

Standardization of Natural Medicine through Integrated Approaches

An Example of Systematic Analysis of Ginseng Drugs



Shu Zhu, Katsuko Komatsu
Institute of Natural Medicine, University of Toyama

Classification of Asian *Panax* by four taxonomists

<p>Hara (1970) (J. Jap. Bot)</p>	<p>Hoo & Tseng (1973) (Acta Phytotax. Sinica)</p>	<p>Zhou, Wu et al. (1975) (Acta Phytotax. Sinica)</p>	<p>Hoo & Tseng (1975) (Flora Reipublicae Popularis Sinicae)</p>
<p><i>Panax ginseng</i> <i>P. pseudo-ginseng</i> subsp. <i>pseudoginseng</i> subsp. <i>himalaicus</i> var. <i>angustifolius</i> var. <i>bipinnatifidus</i> subsp. <i>japonicus</i> var. <i>angustatus</i></p>	<p><i>Panax ginseng</i> <i>P. pseudo-ginseng</i> var. <i>pseudo-ginseng</i> var. <i>elegantior</i> var. <i>angustifolius</i> var. <i>bipinnatifidus</i> var. <i>japonicus</i> var. <i>wangianus</i> var. <i>notoginseng</i></p>	<p><i>Panax ginseng</i> <i>P. pseudo-ginseng</i> <i>P. japonicus</i> var. <i>japonicus</i> var. <i>major</i> var. <i>angustifolius</i> var. <i>bipinnatifidus</i> <i>P. notoginseng</i> <i>P. zingiberensis</i> <i>P. stipuleanatus</i></p>	<p><i>Panax ginseng</i> <i>P. pseudo-ginseng</i> var. <i>pseudo-ginseng</i> var. <i>elegantior</i> var. <i>angustifolius</i> var. <i>bipinnatifidus</i> var. <i>japonicus</i> var. <i>notoginseng</i> <i>P. zingiberensis</i></p>



The taxonomic controversy of original plants made it more difficult to identify their derived crude drugs.

Purpose

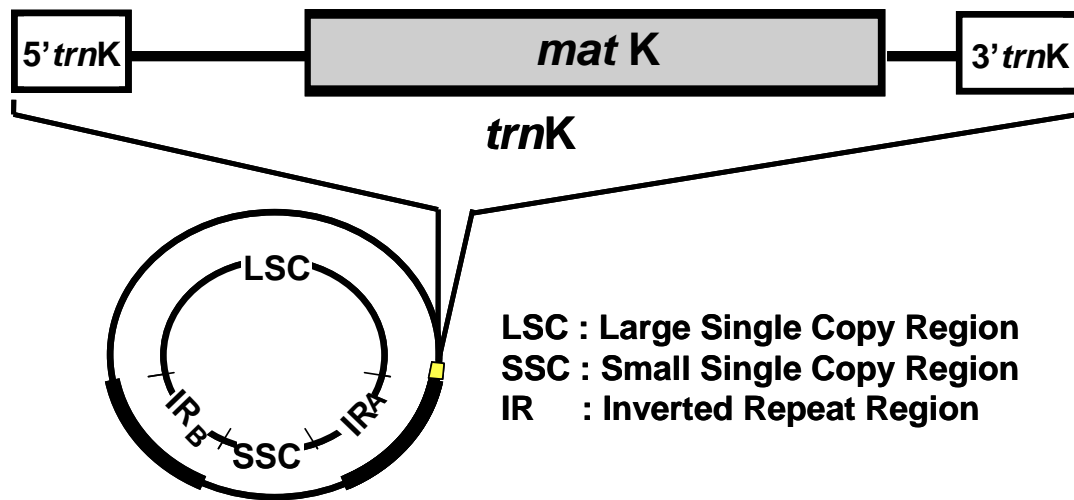
our systematical evaluation of Ginseng drugs aims to achieve molecular and chemical data that could span the obscurity of morphology, providing unambiguous index and leads to integrative understanding of Ginseng drugs.

Four approaches are included:

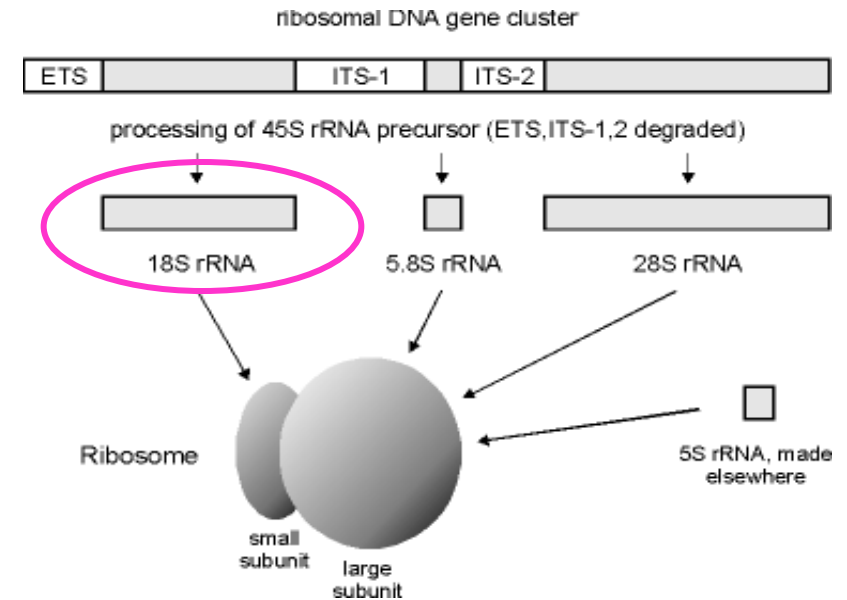
- Phylogenetic analysis based on *trnK* gene and 18S ribosomal RNA gene sequences**
- Development of a DNA microarray for authentication of ginseng drugs and *Panax* species**
- Quality evaluation of Ginseng drugs**
- Investigation of anti-dementia activity of ginseng drugs, further to find out the active ingredients**

Part I

Phylogenetic relationship in genus *Panax*: inferred from chloroplast *trnK* gene and nuclear 18S ribosomal RNA gene sequences



Chloroplast *trnK* gene:
Transfer RNA gene for Lysine (*trnK*) and
matK gene is open reading frame within its intron



Nuclear 18S rRNA gene:
Coding for small subunit of
ribosomal RNA

Materials and methods

Northern Clade

- *Panax ginseng* (8)
- *P. japonicus* (Japan) (5)
- P. quinquefolius* (3)

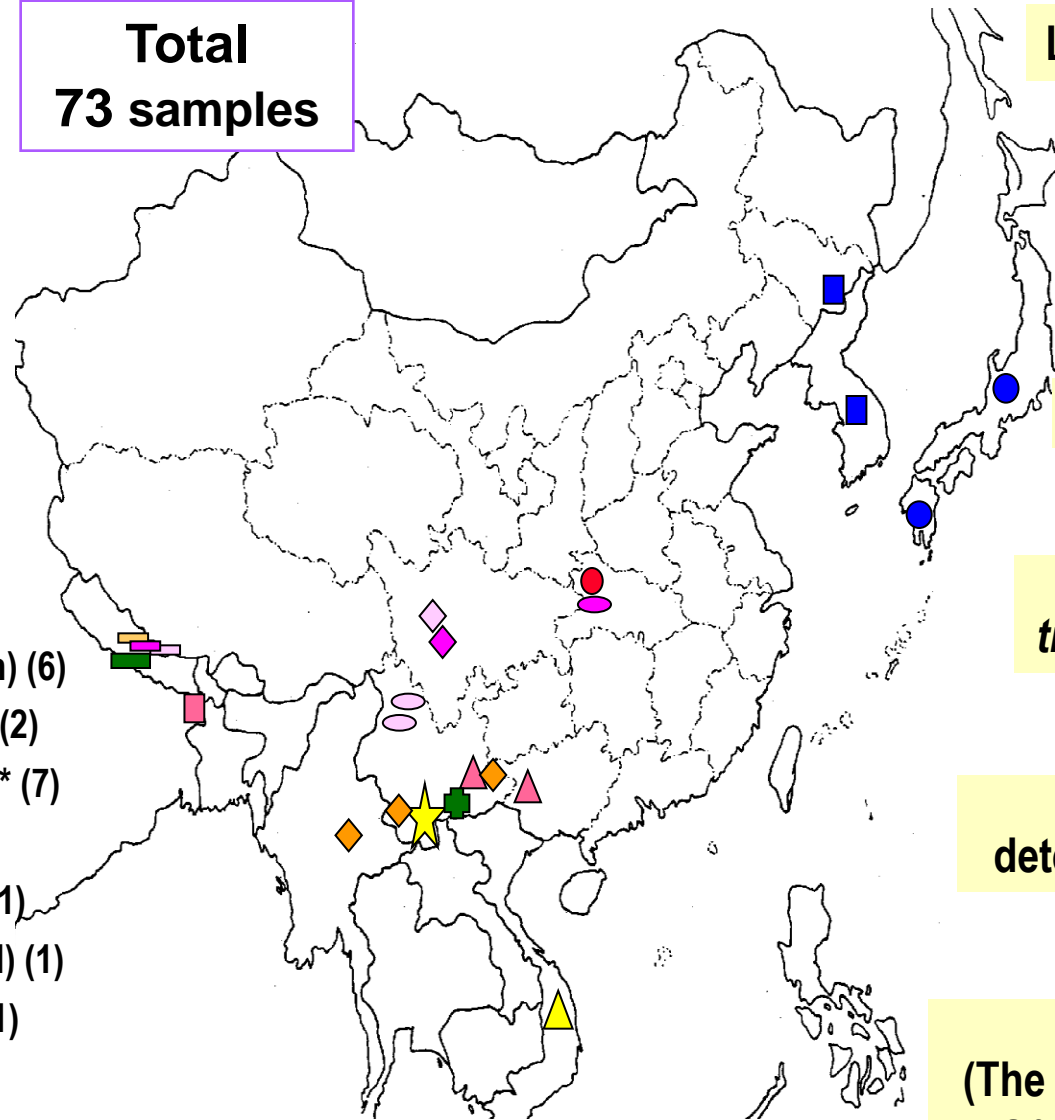
Southern Clade

- ▲ *P. notoginseng* (8)
- ◆ *P. zingiberensis* (7)
- ▲ *P. vietnamensis* (4)
- ★ PVF* (8)
- *P. japonicus* (China) (3)
- PJC* (Hubei, China) (3)
- PJ var. *major** (Yunnan) (6)
- ◆ PJ var. *angustifolius** (2)
- ◇ PJ var. *bipinnatifolius** (7)
- PPH1* (India) (1)
- PPH2* (Chame, Nepal) (1)
- PPH3* (Langtang, Nepal) (1)
- PPH4* (Gokyo, Nepal) (1)

Primitive Clade

- *P. pseudoginseng* (1)
- *P. stipuleanatus* (5)

Total
73 samples



*PJ: *P. japonicus* ; PVF: *P. vietnamensis* var. *fuscidiscus*
PPH: *P. pseudoginseng* subsp. *himalaicus*

Leaves

Underground parts

Powder

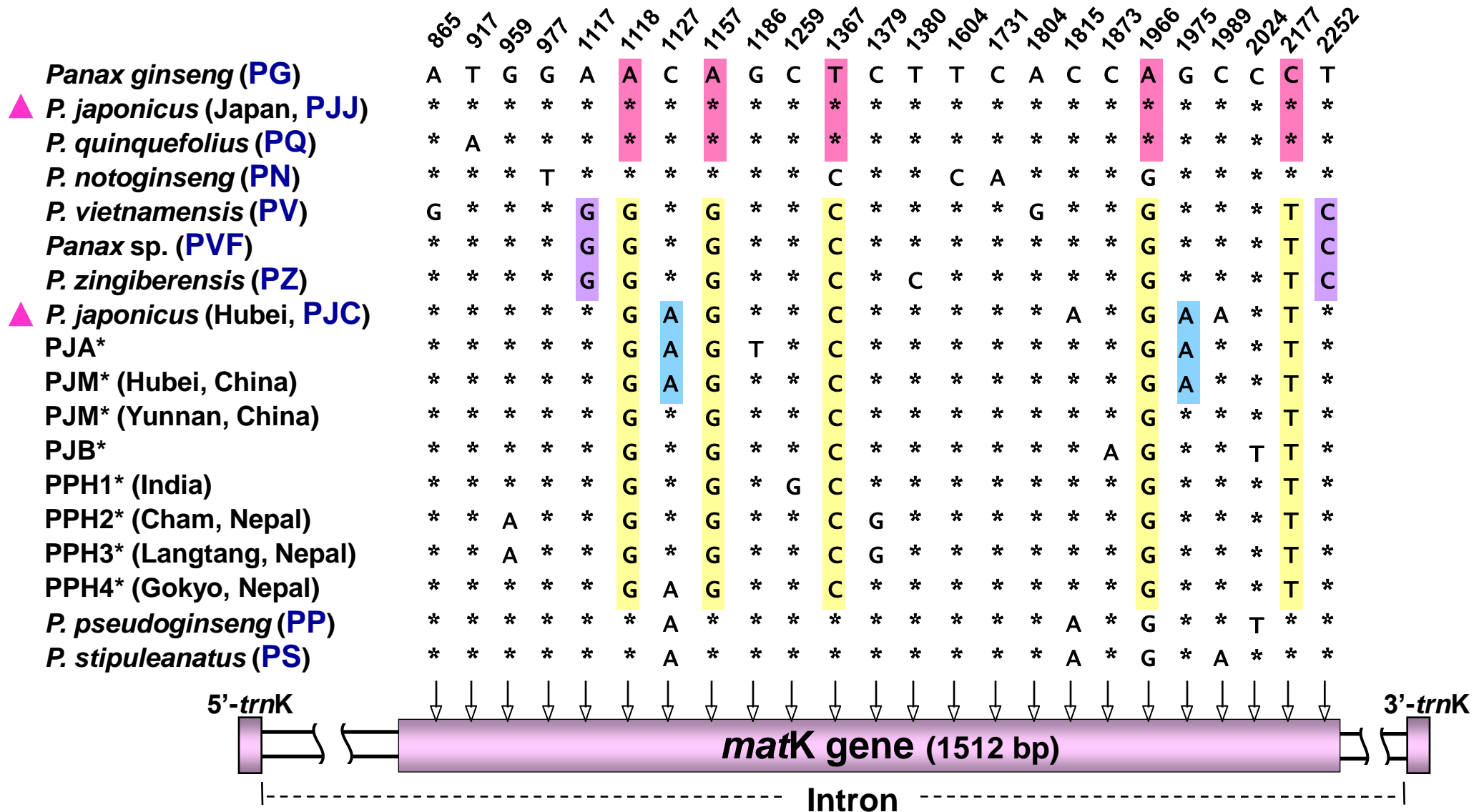
Extraction of total DNA

PCR amplification of
trnK gene and 18S rRNA gene

DNA sequences
determination and comparison

Phylogenetic Analysis
(The maximum parsimony method
and Neighbor-joining method)

Comparison of *matK* gene sequences among 13 *Panax* taxa

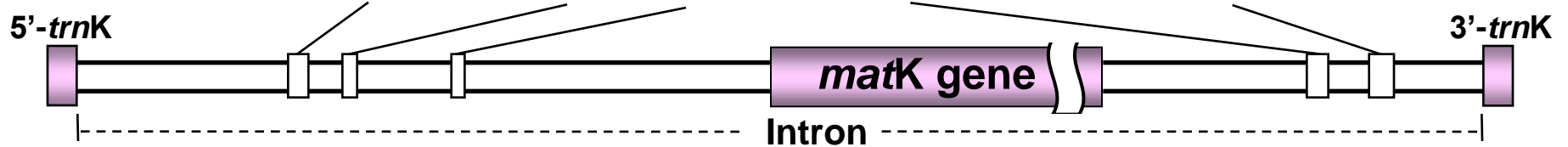


* PJA: *P. japonicus* var. *angustifolius*; PJM: *P. japonicus* var. *major*; PJB: *P. japonicus* var. *bipinnatifidus*;
 PPH: *P. pseudoginseng* subsp. *himalaicus*.

** More substitution sites of *P. pseudoginseng* and *P. stipuleanatus* could not be presented here for spatial limitation.

Comparison of *trnK* intron sequences among 13 *Panax* taxa

	220	229	237	241	319	321	2361	2374	2393	2408	Length (bp)
<i>Panax ginseng</i> (PG)	TTTTGAAACG	AAAAT	GAA	ATCCTTTTTTTATA	GGATGTAGGATGTAGT	2573					
<i>P. japonicus</i> (Japan, PJJ)	*****	*****	***	*****T**	*****	2566					
<i>P. quinquefolius</i> (PQ)	*****	*****	***	*****	*****	2573					
<i>P. notoginseng</i> (PN)	*****	*****	* - *	***** - **	*****	2564					
<i>P. vietnamensis</i> (PV)	*****	*****	***	*****T**	*****	2566					
<i>Panax</i> sp. (PVF)	*****	*****	***	*****	*****	2566					
<i>P. zingiberensis</i> (PZ)	*****	*****	***	*****	*****	2566					
<i>P. japonicus</i> (Hubei, PJC)	*****	*****	***	*****	***A*****	2573					
PJA*	*****	*****	***	*****	***A*****	2573					
PJM* (Hubei, China)	*****	*****	***	*****	***A*****	2573					
PJM* (Yunnan, China)	*****	*****	***	*****	*****	2566					
PJB*	*****	*****	***	*****	*****	2566					
PPH1* (India)	*****	*****	***	*****	*****	2566					
PPH2* (Cham, Nepal)	*****	*****	***	*****	*****	2566					
PPH3* (Langtang, Nepal)	*****	*****	***	*****	*****	2573					
PPH4* (Gokyo, Nepal)	*****	*****	***	*****	*****	2573					
<i>P. pseudoginseng</i> (PP)	*-----*	*-----*	***	*-----*	*-----*	2537					
<i>P. stipuleanatus</i> (PS)	*-----*	*-----*	*G*	*-----*	*-----*	2537					

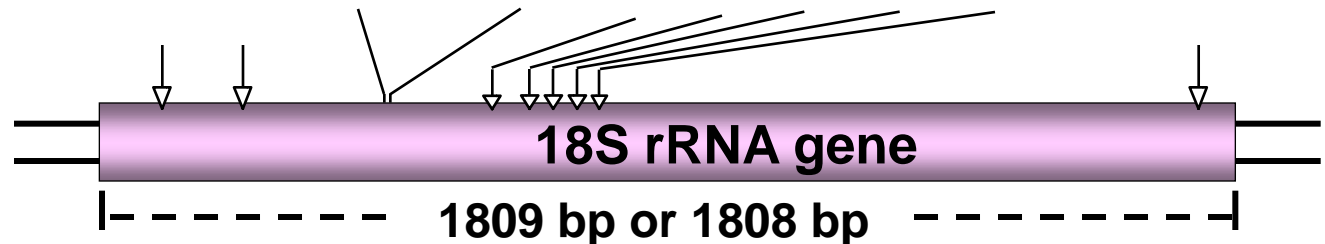


* PJA: *P. japonicus* var. *angustifolius*; PJM: *P. japonicus* var. *major*; PJB: *P. japonicus* var. *bipinnatifidus*; PPH: *P. pseudoginseng* subsp. *himalaicus*. ** More substitution sites could not present here for spatial limited

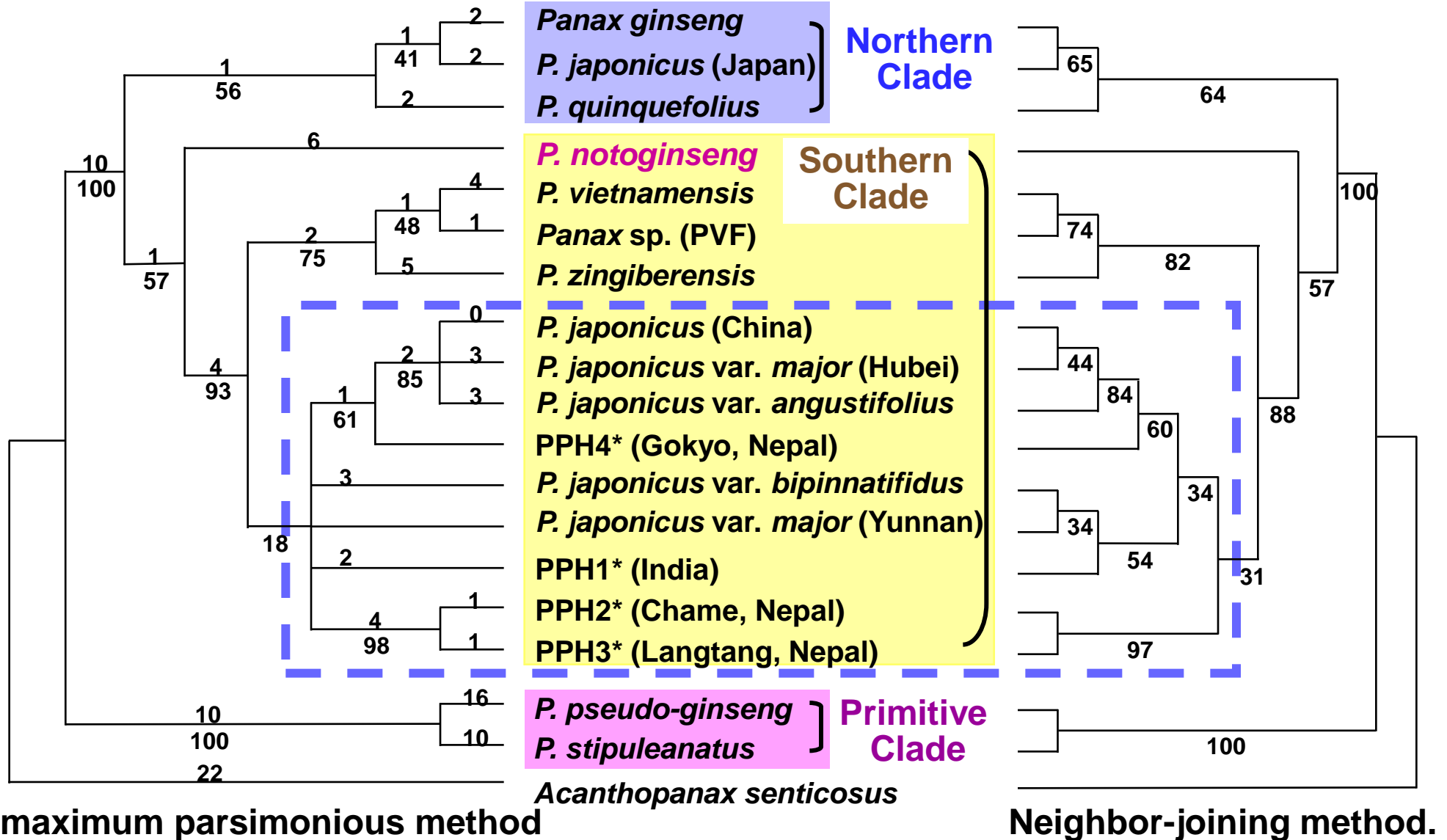
Comparison of 18S ribosomal RNA gene sequences among 13 *Panax* taxa

	191	233	496	502	683	712	714	725	729	1718
<i>Panax ginseng</i> (PG)	C	T	TGATT	CA	A	C	C	C	C	C
<i>P. japonicus</i> (Japan, PJJ)	*	*	*C***	G*	*	T	*	*	*	*
<i>P. notoginseng</i> (PN)	T	*	*C*G*	G*	*	*	*	*	*	T
<i>P. quinquefolius</i> (PQ)	*	*	*C*G*	G*	*	*	*	*	*	*
<i>P. vietnamensis</i> (PV)	*	*	*C*G*	G*	*	*	*	*	*	*
<i>Panax</i> sp. (PVF)	*	*	*C*G*	G*	*	*	*	*	*	*
<i>P. zingiberensis</i> (PZ)	*	*	*C***	G*	*	*	*	*	*	*
<i>P. japonicus</i> (Hubei, PJC)	*	*	*C***	G*	G	*	*	*	*	*
<i>P. japonicus</i> var. <i>angusfolius</i> (PJA)	T	*	*C***	G*	*	*	*	T	*	*
<i>P. japonicus</i> var. <i>major</i> (Hubei, PJM)	*	*	*C***	G*	*	*	*	*	*	*
<i>P. japonicus</i> var. <i>major</i> (Yunnan, PJM)	*	*	*C***	G*	*	*	*	*	*	*
<i>P. japonicus</i> var. <i>bipinnatifidus</i> (PJB)	*	*	*C***	G*	*	*	*	*	*	*
PPH1* (India)	*	*	*C***	G*	*	*	*	*	*	*
PPH2* (Cham, Nepal)	*	*	*C***	G*	*	*	*	*	*	*
PPH3* (Langtang, Nepal)	*	*	*C***	G*	*	*	*	*	*	*
PPH4* (Gokyo, Nepal)	*	*	*C*A*	G*	*	*	*	*	*	*
<i>P. pseudoginseng</i> (PP)	*	-	*C*C*	G*	*	A	T	*	T	*
<i>P. stipuleanatus</i> (PS)	*	-	*C*C*	G*	*	*	*	*	*	*

*PPH: *P. pseudo-ginseng* subsp. *himalaicus*



Phylogenetic tree based on combined *trnK* gene & 18S rRNA gene sequences



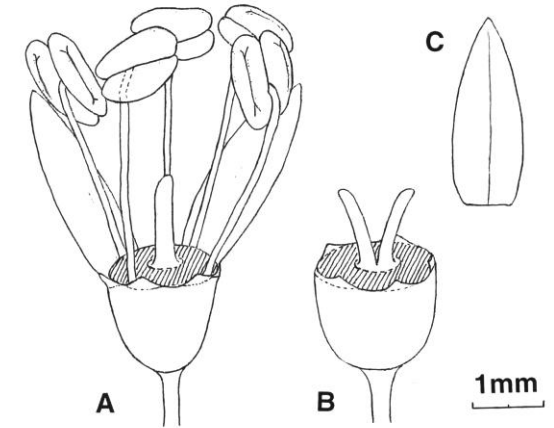
The taxa enclosed in broken-line frame are taxonomically debated taxa. * PPH: *P. pseudoginseng* subsp. *himalaicus*.

A new variety of genus *Panax* from southern Yunnan, China



Holotypus

(Jinping, Yunnan, China; Alt. 1800m)



the disk is flat, fuscous or vaccinous
styles are completely separated in 2-styled flowers

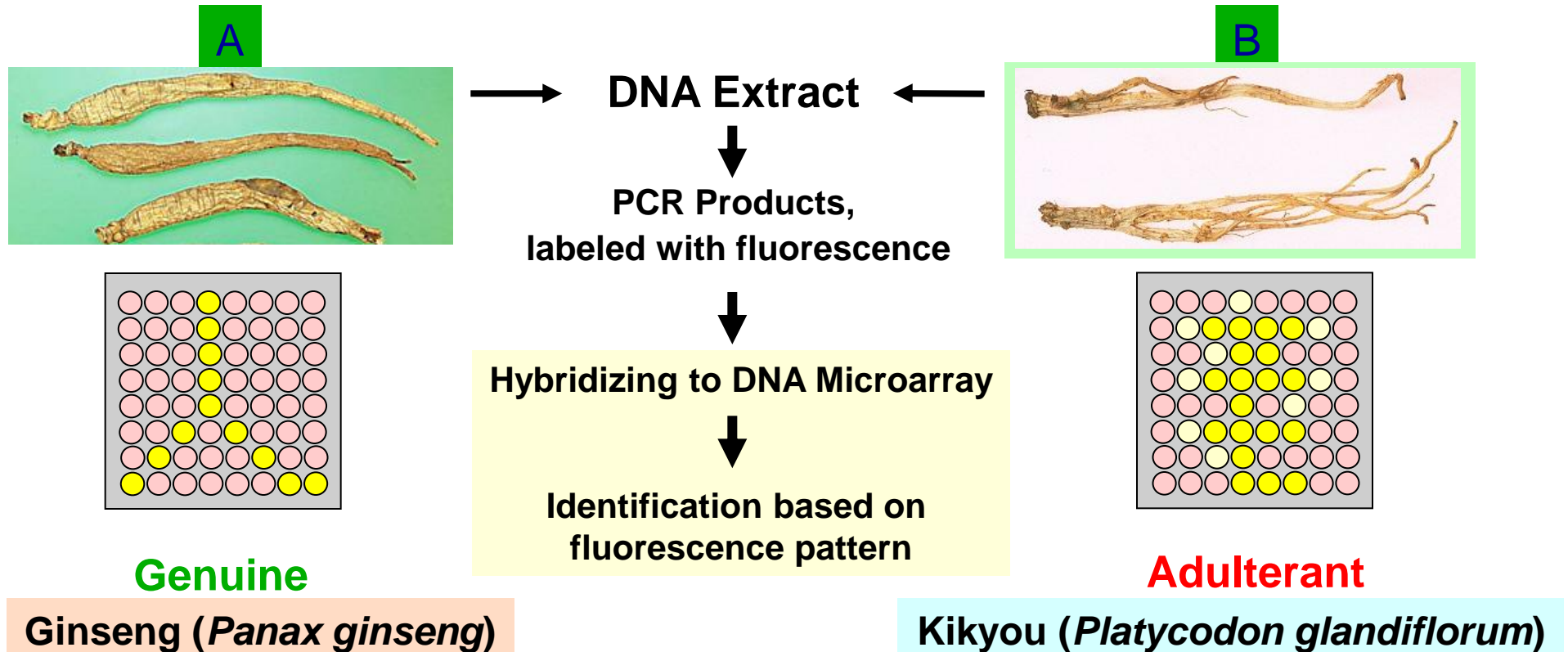


**Chromosome
number
2n=24.**

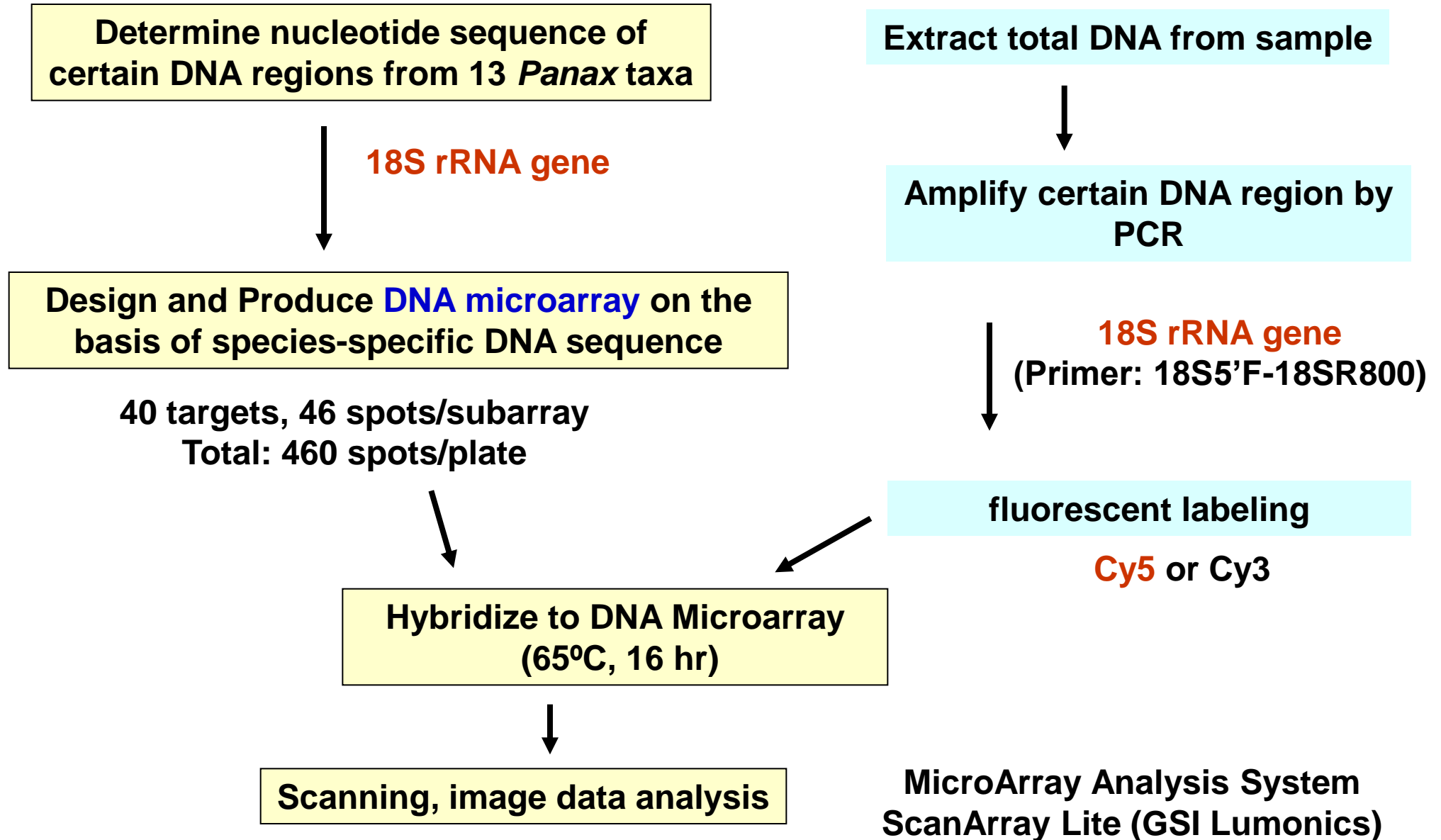
***Panax vietnamensis* Ha & Grushv.
var. *fuscidiscus* K.Komatsu, S.Zhu & S.Q.Cai**

Part II

Development of DNA microarray for authentication of Ginseng drugs



Method

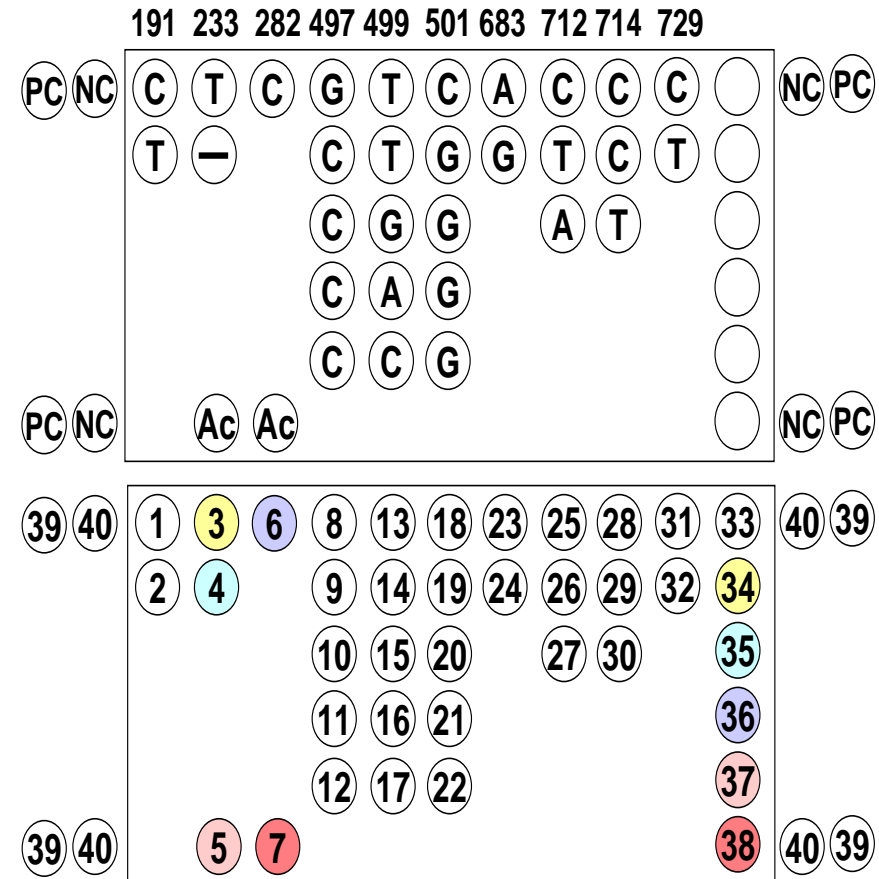
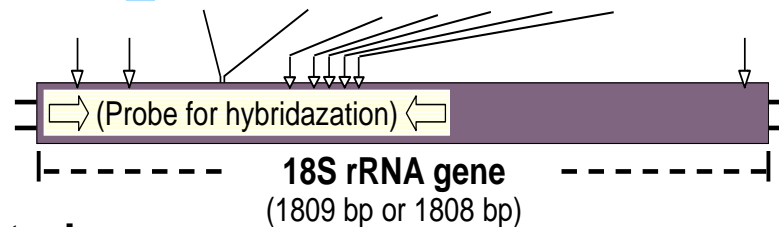


Development of DNA Microarray for Identification of Ginseng Drugs

- 18S rRNA gene -

	191	233	496	502	683	712	714	725	729	1718
<i>Panax ginseng</i>	C	T	TGATT	CA	A	C	C	C	C	C
<i>P. japonicus</i> (Japan)	*	*	*C***	G*	*	T	*	*	*	*
<i>P. notoginseng</i>	T	*	*C*G*	G*	*	*	*	*	*	T
<i>P. quinquefolius</i>	*	*	*C*G*	G*	*	*	*	*	*	*
<i>P. vietnamensis</i>	*	*	*C*G*	G*	*	*	*	*	*	*
<i>P. vietnamensis</i> var. <i>fuscidiscus</i>	*	*	*C*G*	G*	*	*	*	*	*	*
<i>P. zingiberensis</i>	*	*	*C***	G*	*	*	*	*	*	*
<i>P. japonicus</i> (China)	*	*	*C***	G*	G	*	*	*	*	*
<i>P. japonicus</i> var. <i>angustifolius</i>	T	*	*C***	G*	*	*	*	T	*	*
<i>P. japonicus</i> var. <i>major</i> (Hubei)	*	*	*C***	G*	*	*	*	*	*	*
<i>P. japonicus</i> var. <i>major</i> (Yunnan)	*	*	*C***	G*	*	*	*	*	*	*
<i>P. japonicus</i> var. <i>bipinnatifidus</i>	*	*	*C***	G*	*	*	*	*	*	*
PPH1* (India)	*	*	*C***	G*	*	*	*	*	*	*
PPH3* (Langtang, Nepal)	*	*	*C***	G*	*	*	*	*	*	*
PPH4* (Gokyo, Nepal)	*	*	*C***	G*	*	*	*	*	*	*
PPH2* (Chame, Nepal)	*	*	*C*A*	G*	*	*	*	*	*	*
<i>P. pseudoginseng</i>	*	-	*C*C*	G*	*	A	T	*	T	*
<i>P. stipuleanatus</i>	*	-	*C*C*	G*	*	*	*	*	*	*

*PPH: *P. pseudo-ginseng* subsp. *himalaicus*



PC: Positive Control,
NC: Negative Control,
Ac: *Eleutherococcus senticosus*

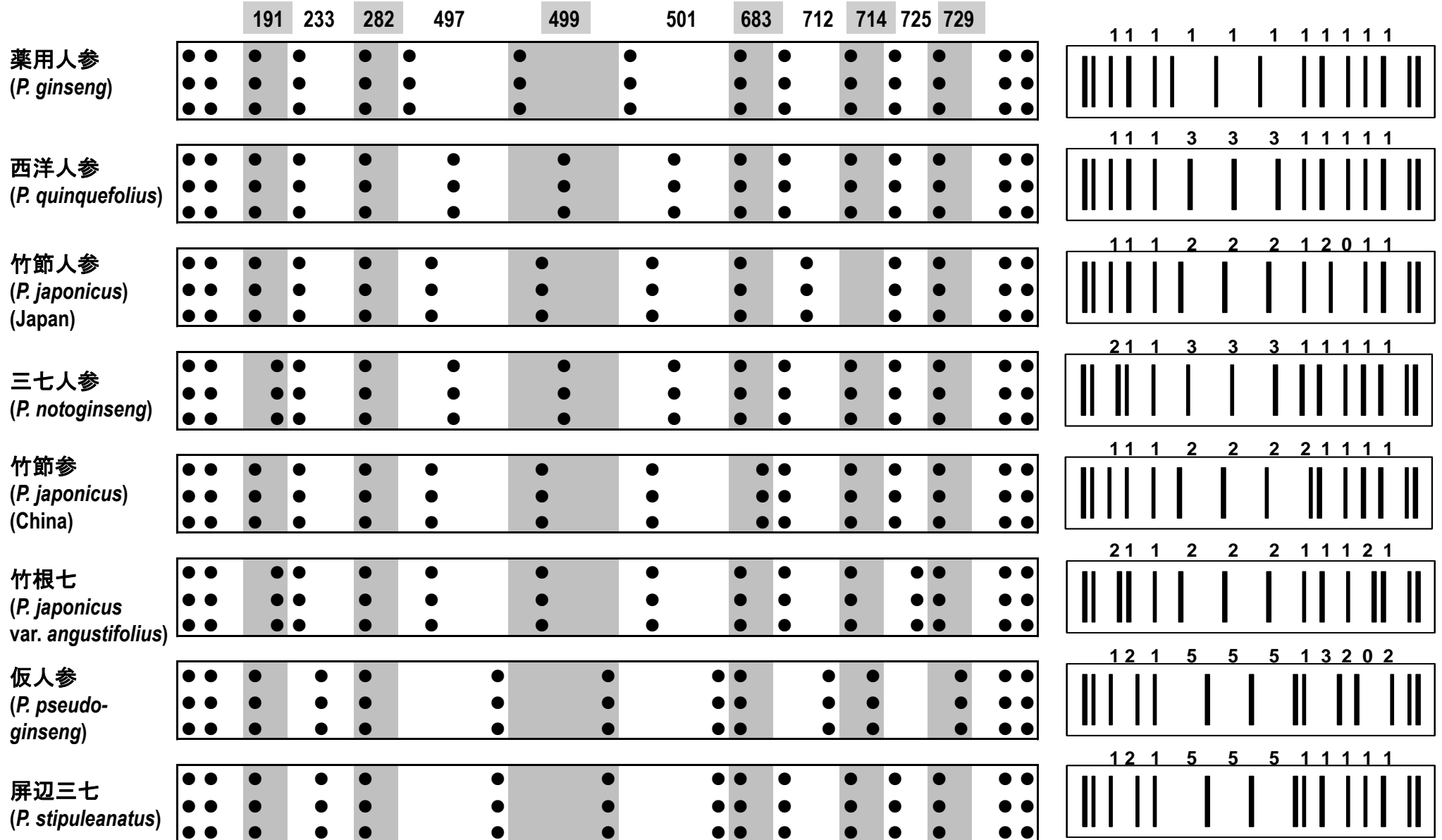
* Spots with the same color are targets designed for detecting the same nucleotide position, but with different length of sequences.

35 specific probe were designed

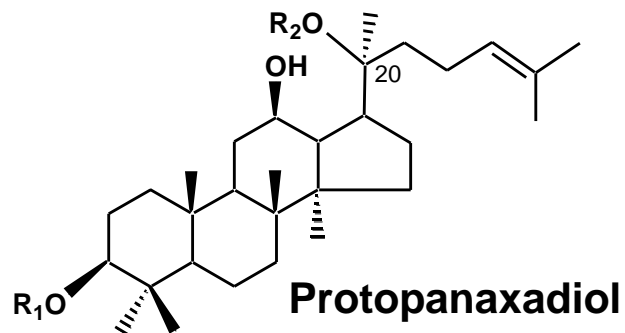
No.	Probe Name	Sequence (5' - 3')
1	P-18S-191-1	GCATCCCTTCCA g AAGTCGGGGTTT
2	P-18S-191-2	GCATCCCTTCCA a AAGTCGGGGTTT
3	P-18S-233-1	GCAACGGGCAG a AGCCCGCGTCGA
4	P-18S-233-2	GCAACGGGCAG a GCCCGCGTCGA
5	P-18S-233-3	GCAACG a GCA ta GCCCGCGTCGA
6	P-18S-282-1	TCGCCGGCACGA g GGCCGTGCGAT
7	P-18S-282-2	TCGCCGGCACGA a GGCCGTGCGAT
8	P-18S-497-1	ACAATACCGGGCT g AT c AGTCTGGT
9	P-18S-497-2	CAATACCGGGCT c AT g AGTCTGGT
10	P-18S-497-3	CAATACCGGGCT c AG g AGTCTGG
11	P-18S-497-4	CAATACCGGGCT c A a T g AGTCTGGT
12	P-18S-497-5	CAATACCGGGCT c A c T g AGTCTGG
13	P-18S-499-1	AATACCGGGCT g AT c AGTCTGGTAA
14	P-18S-499-2	ATACCGGGCT c AT g AGTCTGGTAA
15	P-18S-499-3	ATACCGGGCT c AG g AGTCTGGTA
16	P-18S-499-4	ATACCGGGCT c A a T g AGTCTGGTAA
17	P-18S-499-5	ATACCGGGCT c A c T g AGTCTGGTAA

No.	Probe Name	Sequence (5' - 3')
18	P-18S-501-1	TACCGGGCT g AT c AGTCTGGTAATT
19	P-18S-501-2	ACCGGGCT c AT g AGTCTGGTAATT
20	P-18S-501-3	ACCGGGCT c AG g AGTCTGGTAAT
21	P-18S-501-4	ACCGGGCT c A a T g AGTCTGGTAATT
22	P-18S-501-5	ACCGGGCT c A c T g AGTCTGGTAATT
23	P-18S-683-1	GGTGTGCACCG a TCGTCTCGTCC
24	P-18S-683-2	GGTGTGCACCG g TCGTCTCGTCC
25	P-18S-712-1	CGGCGATGCG c T c CTGTCCTTAA
26	P-18S-712-2	CGGCGATGCG t T c CTGTCCTTAA
27	P-18S-712-3	CGGCGATGCG a T t CTGTCCTTAA
28	P-18S-714-1	CGGCGATGCG c T c CTGTCCTTAACT
29	P-18S-714-2	CGGCGATGCG a T t CTGTCCTTAACT
30	P-18S-725-1	TCCTGTCCTTAA c TGGCCGGGTCGT
31	P-18S-725-2	TCCTGTCCTTAA t TGGCCGGGTCGT
32	P-18S-729-1	TCCTTAACTGG c CGGGTCGTGCCT
33	P-18S-729-2	TCCTTAACTGG t CGGGTCGTGCCT
34	Positive Cont.	atcatcg cagcaacggg cagaagcccg
35	Negative Cont	AGTCAGCCAGTCAGGCACTTCGATA

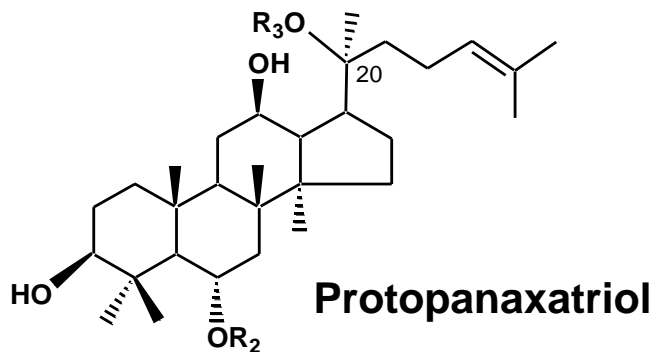
Expected fluorescent patterns of *Panax* species on PNX-array



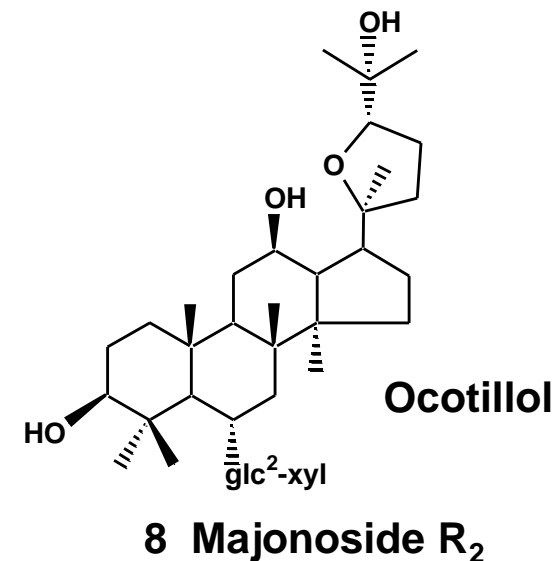
Part III Quality Evaluation of Ginseng Drugs by High Performance Liquid Chromatography (HPLC)



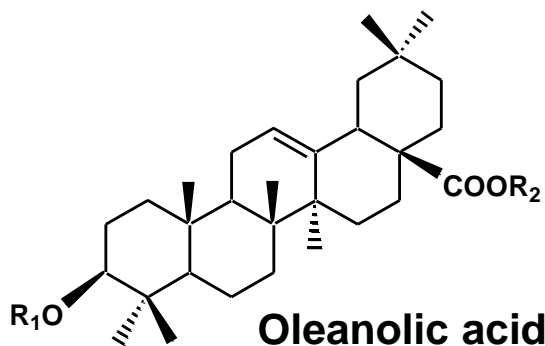
	R ₁	R ₂
1 G-Rb ₁	glc-2-glc	glc-6-glc
2 G-Rc	glc-2-glc	glc-6-araf
3 G-Rd	glc-2-glc	glc
4 C-III	glc-2-glc 6-xyl	H



	R ₁	R ₂
5 G-Re	glc-2-rha	glc
6 G-Rg ₁	glc	glc
7 N-R ₂	glc-2-xyl	H



8 Majonoside R₂



	R ₁	R ₂
9 G-Ro	gluA-2-glc	glc
10 C-IV	gluA-4-araf	glc
11 C-IVa	gluA	glc

G: Ginsenoside

C: Chikusetsusaponin

N: Notoginsenoside

HPLC condition

Column: YMC-Pack, ODS-AQ4.6 × 250mm

Detection wavelength: 196 nm

Column temperature: 40 °C

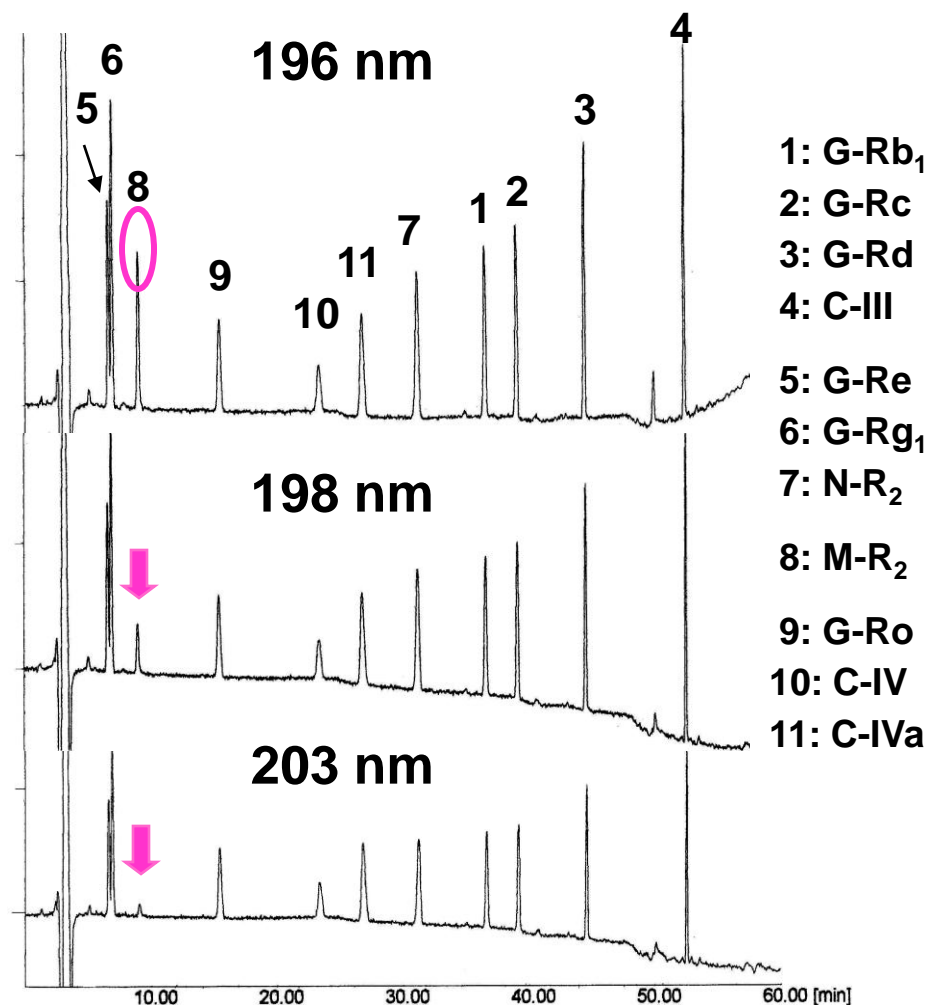
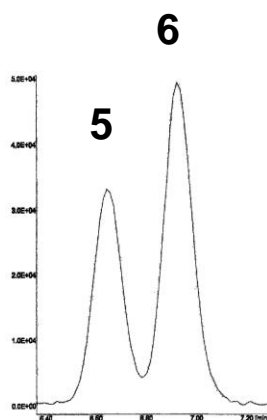
Mobile phase:

A: 10 mM K-phosphate buffer (pH 5.80);

B: CH₃CN C: H₂O

time	A	B	C
0	73	27	0
20	72	28	0
34	66	34	0
44	57	43	0
48	20	80	0
57.5	15	85	0
58	0	85	15
65	0	85	15
70	80	20	0
72	73	27	0
85	73	27	0

Flow rate: 1.0 ml/min



Comparison of chromatograms of 11 saponins under different wavelengths

G: ginsenoside; C: chikusetsusaponin; N: notoginsenoside; M: majonoside

Materials

Ginseng Drugs

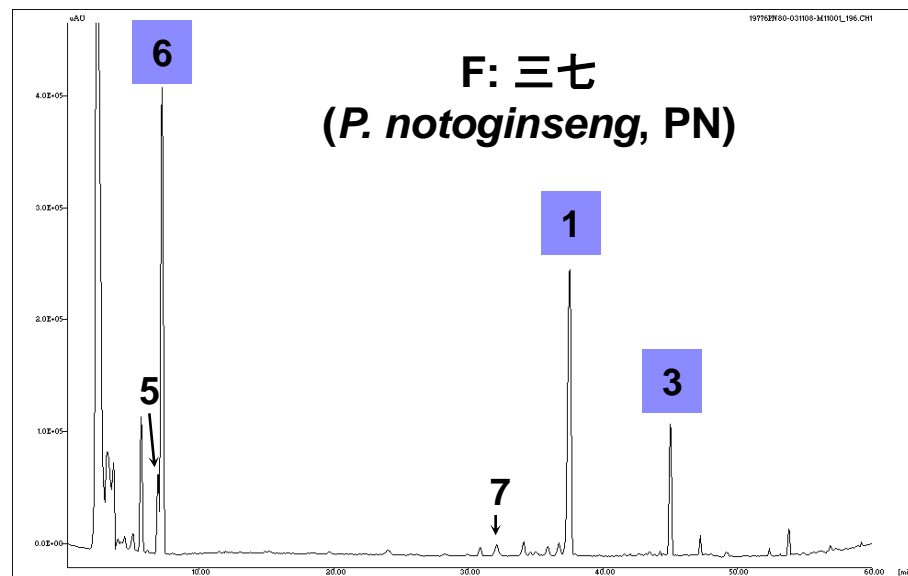
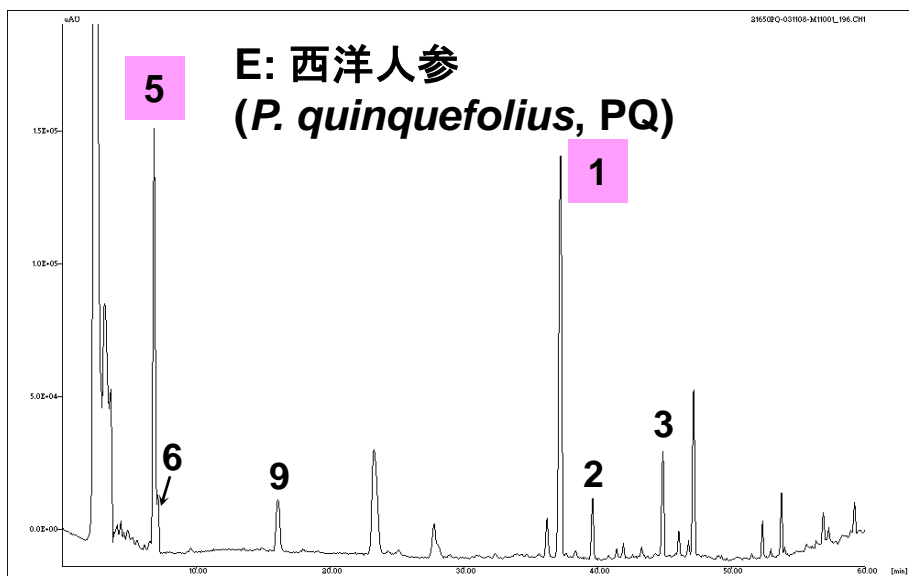
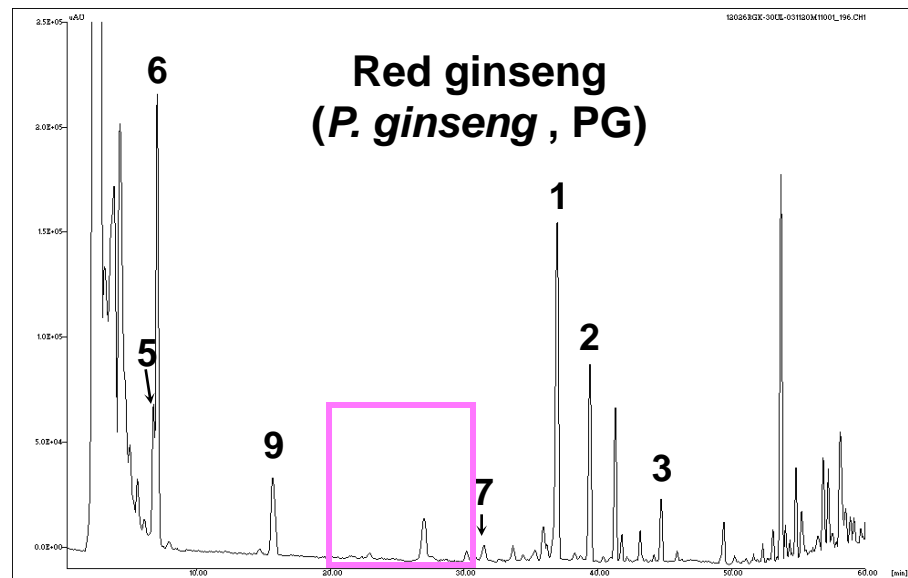
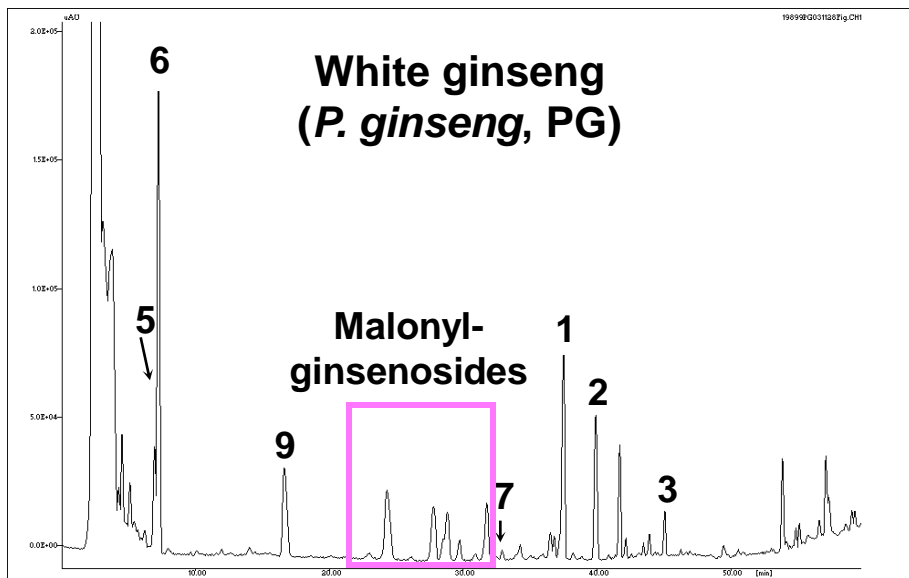
(Analyzed Samples / identified by genetic data)

Original plants

Ginseng (7), Red Ginseng (3)	1) <i>Panax ginseng</i> (PG)
American Ginseng (5/ 2)	2) <i>P. quinquefolius</i> (PQ)
Chikusetsu-ninjin (4/ 1), Satsuma-Ninjin (1/ 1)	3) <i>P. japonicus</i> (Japan) (PJJ)
Notoginseng (8)	4) <i>P. notoginseng</i> (PN)
Ginger Ginseng (2 / 1)	5) <i>P. zingiberensis</i> (PZ)
Vietnamese Ginseng (1/ 1)	6) <i>P. vietnamensis</i> (PV)
Ye-Sanchi (3/ 1)	7) <i>P. vietnamensis</i> var. <i>fuscidiscus</i> (PVF)
Pinbian-Sanchi (2/ 1), Tam That Hoang (2/ 1)	8) <i>P. stipuleanatus</i> (PS)
Bai-Sanchi (2/ 2)	9) <i>P. japonicus</i> (China) (PJC)
Zhugenqi (2/ 2)	10) <i>P. japonicus</i> var. <i>angustifolius</i> (PJA)
Kouziqi (1/ 1), Daye-Sanchi (1), Zhuzisheng (2/ 2)	11) <i>P. japonicus</i> var. <i>major</i> (PJM)
Yuye-Sanchi (1/ 1)	12) <i>P. japonicus</i> var. <i>bipinnatifidus</i> (PJB)

Total 47 samples

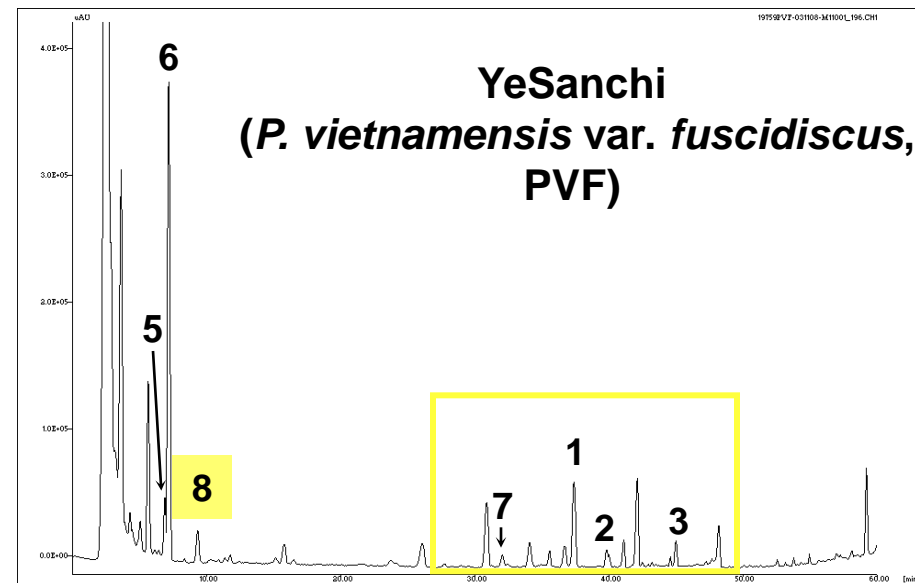
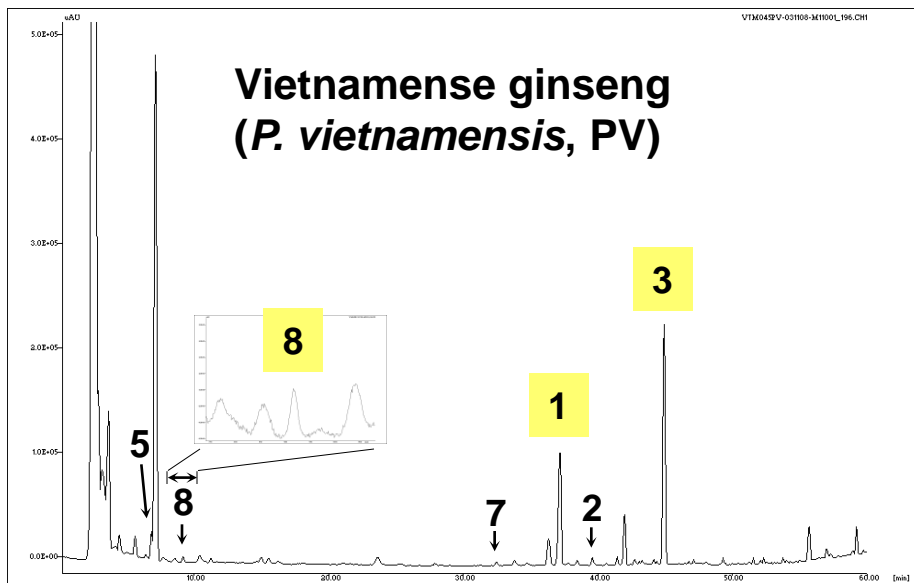
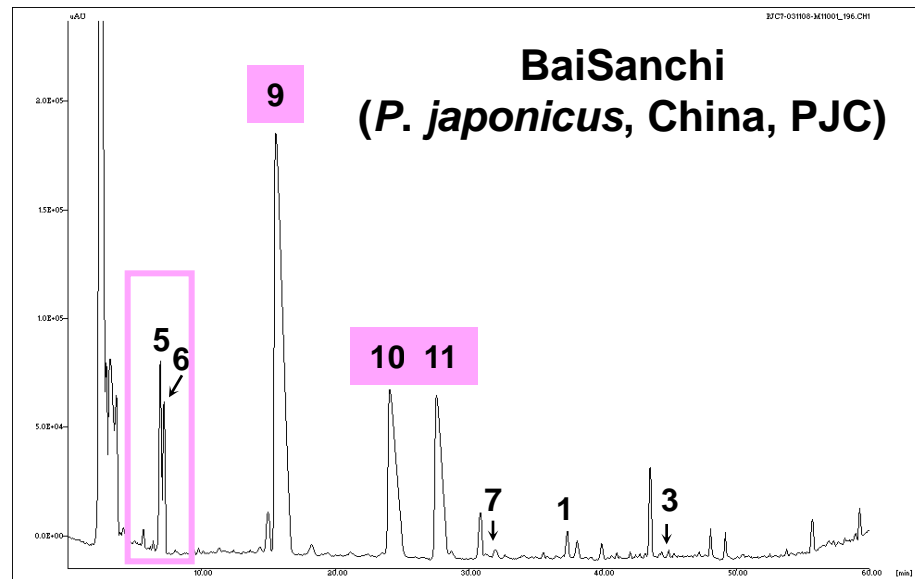
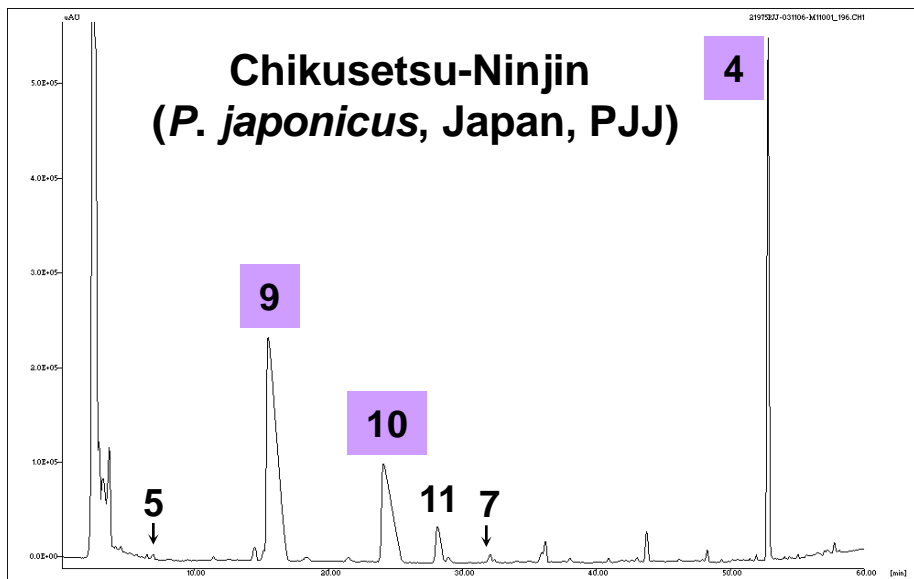
HPLC chromatograms of Ginseng Drugs



- 1: G-Rb₁
- 2: G-Rc
- 3: G-Rd
- 4: C-III
- 5: G-Re
- 6: G-Rg₁
- 7: N-R₂
- 8: M-R₂
- 9: G-Ro
- 10: C-IV
- 11: C-IVa

G: ginsenoside; C: chikusetsusaponin; N: notoginsenoside; M: majonoside

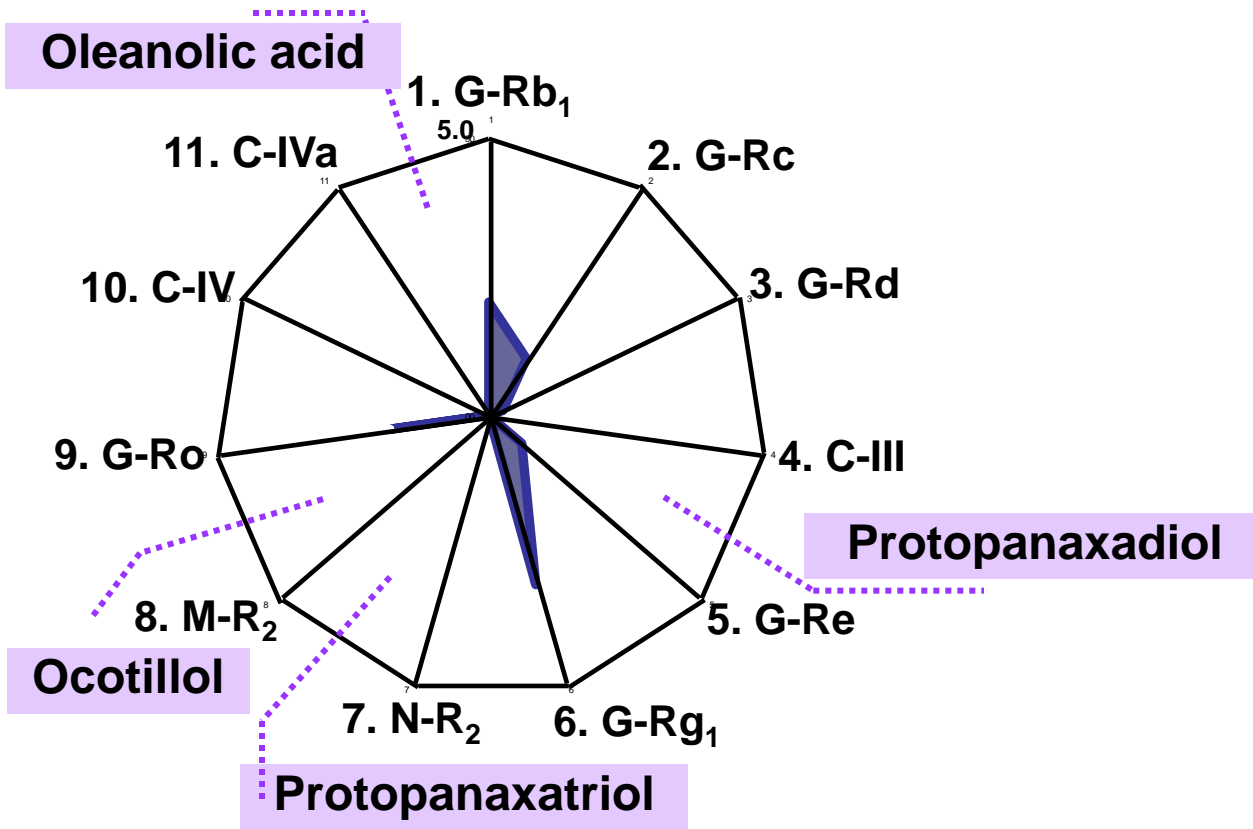
HPLC chromatograms of Ginseng Drugs



- 1: G-Rb₁
- 2: G-Rc
- 3: G-Rd
- 4: C-III
- 5: G-Re
- 6: G-Rg₁
- 7: N-R₂
- 8: M-R₂
- 9: G-Ro
- 10: C-IV
- 11: C-IVa

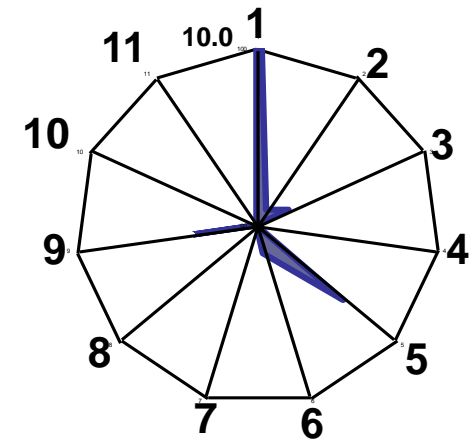
G: ginsenoside; C: chikusetsusaponin; N: notoginsenoside; M: majonoside

Quality Evaluation of Ginseng Drugs based on chemical constituents

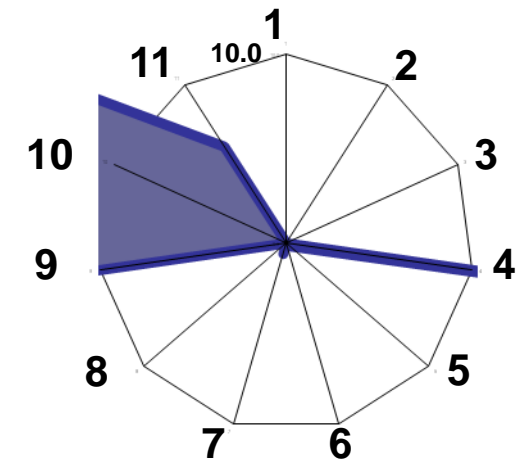


White ginseng (*P. ginseng*, PG)

G: ginsenoside C: chikusetsusaponin
 N: notoginsenoside M: majonoside



B: American ginseng (*P. quinquefolius*, PQ)

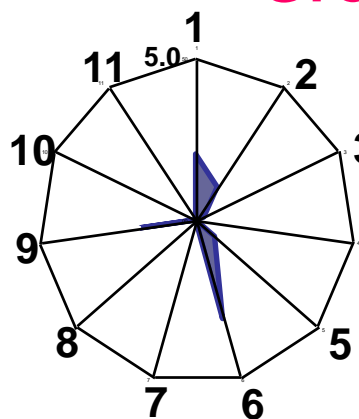


D: Chikusetsu-Ninjin (*P. japonicus*, Japan, PJJ)

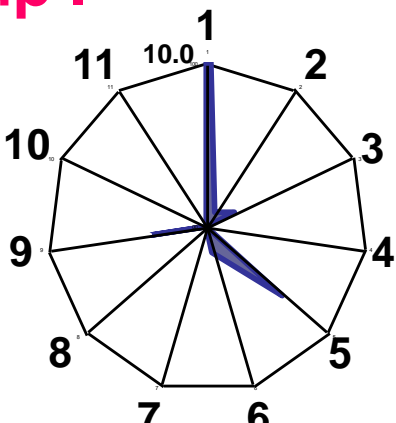
- Zhu S., Zou K., Fushimi H., Cai S. Q. and Komatsu K. *Planta Medica*. **70**: 666-677, 2004.
- Zhu S., Zou K., Cai S. Q., Meselhy R.M. and Komatsu K. *Chemi. Phar. Bull.*, **52**: 995-998, 2004.

Quality Evaluation of Ginseng Drugs based on chemical constituents

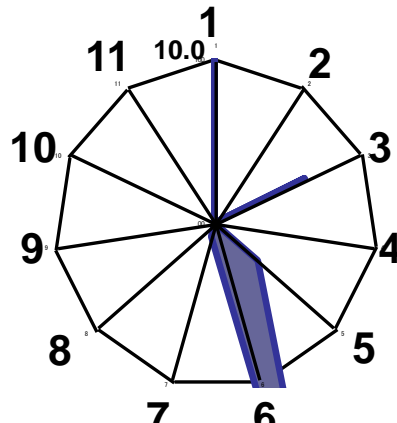
Group I



Ginseng
(*P. ginseng*)

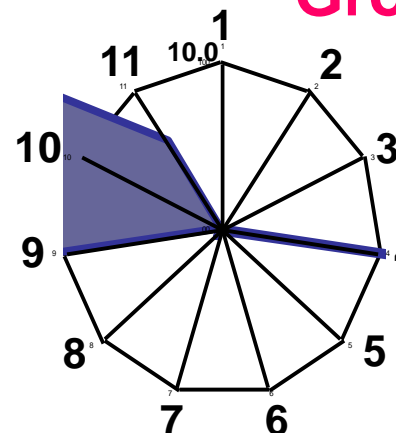


American ginseng
(*P. quinquefolius*)

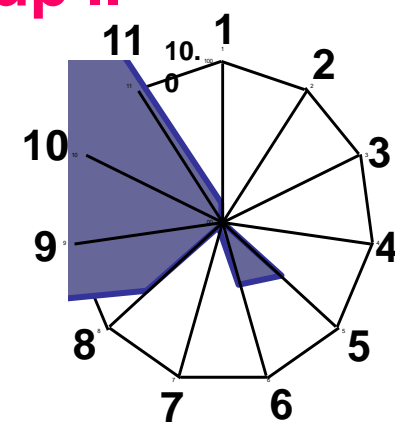


Notoginseng
(*P. notoginseng*)

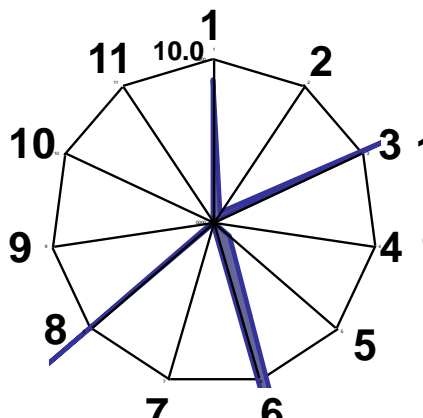
Group II



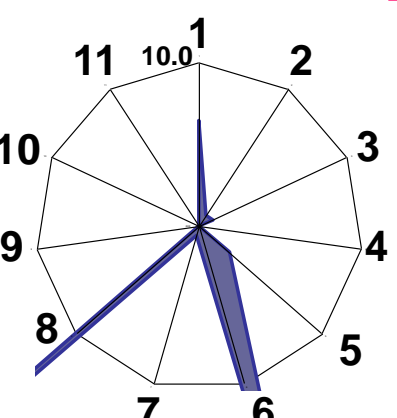
Chikusetsuinjin
(*P. japonicus*, Japan)



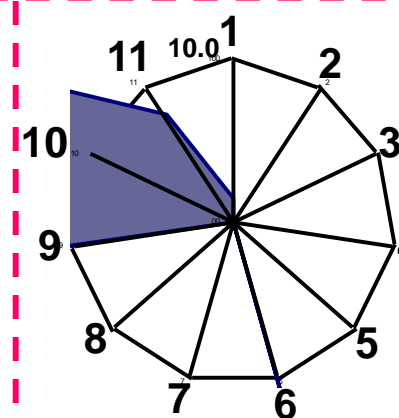
BaiSanchi
(*P. japonicus*, China)



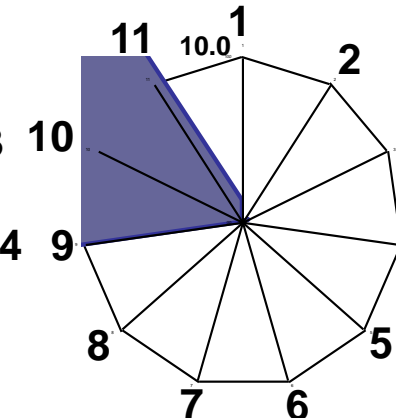
Vietnamense-ginseng
(*P. vietnamensis*)



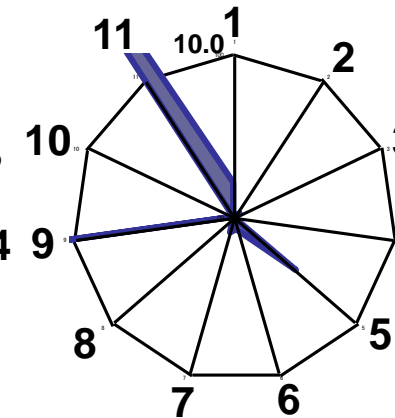
YeSanchi
(*P. vietnamensis*
var. *fuscidiscus*)



JiangSanchi
(*P. zingiberensis*)



Zhugenchi
(*P. japonicus*
var. *angustifolius*)



Kouzichi
(*P. japonicus*
var. *major*, Hubei)

1: G-Rb₁ 2: G-Rc 3: G-Rd 4: C-III 5: G-Re 6: G-Rg₁ 7: N-R₂ 8: M-R₂ 9: G-Ro 10: C-IV 11: C-IVa
G: ginsenoside; C: chikusetsusaponin; N: notoginsenoside; M: majonoside

Acknowledgement

University of Toyama

Assoc. Prof. Fushimi H.

Assoc. Prof. Tohda C.

Emer. Prof. Hattori M.

**Medicinal Plants Research
center of Toyama Pref.**

Mr. Murakami M.

Three Gorge University

Prof. Zou K.

Peking University

Prof. Cai S.Q.

Prof. Chen H.B.