.9
Youmaoyansang	189	59.3	152	55.3	210	62.9
T11	189	59.3	152	55.3	211	62.6
Qianesang 1	189	59.8	152	55.3	213	61.5
Gui 14	189	59.8	152	55.3	209	62.9
Chasang	189	59.3	152	55.3	210	62.9
Average	**189**				**212**	

RESULT

The ITS sequence

The complete sequences of ITS1 and ITS2 (including 5.8 S, and a part of the 18 S and 26 S gene of rDNA) were determined for all mulberry materials. The sequence boundaries between the two ITS regions and three coding regions (18 S, 5.8 S, and 26 S) of rDNA in *Morus* were determined following Shi et al. (2001). The length of ITS1 varied between 181 and 197 bp and the length of ITS2 varied between 208 and 219 bp, while the length of the 5.8 S gene was consistent (152 bp) throughout all the mulberry taxa investigated, except for *M. mongolica* (154 bp) (Table 2). The 18 bp at the 3'-end of the 18 S gene and the 15 bp at the 5'-end of the 26 S gene was included in the phylogenetic analysis. Thus a total of 610 nucleotide positions (a part of 18S, ITS1, 5.8 S, ITS2, and a part of 26 S) was aligned as the ITS data set. Sixty-six variable positions (33.0%) within all materials are found in the ITS1 extended matrix, 47 of which are informative sites. Twenty-three variable positions (11.5%) within *Morus* are found in the ITS1 extended matrix, 19 of which are informative. Eighty-one variable positions (36.5%) within *Morus* are found in the ITS2 extended matrix, 46 of which are autapomorphic. ITS2 has higher sequence diversity than ITS1. The identity between these sequences varies in a range from 51.0 to100%, while the average identity between all mulberry accessions sequence is 96.2%, with a range from 90.0% to 100%. In addition, the identity between mulberry accessions and genus *B. papyrifera* sequence is below 60.0%. The result showed higher genetic similarity among mulberry accessions than with *B. papyrifera.*

Molecular phylogenetic analysis was done using ITS sequence data with *B. papyrifera* as an outgroup species. Strict consensus tree was obtained through the neighbor-joining (NJ) method (Figure 1). The clustering result showed that all species of genus *Morus* grouped together, which indicated that *Morus* is monophyletic. The four major clades formed are indicated in Figure 1.

The *trn*L-F sequence

The *trn*L-F IGS region sequences were determined in this study and their length varied from 360 to 367 bp. The average nucleotide composition was 0.29935(A) 0.37448(T) 0.18379(C) 0.14238(G). The mean G+C content is 0.32617 %, which indicated that the spacer region sequences were characterized by AT-rich fragment. After alignment, there are 372 base positions in the *trn*L-F data matrix, of which 41 nucleotide sites were variable (11.02%) and 23 were parsimony-informative. Within *Morus*, there are 12 variable sites (3.2%), and 9 were potential informative sites. The average identity between these sequences is 98.1%, with a range from 89.9 to100 %, while the average sequence identity within *Morus* is 99.3%, with a range from 98.0 to100%. In addition, the average identity between mulberry accessions and *B. papyrifera* sequence is below 90.2%. The result further proved that among mulberry accessions there is higher genetic similarity, compared with *B. papyrifera.*

Molecular phylogenetic analysis was done using *trn*L-F sequence data of each region with genus *B. papyrifera* as an outgroup species. Strict consensus tree was obtained through the neighbor-joining method (Figure 2). The clustering result also showed that all species of *Morus*

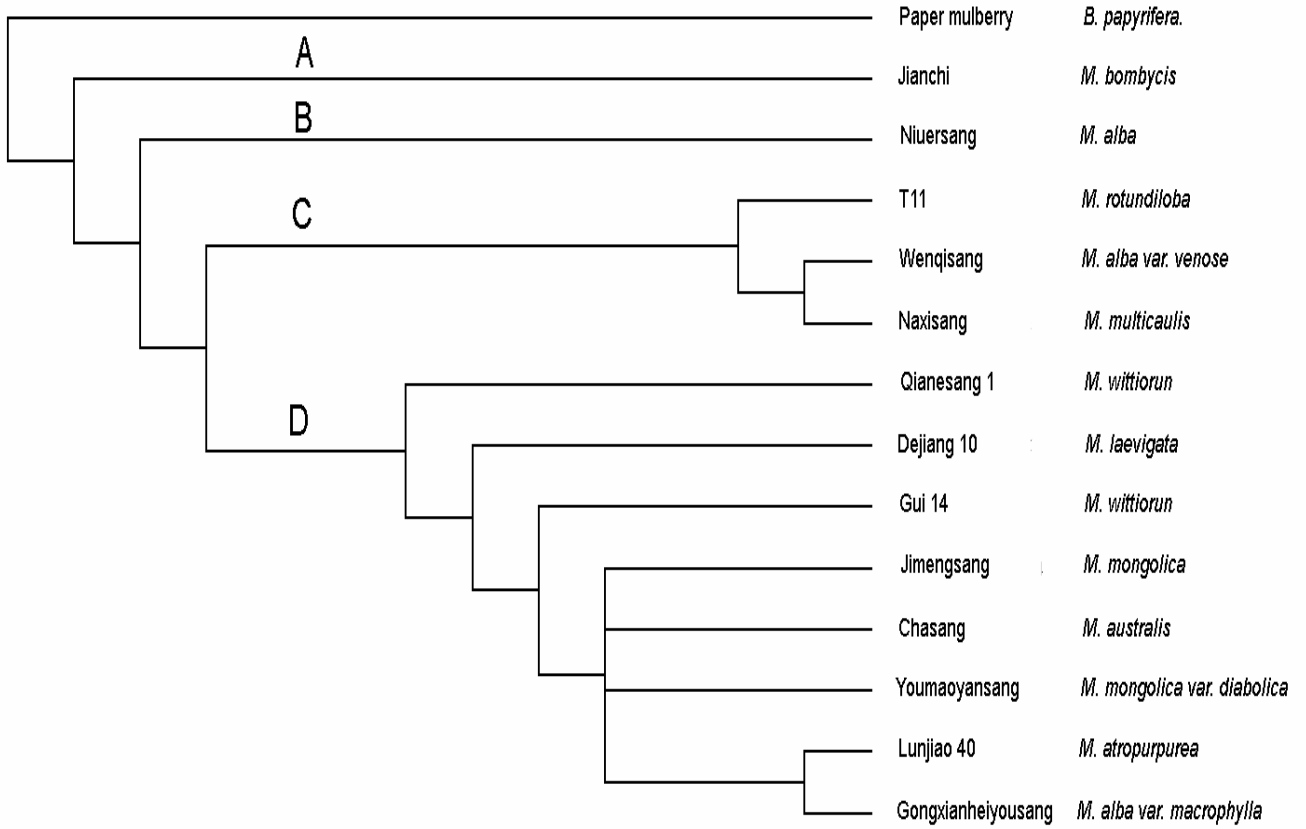

Figure 1. Strict consensus phylogenetic trees obtained from the analysis of ITS data alone through the neighbor-joining method.

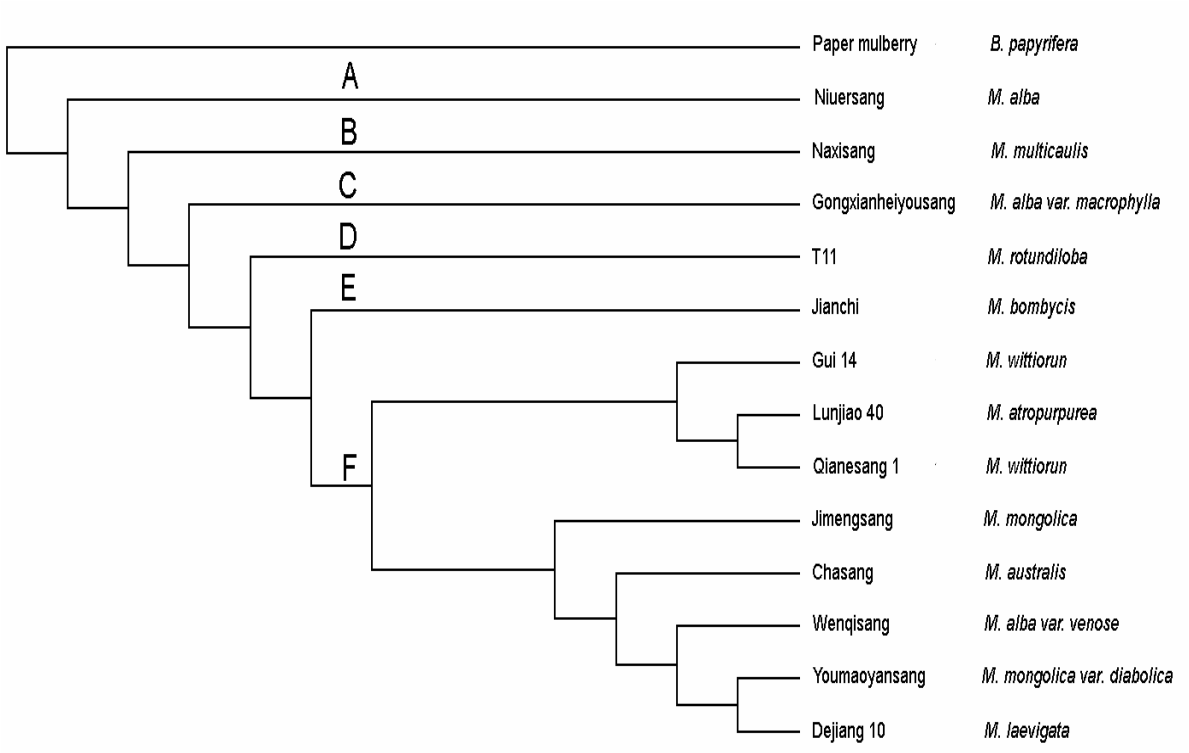

Figure 2. Strict consensus phylogenetic trees obtained from the analysis of *trn*L-F data alone through the neighbor-joining method.

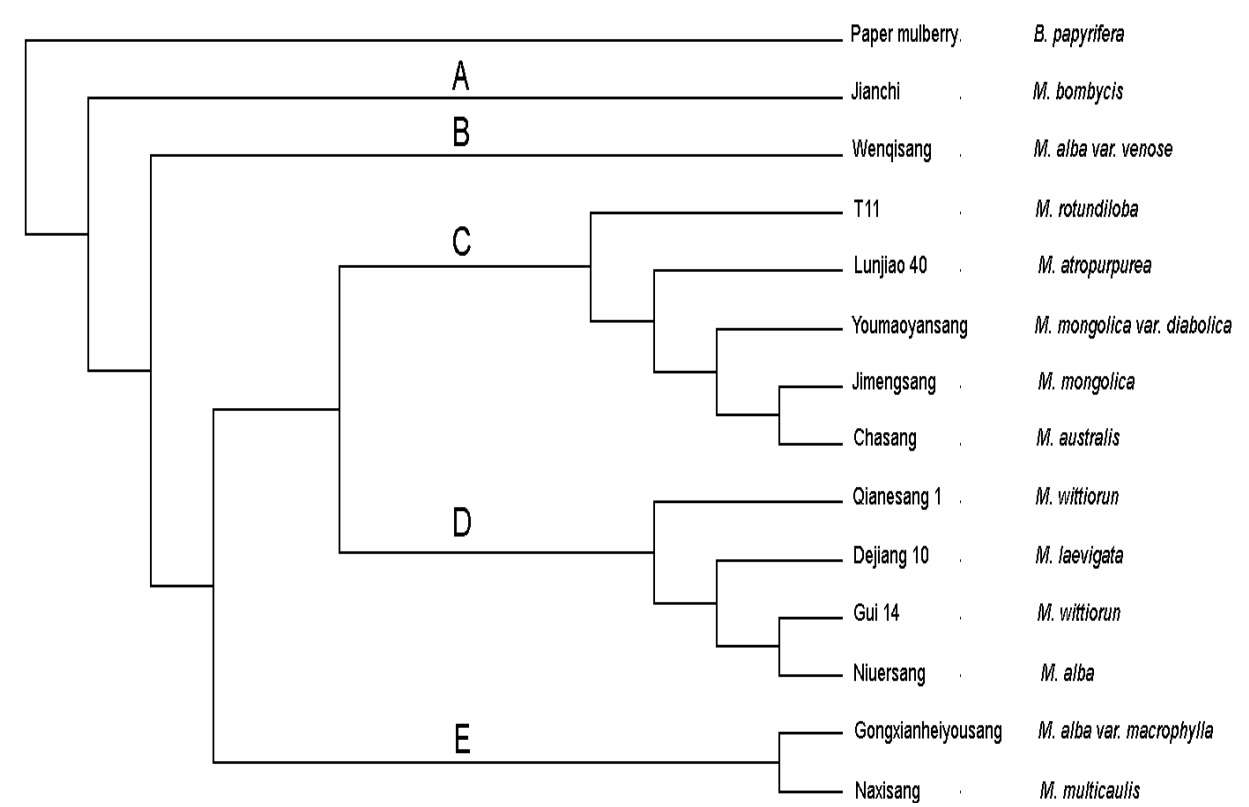

Figure 3. Strict consensus phylogenetic trees obtained from the analysis based on the combined *trn*L-F and ITS data through the neighbor-joining method.

grouped together, which indicated that *Morus* is monophyletic. Six major clades were formed, as indicated in Figure 2. The relationships among those clades resolved by the *trn*L-F has some difference from those derived from the ITS data (Figure 1). However, the clade F in the trnL-F tree was basically consistent with the clade D in the ITS tree, all including *M. wittiorum, M. atropurpurea, M. mongolica, M. mongolica* var. *diabolica, M. laevigata, M. australis.*

Combined analysis

Because the phylogenetic signal recovered from both datasets is mostly concordant and there are no strongly conflicting placements within the ingroup, we assumed that the two sequenced molecules have evolved in parallel along the lineages that resulted in the present day taxa and that their respective data matrices could be combined into a single dataset and used for simultaneous analysis. The partition homogeneity test indicates that there is no significant incongruence between them. Therefore, the sequence data of ITS and trnL-F were combined for additional analysis. The combined *trn*L-F and ITS matrix was 982 bp long. Strict consensus tree was also obtained through the Neighbor-joining method (Figure 3). The clustering result also proved that *Morus* is a monophyletic. Five major clades were formed, as indicated in Figure 3.

DISCUSSION

Mulberry is a perennial, heterogeneous outbreeding tree, the leaves of which are the exclusive food of the silk secreting insect, *B. mori*. Outbreeding in cultivated mulberry species is common and inter-species hybridization is often observed. Because of phenotypic plasticity, the occurrence of interspecific hybridization, mutation, and the absence of an unambiguous set of criteria for designating a true species, there is confusion in the systematic classification of mulberry. The present work evaluates the phylogenetic relationships of genus *Morus* using *trn* L-F and ITS sequences.

Within *Morus*, the sequences of ITS is much longer than that of *trn*L-F. Twenty-three variable positions (11.5%) were found in the ITS1, Eighty-one variable positions (36.5%) were found in the ITS2, but there are only 12 variable sites (3.2%) in the *trn*L-F region. The three strict consensus trees from ITS, trnL-F and combined data showed that all species of *Morus* grouped together, which indicated that *Morus* is monophyletic.

This result obtained from DNA sequences is consistent with previous research basing on study on isozymes and RAPD marker (Hirano 1982; Zhao et al, 2000). In addition, because there is no significant difference between the two data sets and the combined data analysis shows better resolution, the phylogenetic discussions below are based on this combined consensus tree.

Strict consensus tree obtained through the neighbor-joining method can be divided into five major clades, according to combined sequence data. *M. bombycis* and *M. alba* var. *venose* formed clades A and B, respectively. Clade C comprises of 5 species with the character of early budding; *M. rotundiloba, M. atropurpurea, M. mongolica, M. australi,* and *M. mongolica* var. *diabolica*. All species belong to Dolichostylae in the the morphological classification of *Morus* (Koidzumi, 1917), except *M. atropurpurea*. Clade D comprises of 3 species*; M. wittiorum, M. laevigata,* and *M. alba*, all of which belong to Macromorus Papillosae in the the morphological classification. Clade E comprises of *M. multicaulis* and *M. alba* var. *macrophylla,* also belonging to Macromorus Papillosae in the morphological classification. The results from cluster analysis were basically in agreement with the existing morphologic classification.

In summary, the phylogeny of *Morus* inferred from the ITS and *trn*L-F 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.

ACKNOWLEDGMENTS

We express our thanks to Assoc. Prof. Zhang Lin, Mr. Liu Li and Mr. Cheng Yan for their assistance in conducting the experiments.

REFERENCES

Airy Shaw HK (1993). A dictionary of flowering plants and ferns. 8th edition (JC Willis, revised by H. K.Airy Shaw) Cambridge University Press, London.

Akiko S, Hidetoshi N (2004). Phylogenetic analysis of Asian *Symplocos* (Symplocaceae) based on nuclear and chloroplast DNA sequences. J. Plant Res.

Chassot P, Nemomissa S, Yuan YM, Küpfer P (2001). High paraphyly of *Swertia* L. (Gentianaceae) in the *Gentianella*-lineage as revealed by nuclear and chloroplast DNA sequence variation. Plant Syst. Evol. 229: 1-21.

Carine B, Ludovic G, Doyle McKey (2004). Phylogenetic relationships in the genus *Leonardoxa* (Leguminosae: Caesalpinioideae) inferred from chloroplast *t*rnL intron and *t*rnL-*t*rnF intergenic spacer sequences. Am. J. Bot. 88:143-149

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19:11-15

Dandin SB (1998). Mulberry a versatile biosource in the service of mankind. Acta Sericol. Sin. 24(2):109-113.

Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791.

Gang H, Yong-Ming Y, Chi-Ming H, Xue-Jun G, Nan-Xian Z (2004). Molecular phylogeny of *Lysimachia* (Myrsinaceae) based on chloroplast *trn*L-F and nuclear ribosomal ITS sequences. Mol. Phylogenet. Evol. 31(1): 323-339.

Hotta T (1958). Taxonomical studies on the *Morus* plants and their distributions in Japan and its viscinties. Japanese Society For Promotion of Science Ueno Park, Tokyo, Pp. 1-161.

Hou YJ (1994). Mulberry breeding. Sericulture Department, Zhejiang Agriculture University, Hangzhou, China, p.4.

Hirano H (1982). Thremmatological studies of protein variation in mulberry (in Japanese). Bull. I mp. Sericult. Exp. Sta. 28: 67-186.

Koidzumi G (1917). Taxonomical discussion on *Morus* plants .(in Japanese) Bull. Imp. Sericult. Exp. Sta. 3:1-62.

Linnaeus C (1753). Species Plantarum, Vol. 2. Stockholm. p. 986.

Koidzumi G (1917). Taxonomical discussion on *Morus* plants. Bull. Imp. Sericult. Exp. Stat. 3:1-62.

Koidzumi G (1923). Synopsis specierum generis *Mori*. Bull. Imp. Sericult. Exp. Stat. 11:1-50

Katsumata T (1972). Mulberry species in west Jawa and their peculiarities. J. Sericult. Sci. Jpn. 42: 213-23.

Mast A, Kelso S, Richard AJ, Lang DJ, Feller DMS, Conti E (2001). Phylogenetic relationships in *Primula* L. and related genera (Primulaceae) based on noncoding chloroplast DNA. Int. J. Plant Sci. 162: 1381-1400.

Pan YL. (2000). Progress and prospect of germplasm resources and breeding of mulberry. Acta Sericol. Sin. 26(Suppl): 1-8.

Shi QL (2001). Preliminary study on ITS sequencing and characteristic of Mulberry. Acta Sericol. Sin. 27: 140-141.

Taberlet P, Gielly L, Pauton G, Bouvet J (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol. Biol. 17: 1105-1109.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The Clustal-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876-4882.

Zhao WG, Pan YL (2002). RAPD analysis for the germplasm resources of genus mulberry. Acta Sericol. Sin. 26(4):1-8.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M, Gelfand D, Sninsky J, White T (Eds.) 1990. PCR Protocols: A Guide to Methods and Applications, Academic Press, San Diego, CA. pp. 315-322.

Wang ZY, Tanksley SD (1989). Restriction fragment length polymorphism in *Oryza sativa* L. Genome 32: 1113-111.