Effect of Cell Culture on 18S rRNA Gene Sequences in the Cultural Course of *Taxus chinensis* Cells

Fu Xiang[a], Long J. Yu[a,*], Wu Chen[b], and Zhi Liu[a]

[a] College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China. E-mail: xiangfu@smail.hust.edu.cn
[b] Wuhan University of Science and Engineering, Wuhan 430074, China

* Author for correspondence and reprint requests

Z. Naturforsch. **63c**, 127–132 (2008); received July 5/August 14, 2007

Cell culture is an effective technology for taxol production. This paper discusses the effect of *Taxus* cell cultures on the 18S rRNA gene sequences based on the phylogenetic analysis of cultured *T. chinensis* cells and related species. The phylogenetic tree is reconstructed using the maximum parsimony method and the relative rate test to test the hypothesis of a molecular clock. The phylogenetic analysis indicates that cell culture changes the phylogenetic position of cultured *T. chinensis* cells. More than that, the 18S rRNA gene of cultured *T. chinensis* cells has a faster rate of substitution than that of *T. chinensis*. With *T. media* as reference, the divergence time of the cultured *T. chinensis* cells is 7 Ma (million years) more than that of the *T. chinensis* cells based on the 18S rRNA gene sequences.

Key words: 18S rRNA Gene, Cultured Cells, *Taxus chinensis*

Introduction

Taxol is a natural anticancer drug produced by *Taxus* species. The traditional production method, which draws taxol from the plants directly, has a low producing rate and destroys wild resources of *Taxus*. Tissue and cell culture is a kind of extremely effective technology for taxol production (Hirasuna *et al.*, 1996). The effect of cell culture on the chromosome in the cultural course of *Taxus* cells had been studied, and the results conflict with each other (Gu *et al*., 1991; Yang *et al*., 1994; Zhang *et al*., 1997; Yu *et al*., 1998; Wang *et al*., 2000a). Here, we try to discuss the effect of cell culture on *Taxus* cells further based on the 18S rRNA gene sequences. The 18 rRNA is a highly conserved (least variable) gene. For this reason, genes that encode the rRNA (rDNA) are sequenced to identify an organism's taxonomic group, to calculate related groups, and to estimate rates of species divergence. Based on the phylogenetic analysis of cultured *T. chinensis* cells and related species, we will show the effect of the cell culture on the 18S rRNA gene sequences in the cultural course of *T. chinensis* cells.

Materials and Methods

Phylogenetic analysis

The species *T. chinensis* is 500 years old and from XianNing city. HG-1 are the cultured *T. chinensis* cells. The species *T. media* is the natural hybrid of *T. cuspidate* and *T. baccata*. We sequenced the 18S rRNA gene sequences of *T. media*, *T. chinensis* and HG-1 following the same protocol (Chaw *et al*., 1997; Sasaki *et al*., 2002). They have been deposited at GenBank under accession numbers AY544989, AY544988, and AY679156, respectively. The 18S rRNA gene sequences of other species were retrieved from GenBank by the homology search program MegaBLAST (Altschul *et al*., 1990) as shown in Table I. These sequences are from Taxaceae, Pinaceae, Taxodiaceae, Cupressaceae, Cephalotaxaceae, Podocarpaceae, and Cycadaceae. *C. taitungensis* in Cycadaceae was used as an outgroup.

Sequences were aligned by T-COFFEE (Notredame *et al*., 2000). The aligned sequences were 1,866 bp in length. A few areas of ambiguous alignment remained, and these regions and terminal priming sites (totaling 213 bp) were omitted for the phylogenetic analysis.

The close-neighbour-interchange (CNI) parsimony analysis was conducted using MEGA3 software (Kumar *et al*., 2004). For the maximum parsimony (MP) method, the CNI search started with a tree generated by the random addition of sequences. This process was repeated 10 times to find the MP tree. These initial searches found 24 trees. To assess the support for the inferred rela-

0939–5075/2008/0100–0127 $ 06.00

tionships, bootstrap analysis with 1,500 replicates was conducted.

Test of molecular clock hypothesis

The constancy of the molecular evolutionary rate was assessed using the relative rate test (Sarich and Wilson, 1973; Wu and Li, 1985; Li and Tanimura, 1987; Robinson *et al*., 1998) in PHYLTEST software (Kumar, 1996) and the distance estimation method was Kimura 2-parameter (Kimura, 1980). In the test, the constancy of the molecular evolutionary rate was examined for two lineages when an outgroup lineage was given. If L_a and L_b are the averages of observed numbers of substitutions per site (branch lengths) from the common ancestor of clusters A and B, then $L_a = L_b$ is the null hypothesis under the constancy of molecular clock, *i.e.*, $\delta = L_a - L_b = 0$. It is clear that δ will become negative if lineage A is evolving slower than B. Because the variance of δ can be estimated, we could test the deviation of δ from 0 by a two-tailed normal deviate test. The sequences that did not satisfy the hypothesis of a molecular clock for the data set in Table I at 5% level were eliminated. Then, a tree was constructed for a given topology for the remaining sequences under the assumption of rate constancy using the UPGMA method in MEGA3 software (Kumar *et al*., 2004) with 2,000 replicates of bootstrap analysis and the nucleotide substitution model was the Kimura 2-parameter. This tree was called a linearized tree (Takezaki *et al*., 1995). Based on the divergence time between *Pinus* and *Larix* of 140 Ma (Savard *et al*., 1994; Wang *et al*., 2000b), the linearized tree was used for estimating the divergence time for pairs of sequences.

Table I. 18S rRNA gene sequences used in this study.

Family/species	Accession number in GenBank
Cycadaceae	
Cycas taitungensis	D85297
Pinaceae	
Pinus wallichiana	X75080
Pinus elliottii	D38245
Abies lasiocarpa	X79407
Larix leptolepis	D85294
Taxodiaceae	
Taiwania cryptomerioides	D38250
Cryptomeria japonica	D85304
Cupressaceae	
Calocedrus decurrens	D85293
Calocedrus formosana	D85298
Juniperus chinensis	D38243
Taxaceae	
Amentotaxus formosana	D38248
Torreya nucifera	D38249
Taxus mairei	D16445
Taxus x *media*	AY544989
Taxus chinensis	AY544988
HG-1[a]	AY679156
Cephalotaxaceae	
Cephalotaxus wilsoniana	D38241
Podocarpaceae	
Podocarpus novae-caledoniae	AF342766
Podocarpus elatus	AF051796
Podocarpus costalis	D38473
Nageia nagi	D16447
Dacrycarpus imbricatus	D38247

[a] Cultured *T. chinensis* cells.

Results

Phylogenetic analysis

The close-neighbour-interchange searches found 24 maximum parsimony trees [consistency index (CI) = 0.775; retention index (RI) = 0.834]. The strict consensus of the 24 trees is shown in Fig. 1.

As shown in Fig. 1, using *C. taitungensis* as outgroup, all species fell into 5 major clades: a clade of Podocarpaceae, a clade of Pinaceae (bootstrap value = 100%), a clade of Taxaceae (bootstrap value = 100%), a clade of Cephalotaxaceae + Torreya + Amentotaxus (bootstrap value = 100%) and a clade of Taxodiaceae + Cupressaceae (bootstrap value = 100%); support for *Nageia* + *Dacrycarpus* is 100%, support for *Larix* + *Abies* is 100%, and support for *Torreya* + *Amentotaxus* is 83%.

HG-1 fell into the Taxaceae clade, and it is the sister to *T. chinensis* + *T. mairei* with a bootstrap support equal to 50%.

Test of molecular clock hypothesis

With *C. taitungensis* used as the reference taxon, the result of relative rate test is listed in Table II. The negative value indicating the taxon on the left side of the pairwise comparison has a slower rate of substitution, while a positive value indicates a faster rate of substitution. It is clear that HG-1 only satisfies the hypothesis of molecular clock with *T. media* at 5% level. In part E of Table II, the lineages HG-1/*T. mairei* and HG-1/*T. chinensis* do not satisfy the constancy of evolutionary rates for the Z-statistic's values which are 30.4473 and 2.43587, respectively; they are greater than the Z-statistic's critical value for the 2-tailed test

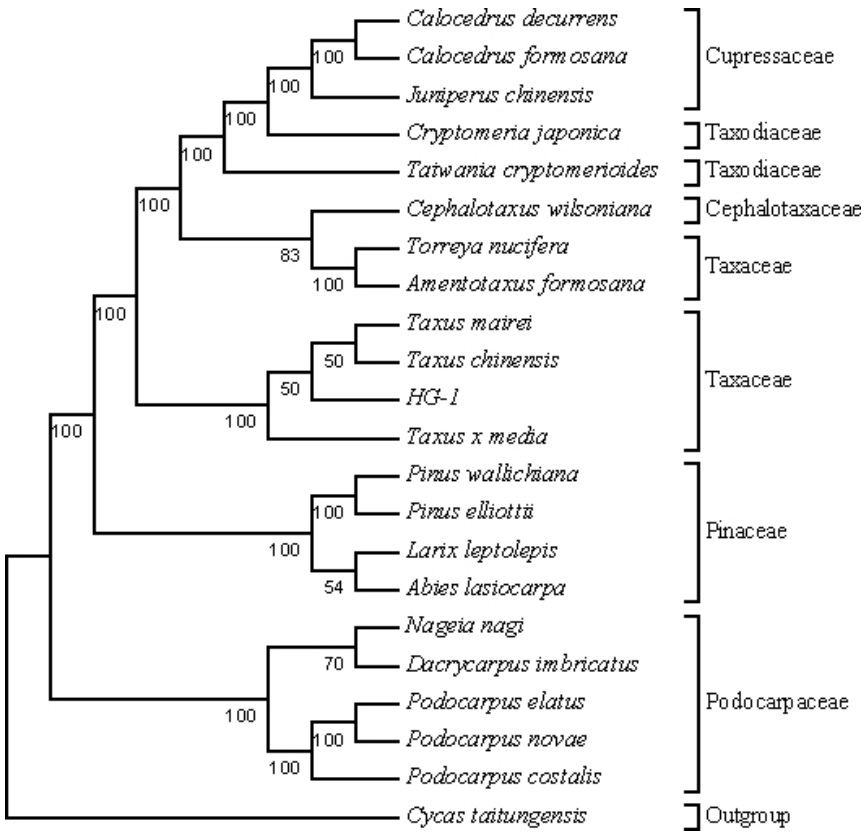

Fig. 1. Strict consensus of 24 maximum parsimony trees based on parsimony analysis. Numbers above branches are bootstrap values calculated from 1,500 replicates of bootstrapping. Consistency index = 0.775, retention index = 0.834.

Table II. Relative rate test[a].

L_a/L_b	δ^b	Z	Constancy at 5% level
A			
media/mairei	0.00164488 ± 0.000929855	1.76896	y
media/pin1	−0.00660784 ± 0.00492464	1.34179	y
media/larix	−0.00341609 ± 0.00478346	0.714146	y
media/nageia	−0.00394513 ± 0.00473132	0.833834	y
media/dacry	−0.00270411 ± 0.00491747	0.549899	y
mairei/pin1	−0.00825272 ± 0.00477719	1.72752	y
mairei/larix	−0.00506096 ± 0.00462977	1.09313	y
mairei/nageia	−0.00559001 ± 0.00457823	1.221	y
mairei/dacry	−0.00434899 ± 0.00476251	0.913171	y
pin1/larix	0.00319176 ± 0.00382066	0.835394	y
pin1/nageia	0.00266271 ± 0.00532066	0.500447	y
pin1/dacry	0.00390373 ± 0.00540155	0.722705	y
larix/nageia	−0.000529048 ± 0.00493606	0.10718	y
larix/dacry	0.000711973 ± 0.00502288	0.141746	y
nageia/dacry	0.00124102 ± 0.00158078	0.785068	y

Table II (continued).

L_a/L_b	δ^b	Z	Constancy at 5% level
B			
mairei/chinensis	0.000934099 ± 0.00157153	0.594389	y
mairei/pin1	−0.00825272 ± 0.00477719	1.72752	y
mairei/larix	−0.00506096 ± 0.00462977	1.09313	y
mairei/nageia	−0.00559001 ± 0.00457823	1.221	y
mairei/dacry	−0.00434899 ± 0.00476251	0.913171	y
chinensis/pin1	−0.00918682 ± 0.00507955	1.80859	y
chinensis/larix	−0.00599506 ± 0.00494213	1.21305	y
chinensis/nageia	−0.00652411 ± 0.00489357	1.3332	y
chinensis/dacry	−0.00528309 ± 0.00507296	1.04142	y
pin1/larix	0.00319176 ± 0.00382066	0.835394	y
pin1/nageia	0.00266271 ± 0.00532066	0.500447	y
pin1/dacry	0.00390373 ± 0.00540155	0.722705	y
larix/nageia	−0.000529048 ± 0.00493606	0.10718	y
larix/dacry	0.000711973 ± 0.00502288	0.141746	y
nageia/dacry	0.00124102 ± 0.00158078	0.785068	y
C			
media/chinensis	0.00257897 ± 0.00187775	1.37344	y
media/pin1	−0.00660784 ± 0.00492464	1.34179	y
media/larix	−0.00341609 ± 0.00478346	0.714146	y
media/nageia	−0.00394513 ± 0.00473132	0.833834	y
media/dacry	−0.00270411 ± 0.00491747	0.549899	y
chinensis/pin1	−0.00918682 ± 0.00507955	1.80859	y
chinensis/larix	−0.00599506 ± 0.00494213	1.21305	y
chinensis/nageia	−0.00652411 ± 0.00489357	1.3332	y
chinensis/dacry	−0.00528309 ± 0.00507296	1.04142	y
pin1/larix	0.00319176 ± 0.00382066	0.835394	y
pin1/nageia	0.00266271 ± 0.00532066	0.500447	y
pin1/dacry	0.00390373 ± 0.00540155	0.722705	y
larix/nageia	−0.000529048 ± 0.00493606	0.10718	y
larix/dacry	0.000711973 ± 0.00502288	0.141746	y
nageia/dacry	0.00124102 ± 0.00158078	0.785068	y
D			
media/hg	-0.00129453 ± 0.000961108	1.34692	y
media/pin1	−0.00660784 ± 0.00492464	1.34179	y
media/larix	−0.00341609 ± 0.00478346	0.714146	y
media/nageia	−0.00394513 ± 0.00473132	0.833834	y
media/dacry	−0.00270411 ± 0.00491747	0.549899	y
hg/pin1	−0.00531331 ± 0.00484025	1.09773	y
hg/larix	−0.00212155 ± 0.00469674	0.451707	y
hg/nageia	−0.0026506 ± 0.00464086	0.571144	y
hg/dacry	−0.00140958 ± 0.00483104	0.291776	y
pin1/larix	−0.00319176 ± 0.00382066	0.835394	y
pin1/nageia	0.00266271 ± 0.00532066	0.500447	y
pin1/dacry	0.00390373 ± 0.00540155	0.722705	y
larix/nageia	−0.000529048 ± 0.00493606	0.10718	y
larix/dacry	0.000711973 ± 0.00502288	0.141746	y
nageia/dacry	0.00124102 ± 0.00158078	0.785068	y
E			
hg/mairei	0.00293941 ± 9.6541e-05	30.4473	n
hg/chinensis	0.00387351 ± 0.00159019	2.43587	n

[a] 1. A negative value indicates that the taxon on the left side of the pairwise comparison has a slower rate of substitution, while a positive value indicates a faster rate of substitution. The word "media" denotes *T. media*, while pin1, larix, nagi, dacry, chinensis, mairei, and hg denote *P. wallichiana, L. leptolepis, N. nagi, D. imbricatus, T. chinensis, T. mairei*, and HG-1, respectively. 2. The letter "y" indicates that the two taxa passed the Z-test at 5% level and satisfied the hypothesis of a molecular clock for the data set at 5% level, while the letter "n" indicates the opposite. 3. The reference taxon is *C. taitungensis*.

[b] δ is the difference of the branch lengths from the common ancestors, and its standard error is given.

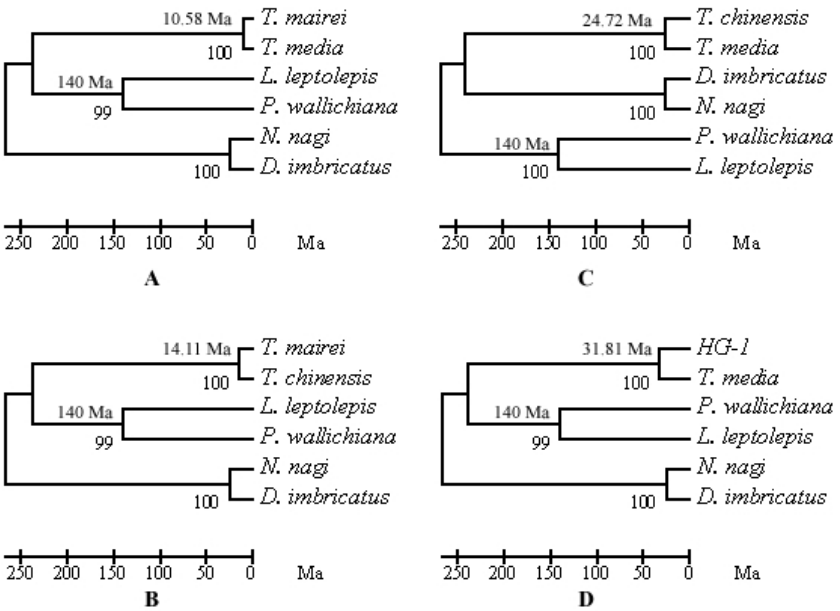

Fig. 2. Linearized trees (A) for *T. mairei* and *T. media*, (B) for *T. mairei* and *T. chinensis*, (C) for *T. chinensis* and *T. media*, (D) for HG-1 and *T. media*. The divergence time estimated and the bootstrap values calculated from 2,000 replicates of bootstrapping are also listed. The reference point is 140 Ma (million years).

(Z = 1.96). Based on the reference point of 140 Ma, the divergence times of *T. mairei, T. chinensis,* HG-1 from *T. media* are 10.58 Ma, 24.72 Ma, and 31.81 Ma, respectively, as shown in parts A, C, D of Fig. 2, and the divergence time between *T. chinensis* and *T. mairei* is 14.11 Ma as shown in part B.

Discussion

As for the clade of Podocarpaceae, the phylogenetic sequences analyses of the mitochondrial ribosome subunit RNA, the nucleus ribosome subunit RNA gene, and chloroplast *rbcL* gene (Chaw *et al*., 2000), the cladistic analysis of *matK* gene (Wang and Shu, 2000), and the phylogenetic analyses of chloroplast *rbcL* gene sequences and *trnL-trnF* intergenic spacer sequences (Wang *et al*., 2002) all suggest that *Dacrycarpus*, *Nageia* and *Podocarpus* form a monophyletic group. *Cephalotaxus* has been taken as a genus of Taxaceae for its close relationship to Taxaceae, and now it has been taken as Cephalotaxaceae (Fu, 1984; Wang and Shu, 2000). It is worth to note the clade of Taxodiaceae + Cupressaceae. Although Taxodiaceae and Cupressaceae had been taken as different families according to their dissimilarities in morphology and anatomy, many researches indicated that they form a monophyletic group and should be treated as a family (Hart, 1987; Brunsfeld *et al*., 1994; Stefanovic *et al*., 1998; Gadek *et al*., 2000; Kusumi *et al*., 2000). Thus, the phylogenetic relationship shown in Fig. 1 is in agreement with previous results.

We can conclude that cell culture changed the phylogenetic position of HG-1 of Fig. 1. In Fig. 1, HG-1 forms a single branch rather than to cluster together with *T. chinensis*. More than that, HG-1 has a faster rate of substitution than *T. chinensis* for $\delta_{\text{HG-1}/T.\ chinensis} > 0$ as shown in Table II. In fact, we can deduce that the rate of substitution is HG-1 > *T. media* > *T. mairei* > *T. chinensis* from Table II. It is obvious that cell culture makes the evolution of HG-1 faster.

In addition, the fact that HG-1 dissatisfies the hypothesis of a molecular clock with *T. chinensis* at 5% level as shown in Table II also makes clear that cell culture has an effect on the evolution of 18S rRNA gene of *T. chinensis* despite the 18S rRNA is the most conserved gene. This effect will be discussed further. As shown in Fig. 2, the divergence times of *T. chinensis* and HG-1 from *T. media* are 24.72 Ma and 31.81 Ma, respectively. It is clear that the divergence time of HG-1 is 7 Ma more than that of *T. chinensis* with *T. media* as reference. This effect occurred in cell culture.

Altschul S. F., Gish W., Miller W., Myers E. W., and Lipman D. J. (1990), Basic local alignment search tool. J. Mol. Biol. **215**, 403–410.

Brunsfeld S. J., Soltis P. S., Soltis D. E., Gadek P. A., Quinn C. J., Strenge D. D., and Ranker T. A. (1994), Phylogenetic relationships among the genera of Taxodiaceae and Cupressaceae: evidence from rblL sequences. Syst. Bot. **19**, 253–262.

Chaw S. M., Zharkikh A., Sung H. M., Lau T. C., and Li W. H. (1997), Molecular phylogeny of extant gymnosperms and seed plant evolution:analysis of nuclear 18S rRNA sequences. Mol. Biol. Evol. **14**, 56–58.

Chaw S. M., Parkinson C. L., Cheng Y., Vincent T. M., and Palmer I. D. (2000), Seed plant phylogeny inferred from all three plant genomes: Monophyly of extant gymnosperms and origin of Gnetales from conifers. Proc. Natl. Acad. Sci. USA **97**, 4086–4091.

Fu L. G. (1984), The research of *Cephalotaxus*. Acta Phytotaxon. Sin. **22**, 277–288.

Gadek P. A., Alpers D. L., Heslewood M. M., and Quinn C. I. (2000), Relationships within Cupressaceae *sensu lato*: a combined morphological and molecular approach. Am. J. Bot. **87**, 1044–1057.

Gu S. R., Gui Y. L., Liu S. Q., and Xu T. Y. (1991), Suspension culture of endosperm callus cell and amitosis *in vitro*. Acta Bot. Sin. **33**, 478–481.

Hart J. A. (1987), A cladistic analysis of conifers: Preliminary results. J. Arnold. Arbor. **68**, 269–307.

Hirasuna T. J., Pestchanker L. J., and Srinivasan C. (1996), Taxol production in suspension cultures of *Taxus baccata*. Plant Cell Tissue Organ Cult. **44**, 95–102.

Kimura M. (1980), Simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16**, 111–120.

Kumar S. (1996), PHYLTEST. Version 2.0. Pennsylvania State University, University Park, Pennsylvania.

Kumar S., Tamura K., and Nei M. (2004), MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. **5**, 150–163.

Kusumi J., Tsumura Y., Yoshimaru H., and Tachida H. (2000), Phylogenetic relationships in Taxodiaceae and Cupressaceae *sensu stricto* based on matK gene, chlL gene, trnL-trnF IGS region, and trnL intron sequences. Am. J. Bot. **87**, 1480–1488.

Li W. H. and Tanimura M. (1987), The molecular clock runs more slowly in man than in apes and monkeys. Nature **326**, 93–96.

Notredame C., Higgins D., and Heringa J. (2000), T-COFFEE: A novel method for multiple sequence alignments. J. Mol. Biol. **302**, 205–217.

Robinson M., Gouy M., Gautier C., and Mouchiroud D. (1998), Sensitivity of the relative-rate test to taxonomic sampling. Mol. Biol. Evol. **5**, 1091–1098.

Sarich V. M. and Wilson A. C. (1973), Generation time and genomic evolution in primates. Science **179**, 1144–1147.

Sasaki Y., Fushimi H., Cao H., Cai S. Q., and Komatsu K. (2002), Sequence analysis of Chinese and Japanese *Curcuma* drugs on the 18S rRNA gene and trnK gene and the application of amplification-refractory mutation system analysis for their authentication. Biol. Pharm. Bull. **25**, 1593–1599.

Savard L., Li P., Strauss S. H., Chase M. W., Michaud M., and Bousquet J. (1994), Chloroplast and nuclear gene sequences indicate late Pennsylvanian time for the last common ancestor of extant seed plants. Proc. Natl. Acad. Sci. USA **91**, 5263–5167.

Stefanovic S., Jager M., Deutsch J., Broutin I., and Masselot M. (1998), Phylogenetic relationships of conifers inferred from partial 28S rRNA gene sequences. Am. J. Bot. **85**, 688–697.

Takezaki N., Rzhetsky A., and Nei M. (1995), Phylogenetic test of the molecular clock and linearized trees. Mol. Biol. Evol. **12**, 823–833.

Wang X. Q. and Shu Y. Q. (2000), Chloroplast matK gene phylogeny of Taxaceae and Cephalotaxaceae, with additional reference to the systematic position of Nageia. Acta Phytotaxon. Sin. **38**, 201–210.

Wang X. Q., Tank D. C., and Sang T. (2000b), Phylogeny and divergence within the pine family: evidence from three genomes. Mol. Biol. Evol. **17**, 733–781.

Wang S. H., Qi H. F., and Yuan Y. J. (2000a), Study on mitosis and chromosome stability of callus of *Taxus cuspidata*. Acta Phytophysiol. Sin. **26**, 263–266.

Wang T., Su Y. J., Zheng B., and Li X. Y. (2002), Cladistic analysis of sequences of chloroplast rbcL gene and trnL-trnF intergenic spacer in Taxaceae and related taxa. Acta Scientiarum Naturalium Universitatis Sunyatseni Sin. **41**, 71–74.

Wu C. I. and Li W. H. (1985), Evidence for higher rates of nucleotide substitution in rodents than in man. Proc. Natl. Acad. Sci. USA **82**, 1741–1745.

Yang Y. G., Gui Y. L., Tang W., Zhao R., and Guo Z. C. (1994), Observation on differentiation potential and chromosome stability of callus in subcultures of *Picea wilsonii*. Acta. Bot. Sin. **36**, 934–939.

Yu L. J., Zhang C. H., Mei X. G., and Chen M. (1998), Somaclonal variation in the number of chromosome and the number of nuclei in cultured *Taxus chinensis* cells. J. Huazhong University of Science and Technology Sin. **26**, 100–102.

Zhang Y. J., Qian Y. Q., Cai Q. G., Mu X. J., Wei X. P., and Zhou Y. L. (1997), Somaclonal variation in chromosome number and nuclei number of regenerated plants from protoplasts of *Actinidia eriantha*. Acta Bot. Sin. **39**, 102–105.