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Molecular Identification of *Panax ginseng* C.A. Meyer in Ginseng Commercial Products[+]

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Dedicated to the memory of Professor Ivano Morelli.

Molecular techniques (PCR and RFLP) were used to verify the presence of *Panax ginseng* C.A. Meyer in commercial products containing ginseng. DNA, extracted from four vegetable forms present in marketed products, was amplified with 18df/28ccr primers. The RFLP of the DNA amplified products, obtained using *Inf* I, *Sau* 3*A*1 and *Taq* I endonucleases, allowed the identification of *P. ginseng* and its differentiation from *P. quinquefolium*. *P. ginseng* was detected in 9 out 16 samples tested which, according to the declaration on the labels, contained the drug. Negative results were obtained for products containing the dried extract of the drug. A comparison of the results acquired using the molecular techniques with those using HPLC is also reported.

Keywords: *Panax ginseng*, *P. quinquefolium*, Molecular identification technique, Ginseng commercial products.

Molecular biology constitutes a new frontier for phytochemical analysis, allowing the improvement of previous knowledge, as well as the acquisition of new data. Recently, molecular techniques have been successfully utilised in order to validate plant drugs, overcoming the limitations of traditional analyses [1-4]. In the present study, PCR and RFLP were used in order to authenticate *Panax ginseng* C.A. Meyer (Korean ginseng) in different vegetable forms of ginseng commercial products and to differentiate it from other *Panax* species and from some of their adulterants. A comparison with HPLC identification results was also made.

There is an ongoing question over the labelling of herbal products as "Ginseng". Currently the word "Ginseng" is used to sell a variety of herbs associated with certain claimed therapeutic properties (Table 1). This can be confusing as they neither contain the same constituents nor display the same biochemical properties.

The quality of ginseng commercial products influences their effectiveness and safety of use and depends on the employed raw materials. The most active constituents of *P. ginseng* are steroidal saponins, called ginsenosides. So far 22 ginsenosides have been isolated and characterised, based on triterpene aglycone moieties with dammarane and oleanane structures and on the sugar unit sequences. In the monograph entitled "Ginseng" [5] the European Pharmacopoeia (Ph. Eur.) reports only the whole or cut dried root of *P. ginseng*, that must contain ginsenoside Rf and not less than 0.40% of combined ginsenosides Rg1 and Rb1, calculated with reference to the dried drug. This is, therefore, the only true ginseng (Korean ginseng).

Since the 1990s differentiation and research on the various species of *Panax* have been reported and the

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use of TLC, GLC and HPLC, as well as chemical techniques have allowed efficient separation and isolation of ginsenosides [6-9]. However, as reported in the Ph. Eur. [5], TLC and HPLC are the official tools to detect the presence of ginsenosides in root commercial samples. These methods need time (two working days), and a large quantity of plant material, as well as reference standards, that are often difficult to obtain.

Table 1: Main recoverable species in ginseng commercial products other than the European Pharmacopoeia species.

Botanical Name	Common names
Panax ginseng C.A. Meyer (Araliaceae) Ph. Eur. species	Korean ginseng Asian ginseng Chinese ginseng Ren shen Panax schinseng Jiln ginseng
Panax quinquefolium Linn	American ginseng
Panax notoginseng Burkill	San-chi ginseng
Panax pseudoginseng N. Wallich	Himalayan ginseng, Tien-chi ginseng
Panax japonicus or *P. japonicum* C.A. Meyer	Japanese ginseng, Ginseng bamboo
Panax trifolium Linn	Dwarf ginseng
Panax zingiberensis C.Y. Wu & Feng	Ginger ginseng
Panax stipuleanatus Tsai & Feng	Pingbiann ginseng
Panax vietnamensis Ha Thi Dung & I.V. Grushvitskii	Vietnamese ginseng
Other *genera* and families	
Eleutherococcus senticosus Maxim (Araliaceae)	Siberian ginseng, Wujia
Echinopanax horridus Decne & Planch. (Araliaceae)	Alaskan ginseng, Devil's club
Aralia nudicaulis Blume (Araliaceae)	Wild ginseng, Salsaparilla
Rumex hymenosepalus J. Torrey (Polygonaceae)	Red Desert ginseng
Pfaffia paniculata Kuntze (Amaranthaceae)	Brazilian ginseng, Suma
Pseudostellaria heterophylla Pax (Caryophyllaceae)	Sometimes used as a ginseng substitute
Caulophyllum thalictroides Regel (Berberidaceae)	Yellow or Blue ginseng, Blue cohosh
Triosteum perfoliatum Linn. (Caprifoliaceae)	Fever root, sometimes called Ginseng
Codonopsis tangshen Oliver (Campanulaceae)	Sometimes used as a ginseng substitute
Lepidium meyenii Walp. (Cruciferae)	Maca, Peruvian ginseng Andean ginseng
Withania somnifera Dun. (Solanaceae)	Indian ginseng Ashwaganda

The objective of the present study was to develop and standardise a reliable and easy molecular method for authentication of *P. ginseng* in the different forms of commercial ginseng products and to compare these results with HPLC identification made on the same samples [10].

Useful amounts of DNA were extracted from all the considered samples (**1**-**19**). PCR amplification was made using 18df/28ccr primers. The DNA amplified products, digested with the endonucleases *Hinf* I, *Taq* I and *Sau* 3A1, gave fragments of 170 bp; 230 bp and 280 bp; and 120 bp and 580 bp, respectively, characteristic for *P. ginseng*. Similarly, fragments of 60 bp and 100 bp; and 106 bp, 170 bp and 260 bp, were obtained for *P. quinquefolium* (American ginseng) with the use of *Hinf* I and *Taq* I, respectively (Figure 1).

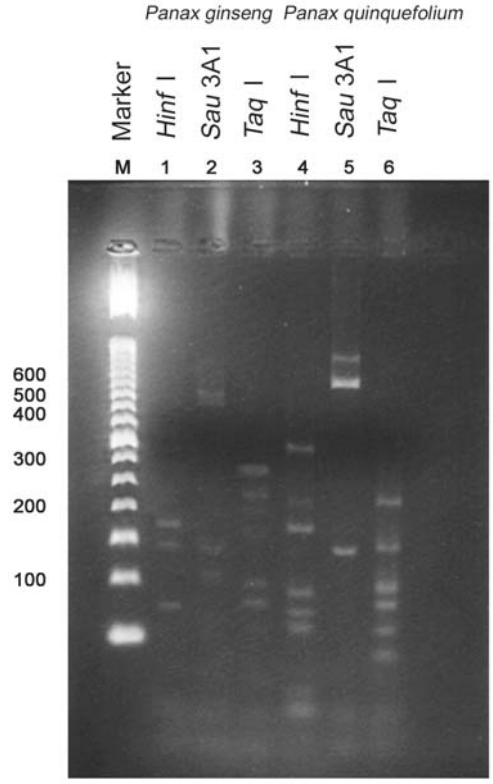

Figure 1: Agarose gel electrophoresis of *Hinf* I, *Sau* 3A1 and *Taq* I restriction fragments obtained from amplified amplicons using the 18df/28ccr primer pair, specific for conserved region 18S-28S of *Panax* species.

For a total of 486 determinations (19 samples, 3 repetitions, 3 extraction protocols, following PCR and restriction with 3 endonucleases), the molecular analysis confirmed the presence of *Panax* species in 12 out of 19 samples tested, as shown in Table 2. Three of the positive samples, named PQ, PQT, PQP (**6**, **11**, **12**), were commercial ginseng products containing *P. quinquefolium* in the form of dried body root, dried root tails and dried root prongs, respectively. Among the samples labelled PG (**1**-**5**, **7**-**10**, **13**-**19**), the presence of *P. ginseng* was confirmed, as reported on the label, in 9 out of 16 samples, but not in three dried body root products (**4**, **5**, **18**) or in four dried extract samples (**15**-**17**, **19**). The presence of adulterants such as *Mirabilis jalapa* L. and *Phytolacca acinosa* Roxb could be excluded in the considered samples since no specific *Sau* 3A1 digestion fragments [11] for either plant were visualised in agarose gel.

Table 2: DNA identification (PCR and RFLP) of *Panax ginseng* and HPLC detection of ginsenoside Rf in ginseng commercial products.

Sample		DNA identification				HPLC
		Body root	Root tails	Root prongs	Dried extracts	Ginsenoside Rf
1	PGB	+				+
2	PGR	+				+
3	PGBO2	+				+
4	PGBH1	–				–
5	PGBH2	–				–
6	PQ	+				–
7	PGBR		+			+
8	PGT99		+			–
9	PGTO3		+			+
10	PGTO4		+			+
11	PQT		+			–
12	PQP			+		–
13	PGRB	+				–
14	PGBDSPR	+				+
15	PGPHRB				–	–
16	PGNGLC				–	–
17	PGRKPS				–	+
18	PGGNST	–				–
19	PGext				–	+

PG: samples labelled as *P. ginseng.*
PQ: samples labelled as *P. quinquefolium*.
1-**12**: raw materials; **13**-**18**: commercial preparations as capsules or tablets; **19**: laboratory hydromethanoholic extract.

HPLC analysis [10], conducted as reported in the Ph. Eur. [5], revealed the presence of all ginsenosides used as reference standards. As required by the Ph. Eur. monograph [5], ginsenosides Rg1 and Rb1 were identified in all tested samples and Rf, characteristic of *P. ginseng*, was detected in only 9 out of the 16 PG samples, which claimed to be based on *P. ginseng*. In particular, ginsenoside Rf was absent from the three PQ samples (**6**, **11**, **12**), as expected for *P. quinquefolium* products, and from seven PG samples (**4**, **5**, **8**, **13**, **15**, **16**, **18**).

The PCR and RFLP results were in accordance with the HPLC data (presence of ginsenoside Rf, characteristic of *P. ginseng*) for the majority of the tested commercial products and in accordance with the species, *P. ginseng*, declared on their labels. PCR and RFLP/HPLC afforded negative results for five products, in the form of dried body root (**4**, **5**, **18**) and of dried extract (**15**, **16**), which excluded the presence of *P. ginseng*, although this was declared on the label. On the other hand, discordance between the molecular results and HPLC data was noted when the preparation was based on dried extracts (**17**, **19**).

Molecular methods have been used to unequivocally allow the authentication to species level of the genus *Panax* and the results were not affected by the nature of the drug. Compared with other methods that detect genome-wise polymorphism simultaneously, such RAPD [12], AP-PCR and AFLP [13], the method applied in this research, based on PCR followed by RFLP, is more reliable for large scale screening of commercial products, is rapid (one working day), and the results are easily readable. However, the procedure failed when the commercial products were dried extracts. A sample of reference *P. ginseng* was processed as described in the European Pharmacopoeia monograph [5], by boiling the root powder in 50% (v/v) aqueous methanol for 1h, to obtain a dried extract (**19**). This prepared extract resulted in a negative result in the molecular analytical procedure, as expected, because DNA molecules are not soluble in the hydromethanolic solvent, whereas the extract gave a positive for Rf on HPLC examination.

Experimental

The analysed commercial ginseng products as raw materials in the form of body root, root tails and root prongs, and as capsules and tablets containing also dried extract were obtained from national health care stores. To protect the Manufacturers' identities the sample sources were labelled as reported in Table 2.

An AB GeneAmp PCR System 9700 thermal cycler was used for the PCR analysis. *P. ginseng* and *P. quinquefolium* dried roots, used as references for the molecular analysis, were kindly provided by the Department of Plant Biology of the University of La Sapienza, Rome, Italy. Chromatography was performed on a Waters chromatographic system equipped with a Waters 600 MS multisolvent delivery system and a Waters 717 Auto sampler. A Waters 996 Photodiode Array Detector was used to monitor the eluates at 203 nm. The chromatographic data were analysed using a Waters Millennium Software version 3.2. Chromatography was performed at room temperature (25° C). Ginsenosides Rg1, Rb1, Rb2, and Rc–Rf, purchased from Extrasynthese, France, were used as reference standards.

Molecular analysis: DNA was extracted from 50-100 mg of each sample using an Invitrogen Easy-DNA Kit [14] and two other molecular protocols [15, 16] in order to compare their effectiveness in the extraction of useful amounts of DNA for molecular analysis. Body roots were previously treated with liquid nitrogen, while the other samples were used directly in the DNA procedures following the Manufacturers' instructions. Sometimes, it was necessary to precipitate with isopropyl alcohol, rinse with 70% ethanol, resuspend in 10 mM TE (Tris-HCl, pH 8.0, 1 mM EDTA) and precipitate a second

time in the presence of 0.3 M sodium acetate and 2 volumes of ethanol. The final pellet, after a second rinse in 70% ethanol, was resuspended in sterile distilled water (50 µL). The PCR amplification was performed on all ginseng DNA samples using oligonucleotide primers 18df/28ccr. This primer pair amplifies the conserved region, 18S-28S, including ITS1 and ITS2, highly variable regions for *Panax* species [17]. Amplification reactions were performed with reaction mixtures and with reaction conditions previously reported [11], using DNA *Taq* polymerase W1 (Invitrogen, Italy). For the RFLP analysis, amplified 18S rDNA fragments (11 µL aliquots) were separately digested in a final volume of 20 µL at 37°C for 3h and 65°C for 16h with 1.5-2 Units for each of the following endonucleases: *Hinf* I, *Sau* 3*A*1, *Taq* I (New England BioLabs, UK). Restriction fragments were analysed by electrophoresis in 1% agarose gels buffered in 0.5 X TBE [TBE buffer: 90 mM Tris (hydroxymethyl)-aminomethane, 90 mM boric acid, 3 mM ethylene-diaminetetraacetate Na salt, pH 8.3] and visualised by UV light after staining with ethidium bromide. The size marker was 50 bp ladder (Invitrogen, Italy).

Chemical analysis: Analysis of the principal active constituents was performed on all available samples by the HPLC method described in the "Ginseng" monograph of the Ph. Eur. [5], using a 5 µm (25 cm x 4.6 mm) Kromasil KR100-5NH2 E6170 column, a mobile phase filtered on an Alltech nylon membrane 47 mm, 0.45 µm, and degassed by a Waters in line degasser, at a flow rate of 1 mL/min.

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