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Research article

Variation in the number of nucleoli and incomplete homogenization of 18S ribosomal DNA sequences in leaf cells of the cultivated Oriental ginseng (*Panax ginseng* Meyer)

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ABSTRACT

Background: Wild ginseng, *Panax ginseng* Meyer, is an endangered species of medicinal plants. In the present study, we analyzed variations within the ribosomal DNA (rDNA) cluster to gain insight into the genetic diversity of the Oriental ginseng, *P. ginseng*, at artificial plant cultivation.

Methods: The roots of wild *P. ginseng* plants were sampled from a nonprotected natural population of the Russian Far East. The slides were prepared from leaf tissues using the squash technique for cytogenetic analysis. The 18S rDNA sequences were cloned and sequenced. The distribution of nucleotide diversity, recombination events, and interspecific phylogenies for the total 18S rDNA sequence data set was also examined.

Results: In mesophyll cells, mononucleolar nuclei were estimated to be dominant (75.7%), while the remaining nuclei contained two to four nucleoli. Among the analyzed 18S rDNA clones, 20% were identical to the 18S rDNA sequence of *P. ginseng* from Japan, and other clones differed in one to six substitutions. The nucleotide polymorphism was more expressed at the positions 440–640 bp, and distributed in variable regions, expansion segments, and conservative elements of core structure. The phylogenetic analysis confirmed conspecificity of ginseng plants cultivated in different regions, with two fixed mutations between *P. ginseng* and other species.

Conclusion: This study identified the evidences of the intragenomic nucleotide polymorphism in the 18S rDNA sequences of *P. ginseng*. These data suggest that, in cultivated plants, the observed genome instability may influence the synthesis of biologically active compounds, which are widely used in traditional medicine.

1. Introduction

Wild ginseng, *Panax ginseng* Meyer, is an endangered species of medicinal plants belonging to the relict family Araliaceae, which has been used as a source of biologically active substances for thousands of years. The domestication and cultivation *in vitro* of *P. ginseng* aim to make up for natural resources of the valuable medicinal remedy for various diseases and a diet supplement for the elderly. However, under conditions of artificial reproduction, the medical properties of ginseng are believed to have become weaker, and the amount of the main biologically active compound—ginsenosides—decreases [1]. The nature of this phenomenon remains insufficiently studied.

In most eukaryotes, the nuclear ribosomal DNA (rDNA) occurs in tandem arrays at one or several loci. Each repeat unit contains the 18S, 5.8S, and 28S rDNA subunits; the internal transcribed spacers; and the intergenic spacer, which separates transcribed units. The molecular evolution of the ribosomal RNA (rRNA) genes fundamentally differs from the evolution of single-copy genes. The individual rDNA repeats do not evolve independently of each other; they homogenize horizontally. Therefore, only small differences are usually observed between the rDNA sequences within the tandem

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unit in any species, while they accumulate between species. The ability of all units to change their rDNA sequences in a highly organized manner was described as concerted evolution [2–6].

The concerted evolution is important to maintain a significant number of the active rDNA units, and homogenization aims to reduce mutational load, and it is supported by selection [7]. However, a lot of studies show that, in many taxa, the homogenization rate may be too low to prevent significant levels of intraspecific polymorphism of the rDNA repeats. This variation is often limited to noncoding regions of the rDNA, such as internal transcribed spacers and intergenic spacer [8–12]. At the same time, there are an increasing number of studies, which revealed the presence of divergent rDNA paralogs and pseudogenes whose coding regions are free from functional obligation in different taxa [12–17]. Polyploidy, interspecific hybridization, loci number, and localization of nuclear-organization regions (NORs) on nonhomologous chromosomes, large genome size, and higher mutation rates in comparison with the rate of sequence homogenization are usually discussed as the possible reasons for the incomplete development of concerted-evolution mechanisms in the rDNA repeats [12,15,16,18–25].

The nucleolus assembles around the clusters of ribosomal gene repeats, and is easily visible in alive or fixed cells by phase-contrast or differential-interference-contrast optics. Currently, the nucleolus is recognized as a multifunctional nuclear subcompartment of the eukaryotic cell, which is involved in many important biological processes, including cellular stress responses and aging, checkpoint control, messenger RNA export and modification, and also protein degradation and sequestration [26–32]. Additionally, the nucleolus is the organizing center for different chromosome domains, and therefore, may be associated with the genetic and epigenetic regulation of eukaryotic genome [26,31].

In the present study, we analyzed variations within the rDNA cluster to gain insight into the genetic diversity of Oriental ginseng, *P. ginseng*, at artificial plant cultivation. This genetic information may be important for our understanding of the mechanisms involved in the loss of ability to actively synthesize ginsenosides by cultivated plants.

2. Materials and methods

2.1. Plant source

The roots of wild *P. ginseng* Meyer plants were sampled from a nonprotected natural population in Sikhote-Alin mountain range, Russian Far East. The collected roots were transferred to an open experimental nursery, and grown under conditions that were similar to the ginseng natural habitat (Spassky District of the Primorsky Region, southern Russian Far East). For investigations, the leaves of 5-yr-old ginseng plants were collected at the beginning of the vegetation period.

2.2. Cytogenetic analysis

The cytogenetic technique was performed as described previously [33]. The leaf tissues (volume: 0.5–1 mL) were pretreated in 0.2% colchicine solution for 2–3 h at room temperature (~22°C), fixed in 3:1 ethanol:acetic acid mixture, and stained with acetohematoxylin. The slides were prepared using the squash technique. A 50% solution of silver nitrate was used to stain the nucleoli [34]. The number of nucleoli was counted in 500–1,000 cells.

2.3. Isolation of total DNA and polymerase-chain-reaction amplification

The isolation of total DNA was performed as described previously [35]. Amplification reactions were performed in 20 μL volumes containing 100 ng template DNA, 10mM Tris-HCl (pH 8.5), 50mM KCl, 2.5mM $MgCl_2$, 0.01% gelatin (w/v), 0.1mM Triton X-100, 0.25mM each deoxynucleotide triphosphate, and 10pM each primer. We also used a mix (1:8) of *Pfu* and *Taq* DNA polymerases (Fermentas, Vilnius, Lithuania) to minimize amplification errors. The polymerase-chain-reaction (PCR) primer sequences and temperature conditions were those as in [36].

2.4. Cloning and sequencing of the 18S rDNA sequences

The PCR products of two independent PCR reactions were separately cloned into the pTZ57R/T plasmid using the InsTAclone PCR product kit (Fermentas). The PCR products were used for cloning procedures without any modification. Clones were generated according to the protocol recommended by the manufacturer and supplied with the cloning kit. The clones were amplified using M13 (−20) (Syntol, Moscow, Russia) primers for colony screening and sequencing. The sequence of each clone was determined by an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Instrumental Centre of Biotechnology and Gene Engineering of the Institute of Biology and Soil Science, Far Eastern Branch of the Russian Academy of Sciences using additional nested primers [37]. The nucleotide-sequence data of 18S rRNA gene were recorded in GenBank nucleotide-sequence databases with the following accession numbers: KC593794–KC593823.

2.5. Statistical analysis and phylogenetic reconstructions

The Basic Local Alignment Search Tool program was used for the sequence analysis, and the sequence assembly and multiple

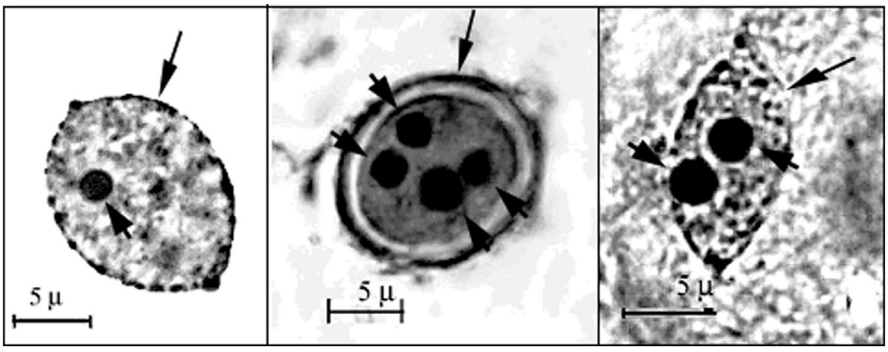

Fig. 1. Nuclei of mesophyll cells in the *Panax ginseng* leaves with different number of nucleoli. The nucleus with four nucleoli was obtained after leaf maceration. Long and short arrows indicate nuclei and nucleoli, respectively.