Introduction

Taxol is currently the best-known drug approved for the treatment of ovarian and breast cancer. For commercial use, taxol was first extracted from the bark of the yew Taxus brevifolia, which was threatened by extinction soon after the discovery of taxol. Cell cultures derived from different Taxus species are an important alternative source of taxol and other taxanes which can be converted chemically to taxol (Sohn and Okos, 1998). Recently, the commercial production of taxol by Taxus cell suspension cultures was established (Zhong, 2002). However, the difficulty of establishing cell cultures and cessation of cell growth and secondary metabolite production after a period of time in suspension culture limits the efficiency of taxol production from cell cultures. The instability of secondary metabolite production owes to changes in the genomic structure of cultured plant cells significantly (Baebler et al., 2005). Interestingly, we had found that cell culture has an effect on the 18S rRNA gene sequences in the cultural course of Taxus chinensis cells (Xiang et al., 2008). The 18S rRNA gene, which is highly conserved, can record the trace of biological evolution. As the most conserved gene, what caused the changes of the 18S rRNA gene sequence and what results were brought about in cell culture? DNA methylation may play a role in such a cultural course, and it prompted us to revisit the changes of 18S rRNA gene sequences in cell culture.

Material and Methods

Sequences

The 18S rRNA gene sequences of T. chinensis (AY544988), T. media (AY544989), and HG-1 (AY679156) were the same as the ones used previously (Xiang et al., 2008). Of these, HG-1 are the cultured T. chinensis cells. The species T. mairei, a naturally occurring variant of T. chinensis, was from Wuhan arboretum. We sequenced the 18S rRNA gene sequence of T. mairei following the same protocol used for T. chinensis, T. media, and HG-1 (Xiang et al., 2008), and it has been deposited at GenBank under accession number AY679157. According to the phylogenetic relationship reported previously (Xiang et al., 2008), the 18S rRNA gene sequence of Amentotaxus formosana (D38248) was used as an outgroup.

Data analysis

Sequences were aligned by ClustalX (Thompson et al., 1997), and low-scoring segments in the
alignment output were realigned manually. The first and last 14 positions were excluded due to the artifacts in sequence AY544988, and 1836 nucleotide positions were obtained.

Neighbour-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods were used to reconstruct phylogenetic trees on these sites. The NJ and MP trees were reconstructed by the methods as implemented in MEGA4 software (Tamura et al., 2007), and the pairwise distances in the NJ method were calculated using the Kimura 2-parameter model (Kimura, 1980).

To assess support for the inferred relationships, bootstrap analysis with 2,000 replicates was conducted using the NJ and MP methods. The ML tree was constructed by the DNAML program in PHYLIP package (Felsenstein, 2004) with 1,000 replicates bootstrap analysis, and the transition/transversion ratio was 3.

The analysis of CpG relative abundance was conducted using DAMBE software (Xia and Xie, 2001).

**Results and Discussion**

The bootstrap consensus MP, NJ, and ML trees are shown in Figs. 1A, B, and C, respectively. It is interesting that HG-1 groups with *T. mairei* and not with *T. chinensis* with 100% bootstrap support in the MP tree, 64% in the NJ tree, and 69% in the ML tree, despite of HG-1 are the cultured *T. chinensis* cells. The phylogenetic position changes of the 18S rRNA gene sequences implied that HG-1, like *T. mairei*, should be a new variety of *T. chinensis*. This result is consistent with that reported previously (Xiang et al., 2008). In other words, cell culture can change the 18S rRNA gene sequence at the level of species despite 18S rRNA is the most conserved gene. So, what caused such a result? In fact, we have found the methylation in the cultural course of *T. chinensis* cells, and it may contribute to the taxol content at some stage (to be published). Is it possible that DNA methylation caused the changes of the 18S rRNA gene sequence at the level of species?

To make sure the role of DNA methylation in the cultural course of *T. chinensis* cells, we analyzed the CpG and TpG+CpA relative abundance and GC content of the 18S rRNA gene sequences of HG-1 (the cultured *T. chinensis* cells), *T. chinensis*, and *T. mairei* (the natural variety of *T. chinensis*). CpG dinucleotides play an important role in cell differentiation and in the regulation of gene expression (Bestor, 1990). As shown in Table I, the CpG dinucleotide relative abundance is normal in all three sequences. It should be noted that the CpG relative abundance of HG-1 is lower than that of *T. chinensis*. Contrarily, *T. mairei*, which is the natural variety of *T. chinensis*, has the same CpG relative abundance as *T. chinensis*. Correspondingly, the TpG+CpA relative abundance has a higher value in the HG-1 sequence compared to sequences of *T. chinensis* and *T. mairei*, and the GC content in the HG-1 sequence is the lowest as shown in Table I.

In general, CpG methylation increases the mutation rate from CpG to TpG or CpA. As methylated CpG deaminates to TpG or CpA dinucleotides, the number of C and G decreases in

### Table I. Relative abundance (RA) of CpG and TpG+CpA and GC content.

<table>
<thead>
<tr>
<th>Species</th>
<th>RA(CpG)</th>
<th>RA(TpG+CpA)</th>
<th>GC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. chinensis</em></td>
<td>0.9762</td>
<td>0.9440</td>
<td>0.4964</td>
</tr>
<tr>
<td><em>T. mairei</em></td>
<td>0.9762</td>
<td>0.9426</td>
<td>0.4972</td>
</tr>
<tr>
<td>HG-1</td>
<td>0.9621</td>
<td>0.9649</td>
<td>0.4936</td>
</tr>
</tbody>
</table>

* RA, odds-ratio measure, i.e., $F(XY)/[F(X) \cdot F(Y)]$ for quantifying the relative abundance of dinucleotides. For nucleotide sequences, $F(X)$ and $F(Y)$ are the frequencies of X and Y, respectively, and $F(XY)$ is the frequency of dinucleotide XY.
this process. This would lead to a lower expected number of CpG dinucleotides in the new sequence compared to the original sequence. In fact, in vertebrates and plants, CpG dinucleotides are massively methylated or demethylated in order to regulate the gene expression activity (Wang et al., 2004), which is connected with a new developed function in evolution history (Yoder et al., 1997).

In the cultural course of \textit{T. chinensis} cells, DNA methylation may contribute to changes of the 18S rRNA gene sequence of HG-1 at species level, which led HG-1, similar to \textit{T. mairei}, become a new variety of \textit{T. chinensis}. This is the reason why HG-1 clusters together with \textit{T. mairei} and not with \textit{T. chinensis} in all three consensus trees, as shown in Fig. 1.


