Standardization of Natural Medicine through Integrated Approaches

An Example of Systematic Analysis of Ginseng Drugs

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Classification of Asian *Panax* by four taxonomists

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

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

Purpose

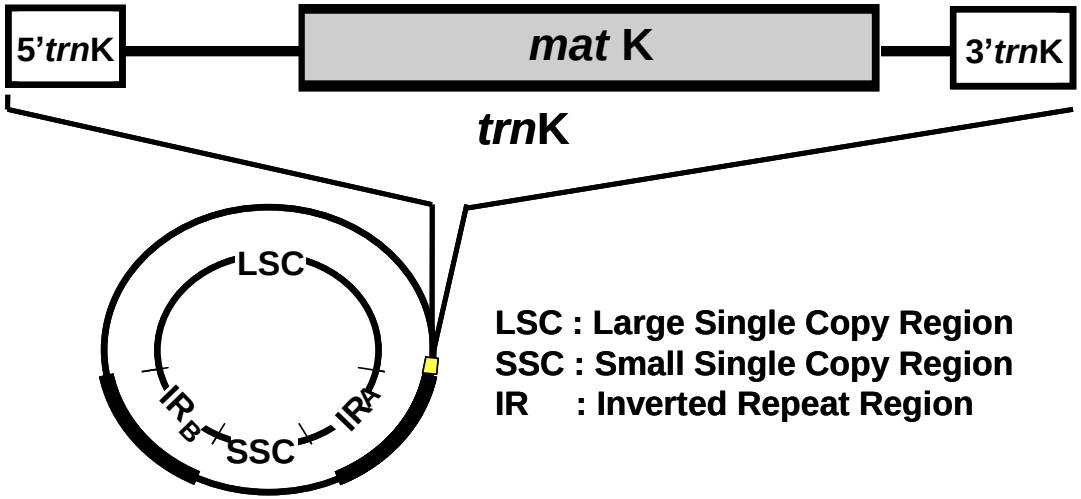
our systematical evaluation of Ginseng drugs aims to achieve molecular and chemical data that could span the obscurity of morphology, providing unambiguous index and leads to integrative understanding of Ginseng drugs.

Four approaches are included:

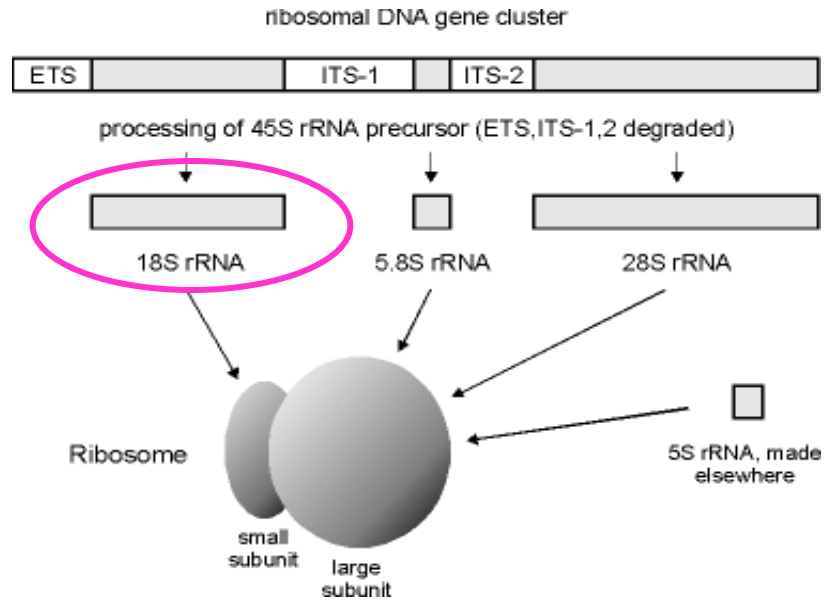
- **Phylogenetic analysis based on *trn*K gene and 18S ribosomal RNA gene sequences**
- **Development of a DNA microarray for authentication of ginseng drugs and *Panax* species**
- **Quality evaluation of Ginseng drugs**
- **Investigation of anti-dementia activity of ginseng drugs, further to find out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

Part I

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



LSC : Large Single Copy Region
SSC : Small Single Copy Region
IR : Inverted Repeat Region



Chloroplast *trn*K gene:
Transfer RNA gene for Lysine (*trn*K) and *mat*K 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)

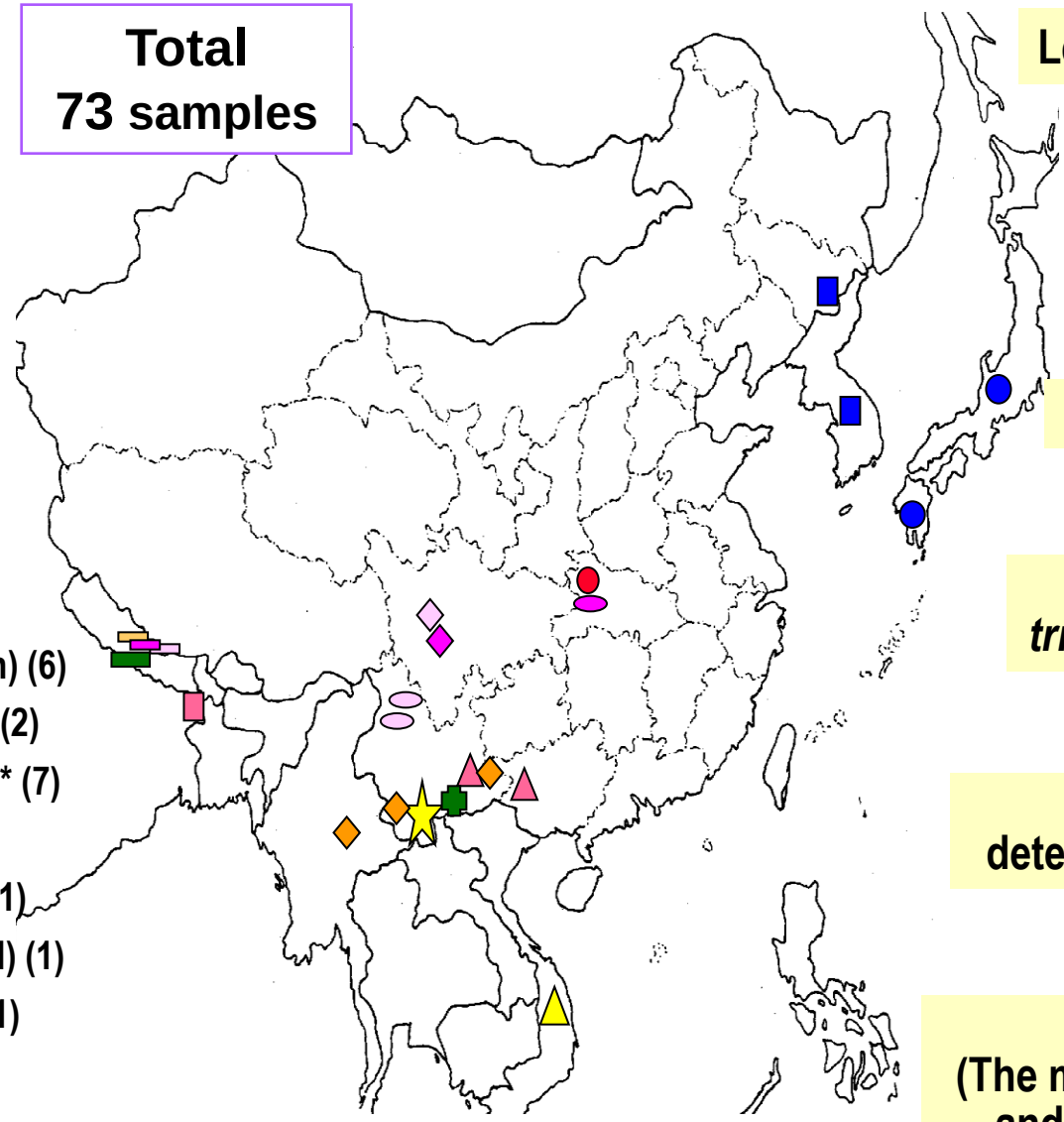
Southern Clade

- *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 *trn*K gene and 18S rRNA gene

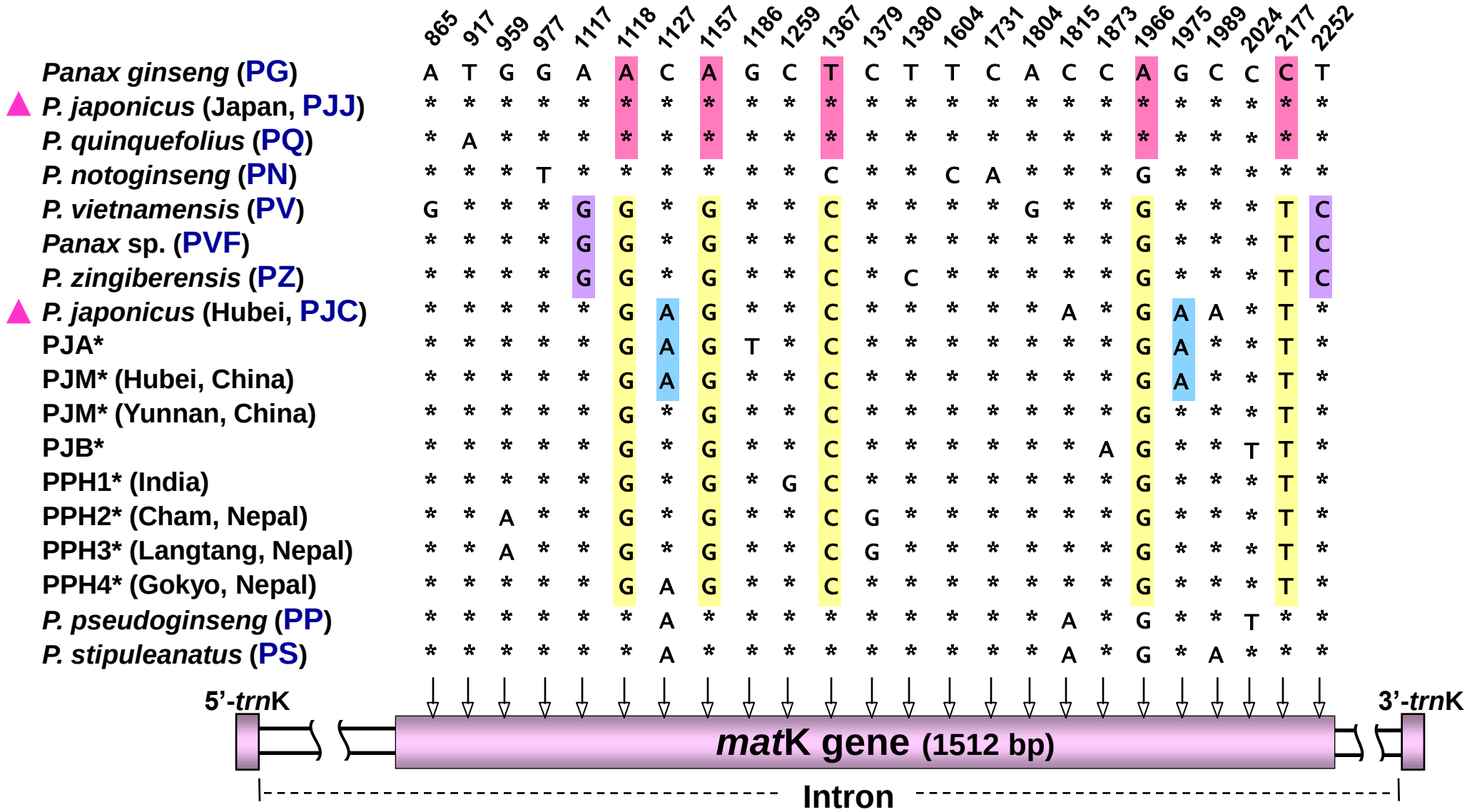
↓

DNA sequences determination and comparison

↓

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

Comparison of *mat*K gene sequences among 13 *Panax* taxa

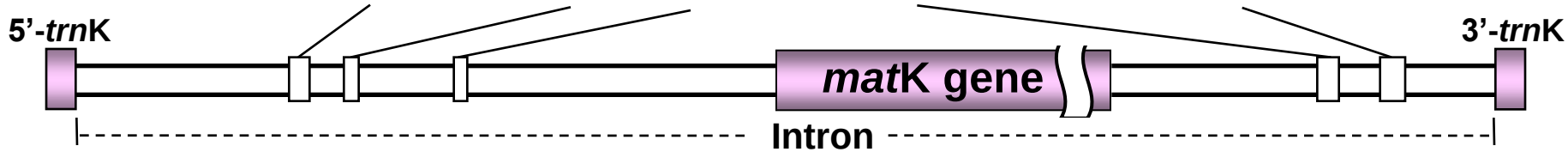


* PJA: *P. japonicus* var. *angustifolius*; PJM: *P. japonicus* var. *major*; PJB: *P. japonicus* var. *bipinnatifidus*; PPH: *P. pseudoginseng* subsp. *himalaicus* .

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

Comparison of *trn*K intron sequences among 13 *Panax* taxa

	220 229	237 241	319 321	2361 2374	2393 2408	Length (bp)
Panax ginseng (PG)	TTTTGAAACG	AAAAT	GAA	ATCCTTTTTTTATA	GGATGTAGGATGTAGT	2573
P. japonicus (Japan, PJJ)	**********	*****	***	**********T**	********-------*	2566
P. quinquefolius (PQ)	**********	*****	***	**********	**********	2573
P. notoginseng (PN)	**********	*****	*-*	**********-***	********-------*	2564
P. vietnamensis (PV)	**********	*****	***	**********T**	********-------*	2566
Panax sp. (PVF)	**********	*****	***	**********	********-------*	2566
P. zingiberensis (PZ)	**********	*****	***	**********	********-------*	2566
P. japonicus (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
P. pseudoginseng (PP)	*--------*	*---*	***	**----------*	*----------*	2537
P. stipuleanatus (PS)	*--------*	*---*	*G*	**----------*	*----------*	2537

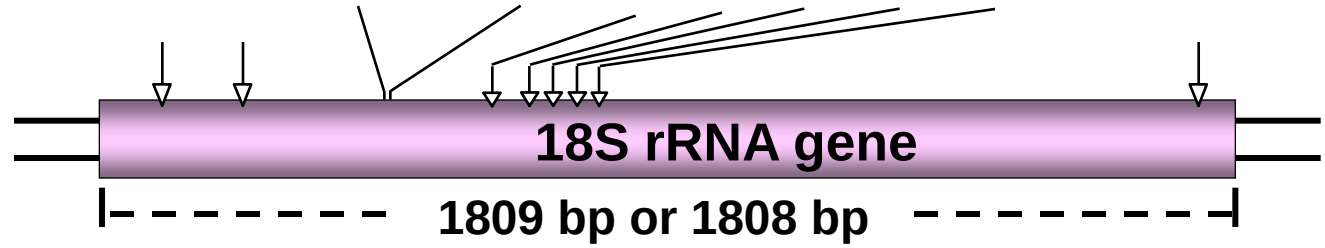


* PJA: *P. japonicus* var. *angustifolius*; PJM: *P. japonicus* var. *major*; PJB: *P. japonicus* var. *bipinnatifidus*; PPH: *P. pseudoginseng* subsp. *himalaicus* . ** More substitution sites could not present here for spatial limited

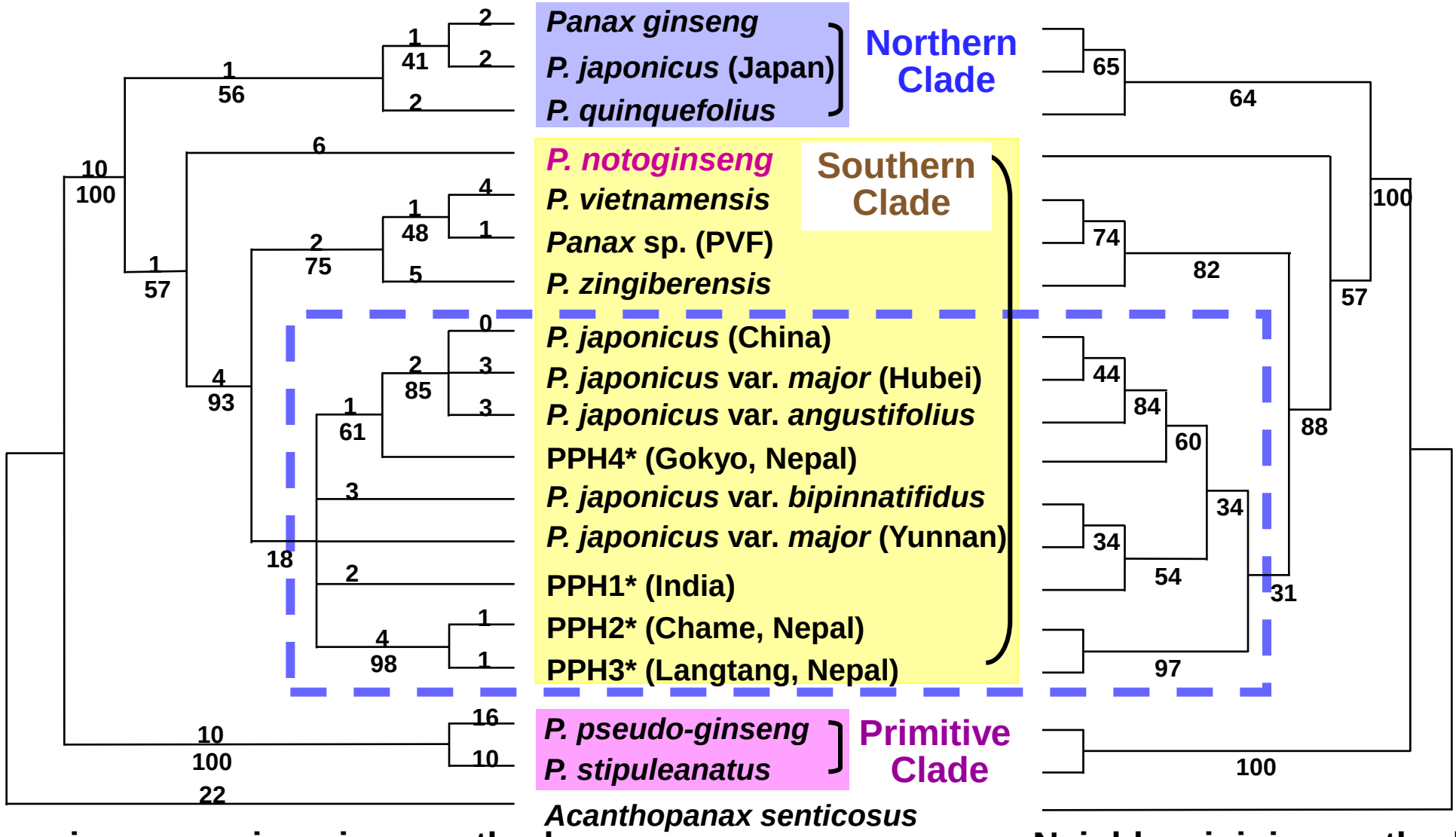
Comparison of 18S ribosomal RNA gene sequences among 13 *Panax* taxa

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

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



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



maximum parsimonious method **Neighbor-joining method.**

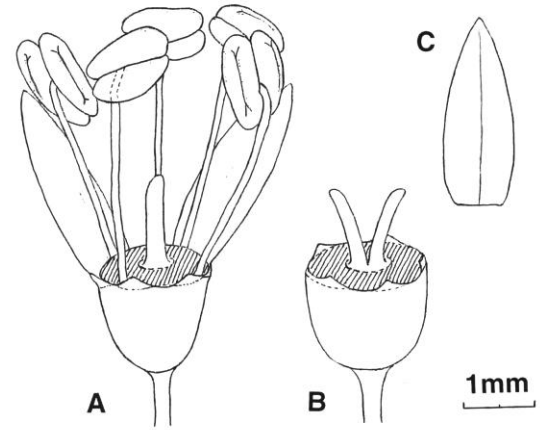
The taxa enclosed in broken-line frame are taxonomically debated taxa. *** PPH: *P. pseudoginseng* subsp. *himalaicus.***

○ **Zhu S.**, Fushimi H., Cai S. Q. and Komatsu K. Planta Medica. **69**: 647-653, 2003.

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.

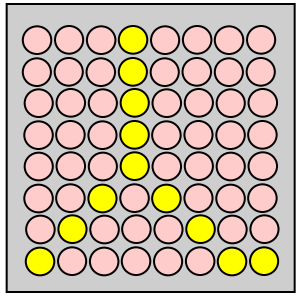
***Panax vietnamensis* Ha & Grushv.**
var. *fuscidiscus* K.Komatsu, S.Zhu & S.Q.Cai

○**Zhu S**., Fushimi H., Cai S. Q., Chen H. B., Komatsu K. The Japanese Journal of Botany. **78**: 86-94, 2003.

Part II

Development of DNA microarray for authentication of Ginseng drugs

A



Genuine

Ginseng (*Panax ginseng*)

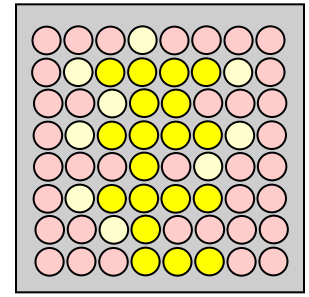
DNA Extract

PCR Products, labeled with fluorescence

Hybridizing to DNA Microarray

Identification based on fluorescence pattern

B



Adulterant

Kikyou (*Platycodon glandiflorum*)

Method

Determine nucleotide sequence of certain DNA regions from 13 *Panax* taxa

18S rRNA gene

Design and Produce DNA microarray on the basis of species-specific DNA sequence

40 targets, 46 spots/subarray
Total: 460 spots/plate

Extract total DNA from sample

Amplify certain DNA region by PCR

18S rRNA gene
(Primer: 18S5'F-18SR800)

fluorescent labeling

Cy5 or Cy3

Hybridize to DNA Microarray
(65°C, 16 hr)

Scanning, image data analysis

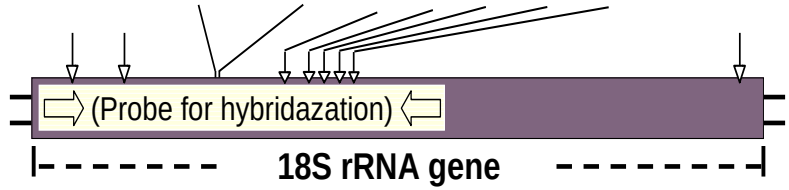
MicroArray Analysis System
ScanArray Lite (GSI Lumonics)

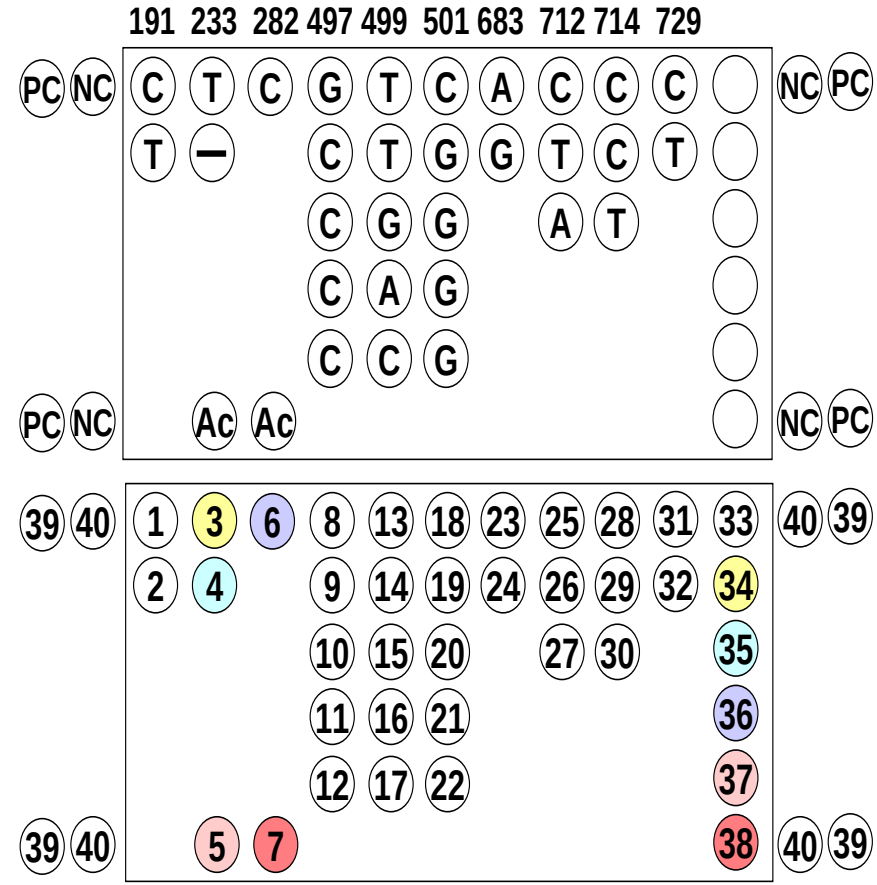
Development of DNA Microarray for Identification of Ginseng Drugs

- 18S rRNA gene -

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

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





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

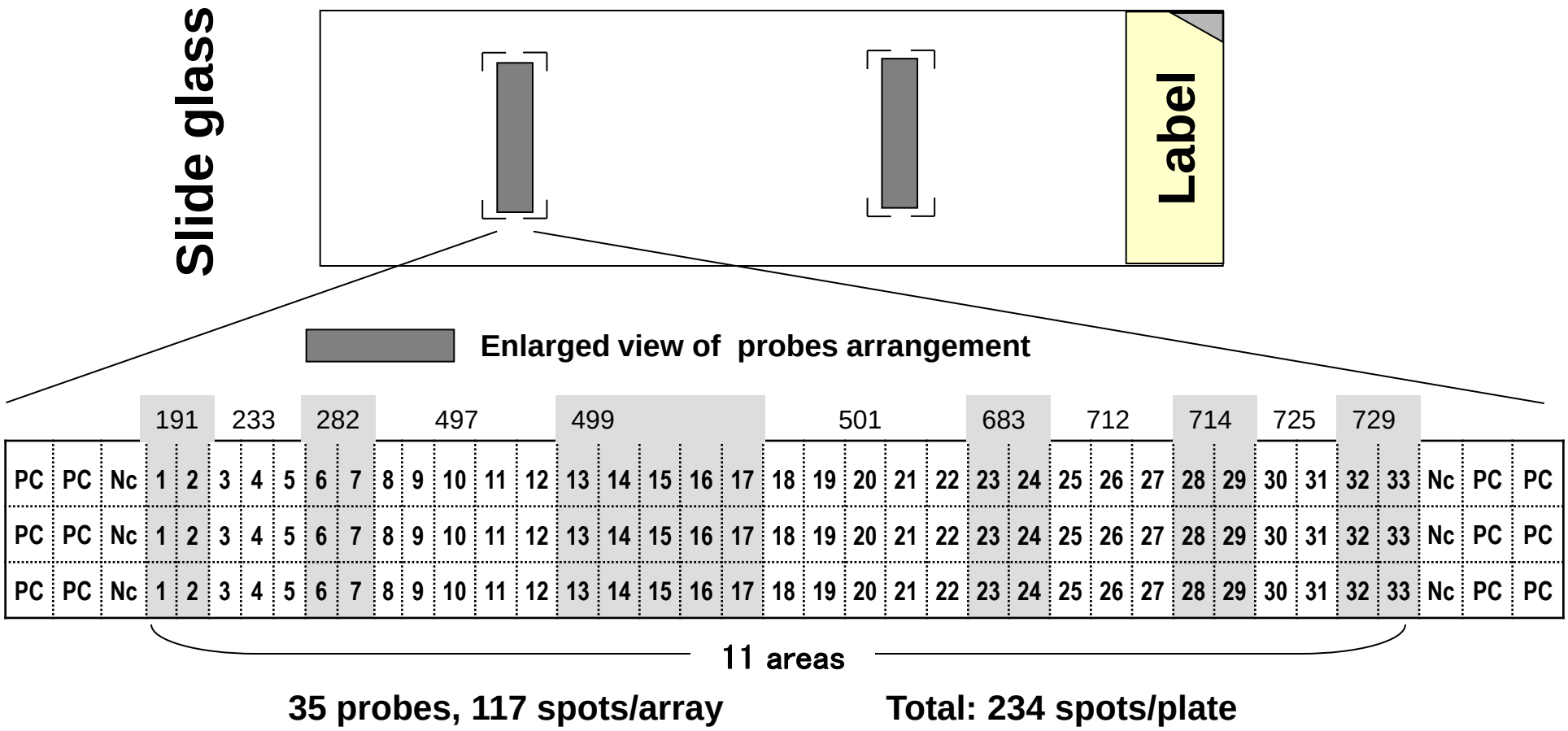
* Spots with the same color are targets designed for detecting the same nucleotide position, but with different length of sequences.

35 specific probe were designed

No.	Probe Name	Sequence (5' - 3')
1	P-18S-191-1	GCATCCCTTCCAgAAGTCGGGGTTT
2	P-18S-191-2	GCATCCCTTCCAaAAGTCGGGGTTT
3	P-18S-233-1	GCAACGGGCAGaaGCCCGCGTCGA
4	P-18S-233-2	GCAACGGGCAGa GCCCGCGTCGA
5	P-18S-233-3	GCAACGaGCA taGCCCGCGTCGA
6	P-18S-282-1	TCGCCGGCACGAgGGCCGTGCGAT
7	P-18S-282-2	TCGCCGGCACGAaGGCCGTGCGAT
8	P-18S-497-1	ACAATACCGGGCTgAtTcAGTCTGGT
9	P-18S-497-2	CAATACCGGGCTcAtTgAGTCTGGT
10	P-18S-497-3	CAATACCGGGCTcAgTgAGTCTGG
11	P-18S-497-4	CAATACCGGGCTcAaTgAGTCTGGT
12	P-18S-497-5	CAATACCGGGCTcAcTgAGTCTGG
13	P-18S-499-1	AATACCGGGCTgAtTcAGTCTGGTAA
14	P-18S-499-2	ATACCGGGCTcAtTgAGTCTGGTAA
15	P-18S-499-3	ATACCGGGCTcAgTgAGTCTGGTA
16	P-18S-499-4	ATACCGGGCTcAaTgAGTCTGGTAA
17	P-18S-499-5	ATACCGGGCTcAcTgAGTCTGGTAA

No.	Probe Name	Sequence (5' - 3')
18	P-18S-501-1	TACCGGGCTgAtTcAGTCTGGTAATT
19	P-18S-501-2	ACCGGGCTcAtTgAGTCTGGTAATT
20	P-18S-501-3	ACCGGGCTcAgTgAGTCTGGTAAT
21	P-18S-501-4	ACCGGGCTcAaTgAGTCTGGTAATT
22	P-18S-501-5	ACCGGGCTcAcTgAGTCTGGTAATT
23	P-18S-683-1	GGTGTGCACCGaTCGTCTCGTCC
24	P-18S-683-2	GGTGTGCACCGgTCGTCTCGTCC
25	P-18S-712-1	CGGCGATGCGcTcCTGTCCTTAA
26	P-18S-712-2	CGGCGATGCGtTcCTGTCCTTAA
27	P-18S-712-3	CCGGCGATGCGaTtCTGTCCTTAA
28	P-18S-714-1	CGGCGATGCGcTcCTGTCCTTAACT
29	P-18S-714-2	CGGCGATGCGaTtCTGTCCTTAACT
30	P-18S-725-1	TCCTGTCCTTAAcTGGCCGGGTCGT
31	P-18S-725-2	TCCTGTCCTTAAtTGGCCGGGTCGT
32	P-18S-729-1	TCCTTAACTGGcCGGGTCGTGCCT
33	P-18S-729-2	TCCTTAACTGGtCGGGTCGTGCCT
34	Positive Cont.	atcatcg cagcaacggg cagaagcccg
35	Negative Cont	AGTCAGCCAGTCAGGCACTTCGATA

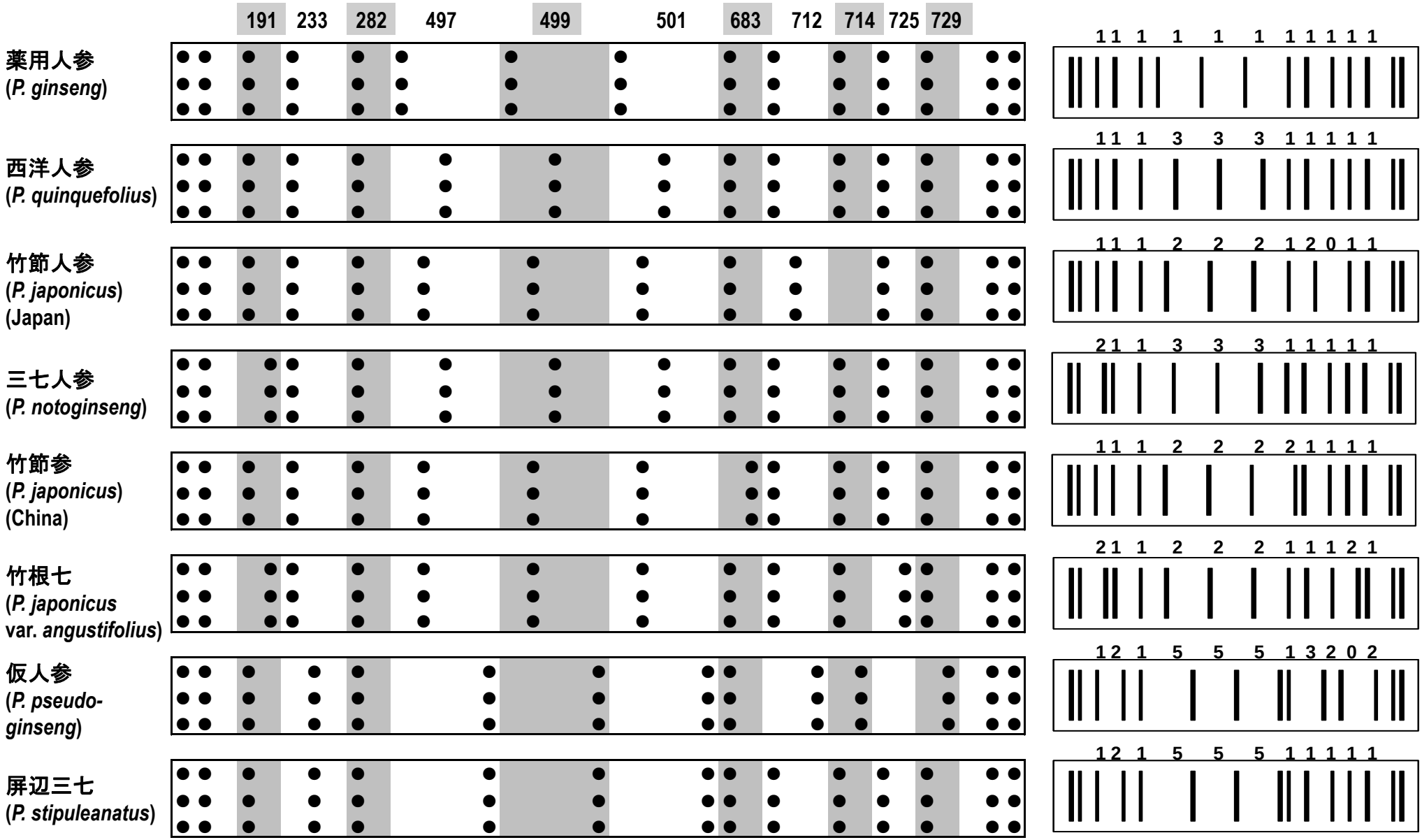
Layout of the DNA microarray



* Number in box is the identity of oligonucleotide probe, corresponding to number shown in table 1. Positive and negative controls are spotted at both left and right ends.

** Number below box indicates the corresponding substitution position in 18S rRNA gene.

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



Result

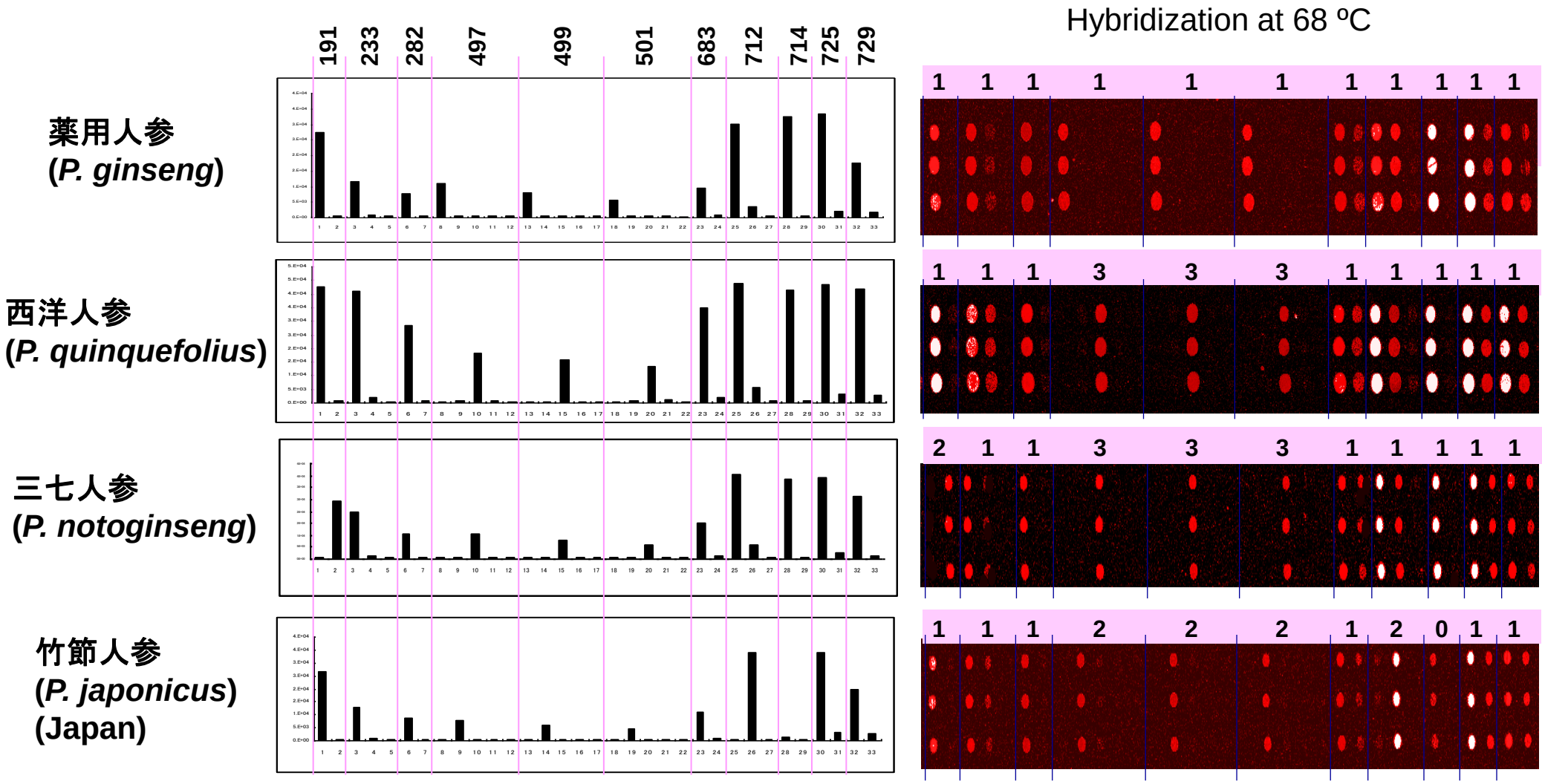
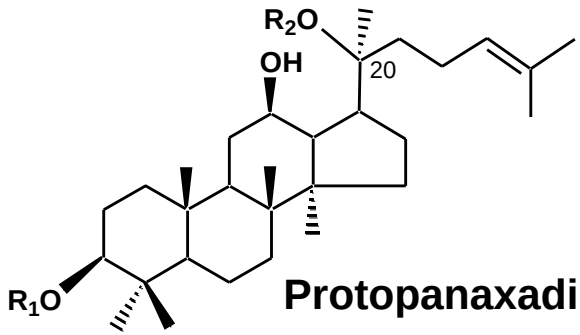


Fig. 3 Fluorescence pattern of PNX-array by using Fluorescently labeled targets from Ginseng, American Ginseng, Notoginseng, Japanese Ginseng.

○ **Zhu S.**, Fushimi H., Komatsu K. J. Agric. Food. Chem., **56**: 3953-3959, 2008.

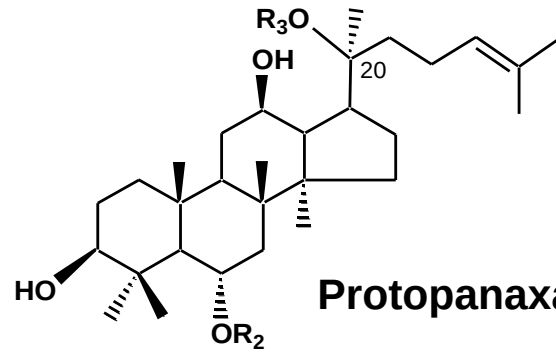
Part III

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



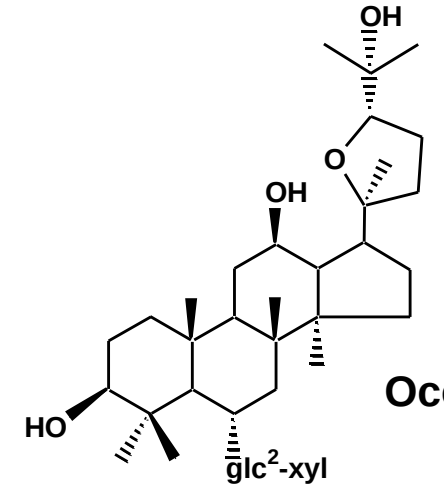
Protopanaxadiol

		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



Protopanaxatriol

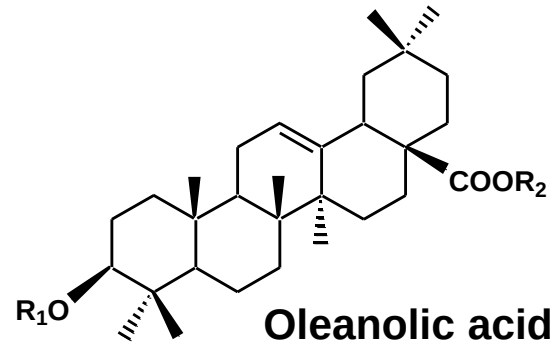
		R_1	R_2
5	G-Re	glc-2-rha	glc
6	G-Rg_1	glc	glc
7	N-R_2	glc-2-xyl	H



Ocotillol

8 Majonoside R_2

G: Ginsenoside
C: Chikusetsusaponin
N: Notoginsenoside



Oleanolic acid

		R_1	R_2
9	G-Ro	gluA-2-glc	glc
10	C-IV	gluA-4-araf	glc
11	C-IVa	gluA	glc

HPLC condition

Colume: YMC-Pack, ODS-AQ4.6×250mm

Detection wavelength: 196 nm

Column temperature: 40 ℃

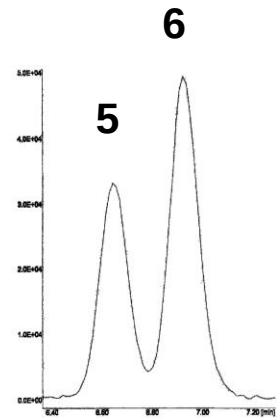
Mobile phase:

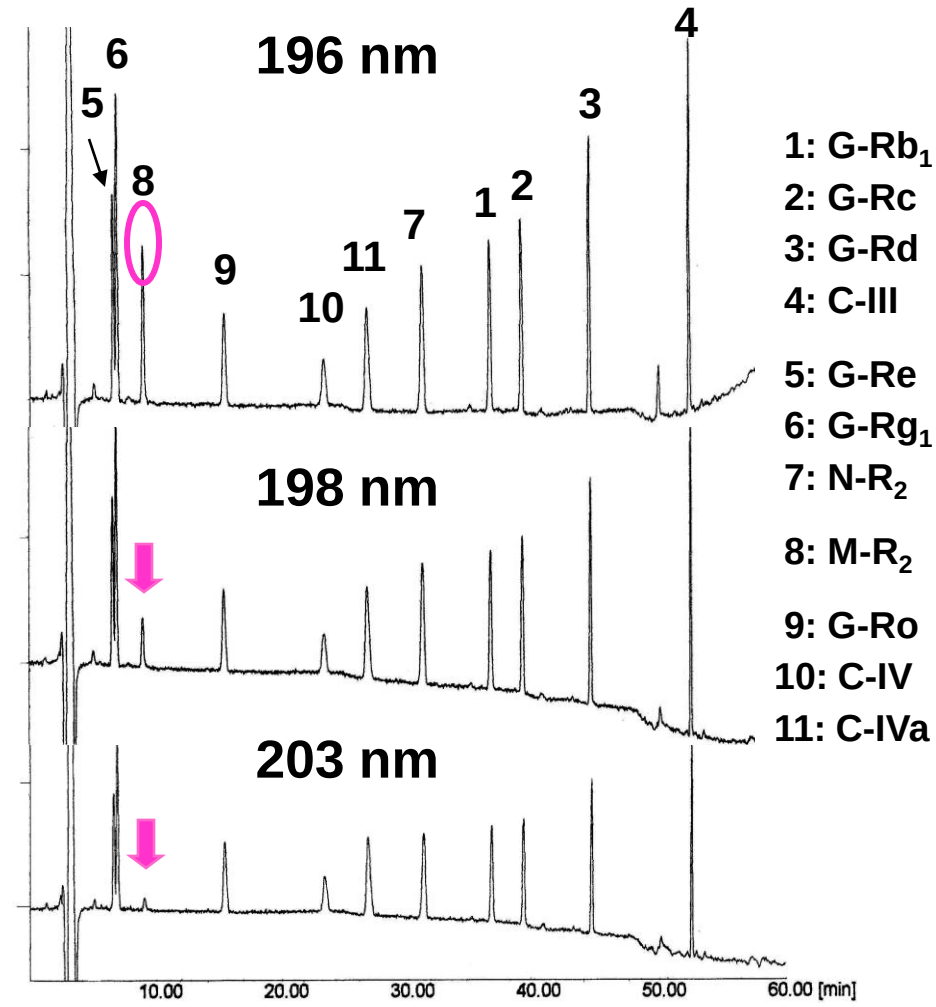
A: 10 mM K-phosphate buffer (pH 5.80);

B: CH_3CN C: H_2O

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

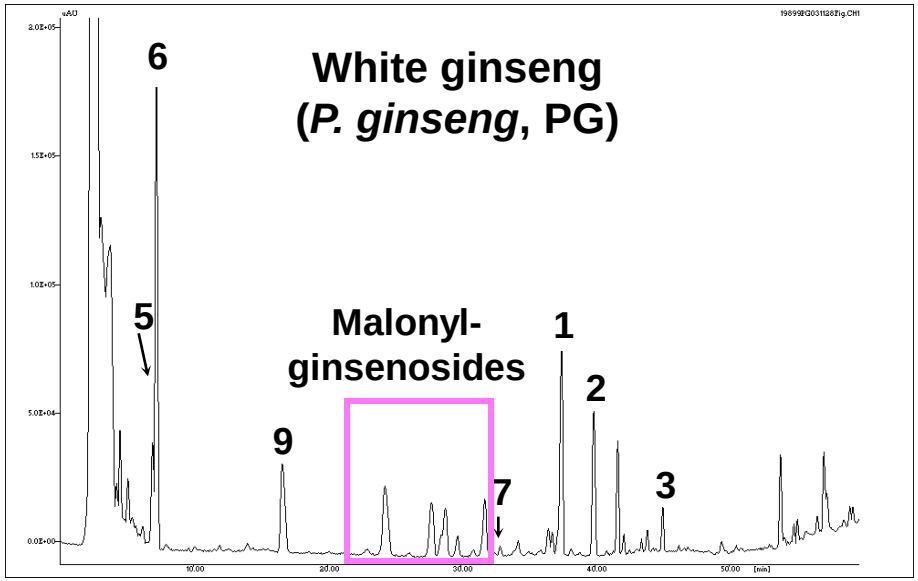
G: ginsenoside; C: chikusetsusaponin; N: notoginsenoside; M: majonoside

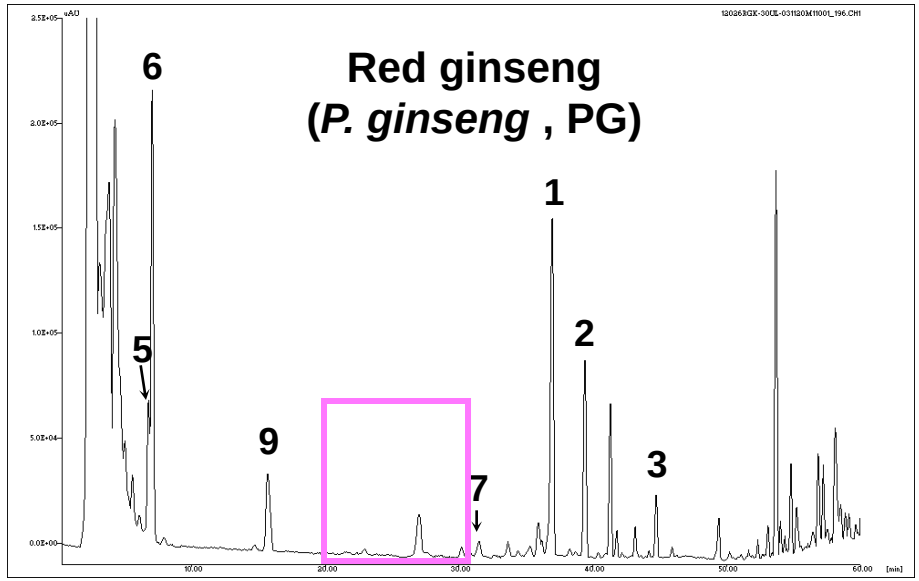
Materials

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

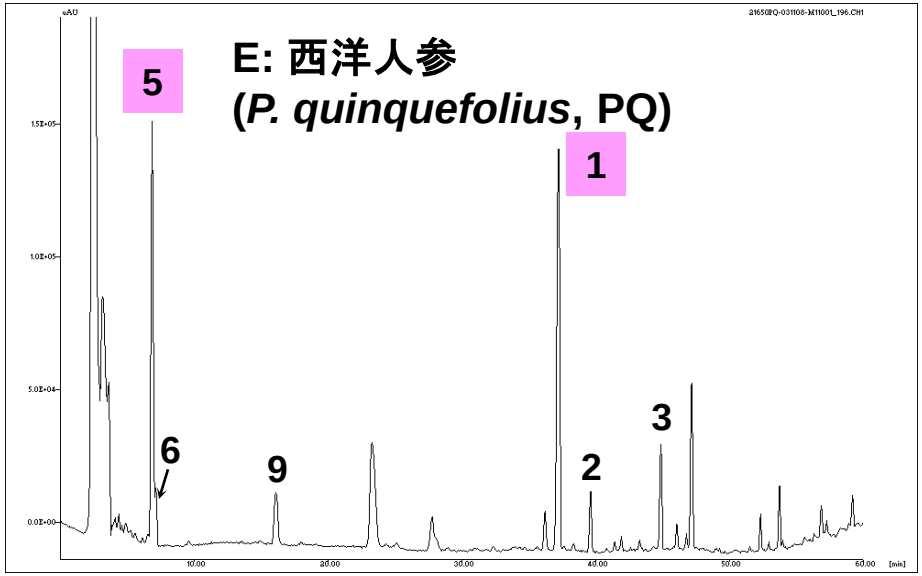
Total 47 samples

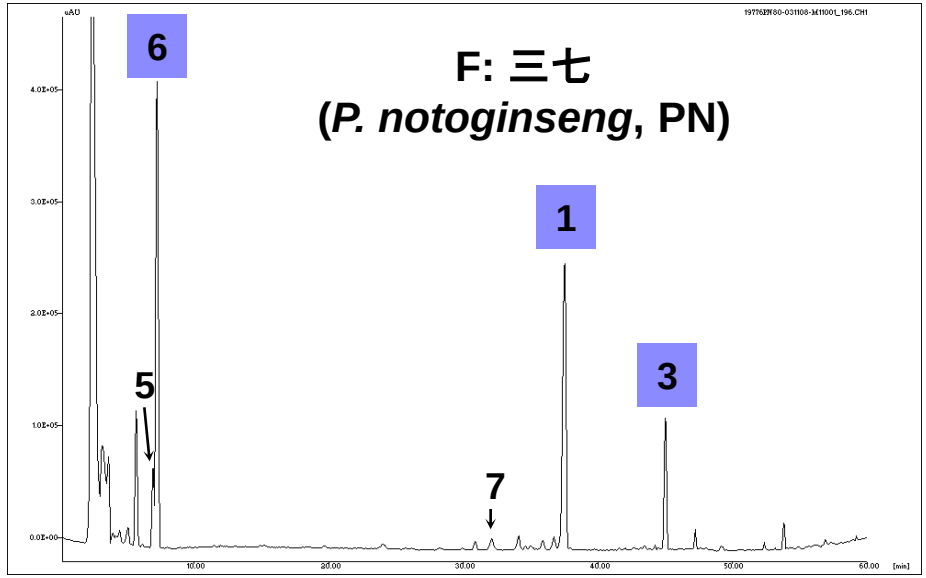
HPLC chromatograms of Ginseng Drugs





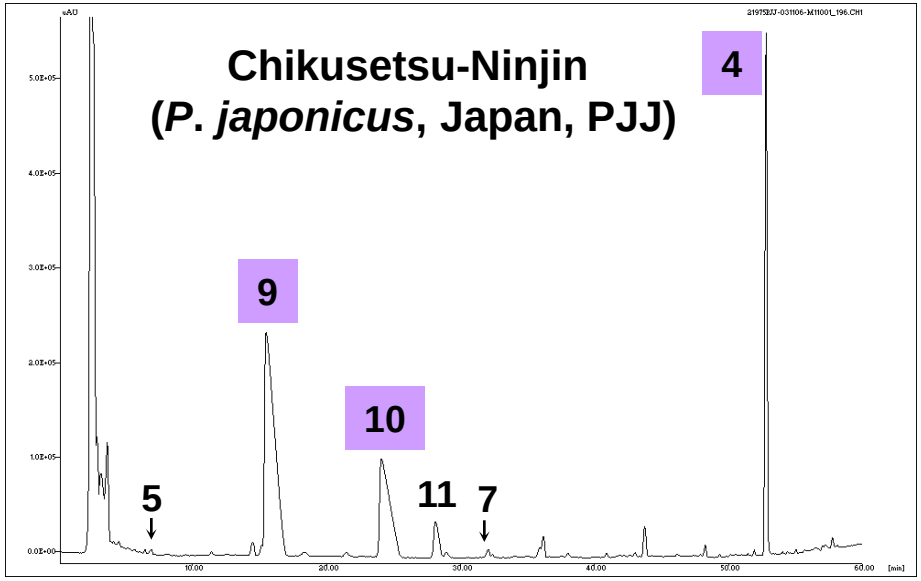
1: G-Rb_1
2: G-Rc
3: G-Rd
4: C-III
5: G-Re
6: G-Rg_1
7: N-R_2
8: M-R_2
9: G-Ro
10: C-IV
11: C-IVa

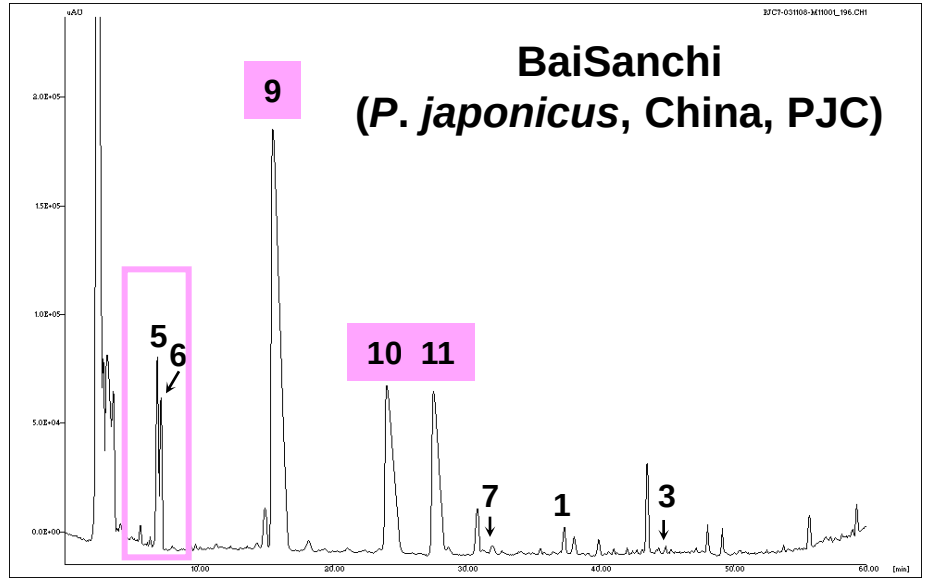


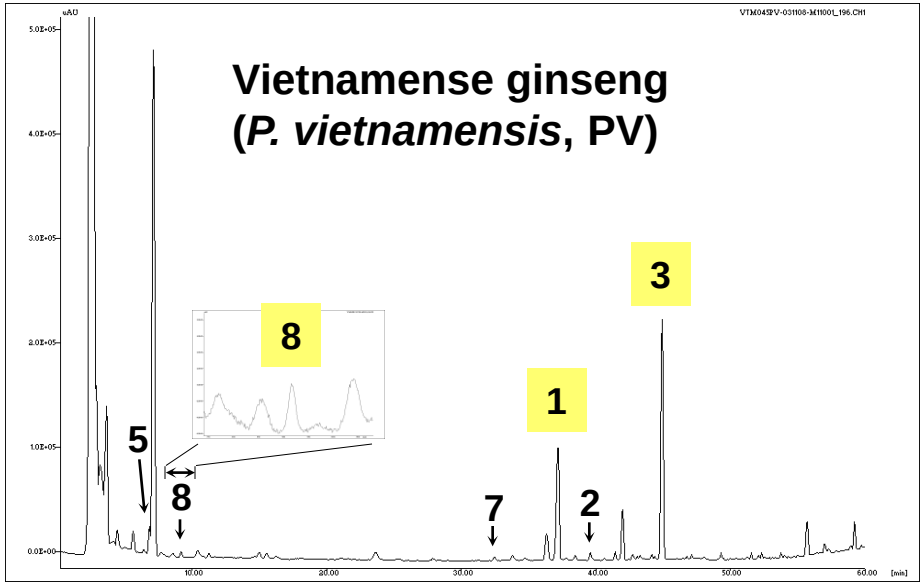


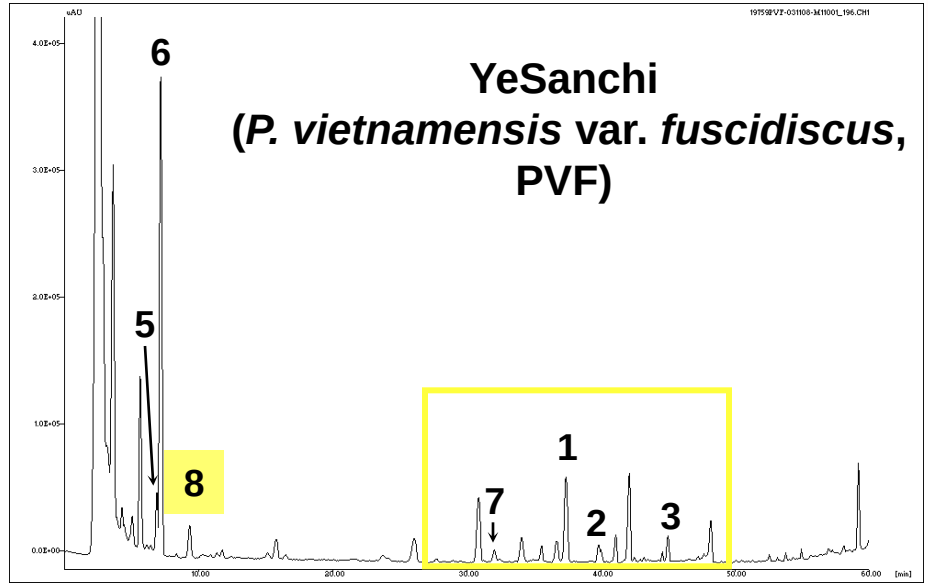
G: ginsenoside; C: chikusetsusaponin; N: notoginsenoside; M: majonoside

HPLC chromatograms of Ginseng Drugs









1: G-Rb_1
2: G-Rc
3: G-Rd
4: C-III

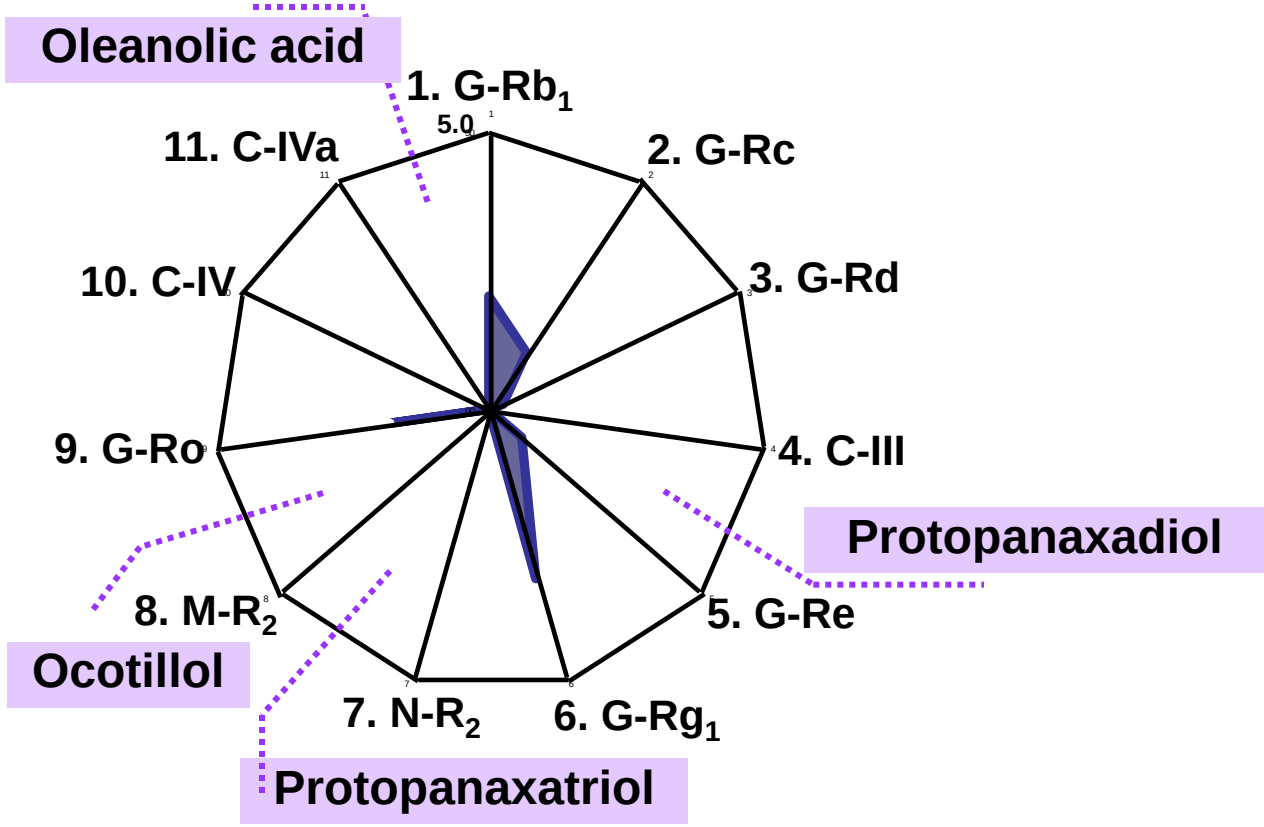
5: G-Re
6: G-Rg_1
7: N-R_2

8: M-R_2

9: G-Ro
10: C-IV
11: C-IVa

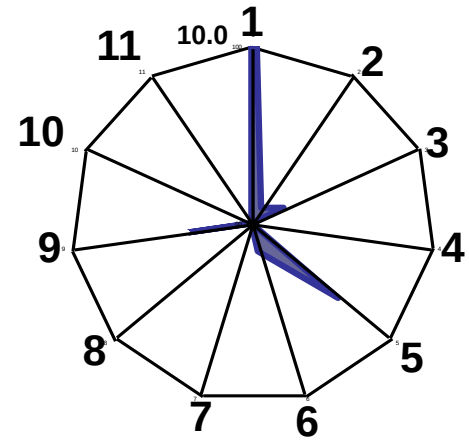
G: ginsenoside; C: chikusetsusaponin; N: notoginsenoside; M: majonoside

Quality Evaluation of Ginseng Drugs based on chemical constituents

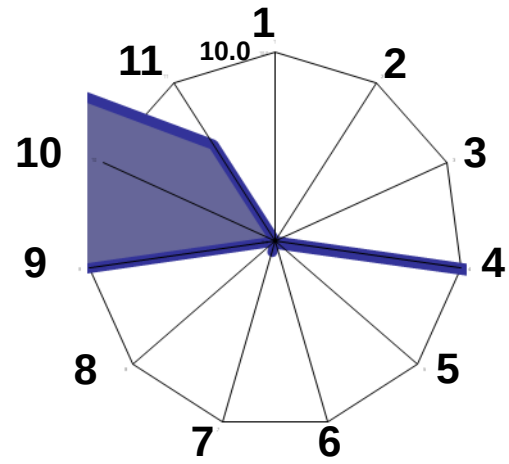


White ginseng (*P. ginseng*, PG)

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



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

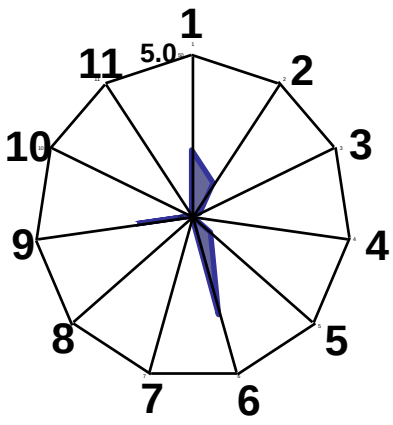


D: Chikusetsu-Ninjin
(*P. japonicus*, Japan, PJJ)

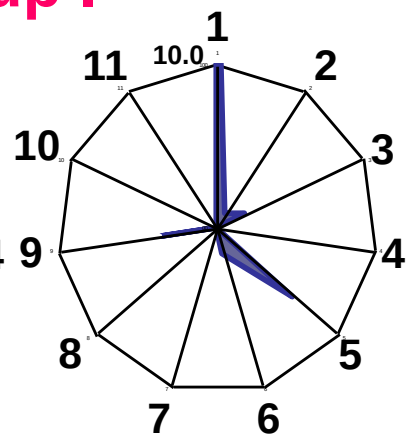
- **Zhu S.**, Zou K., Fushimi H., Cai S. Q. and Komatsu K. Planta Medica. **70**: 666-677, 2004.
- **Zhu S.**, Zou K., Cai S. Q., Meselhy R.M. and Komatsu K. Chemi. Phar. Bull., **52**: 995-998, 2004.

Quality Evaluation of Ginseng Drugs based on chemical constituents

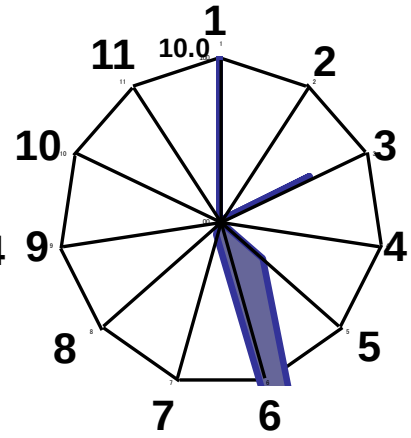
Group I



Ginseng
(*P. ginseng*)

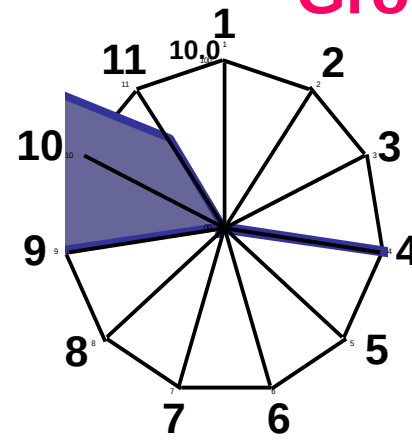


American ginseng
(*P. quinquefolius*)

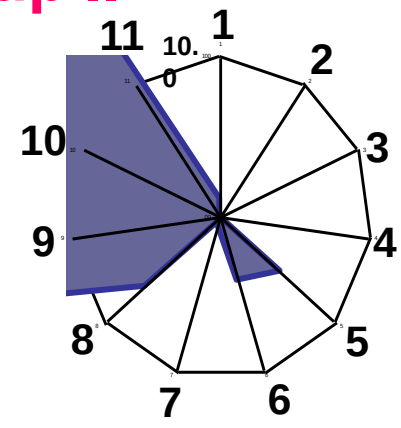


Notoginseng
(*P. notoginseng*)

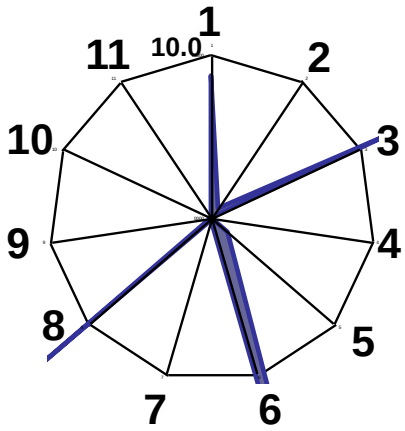
Group II



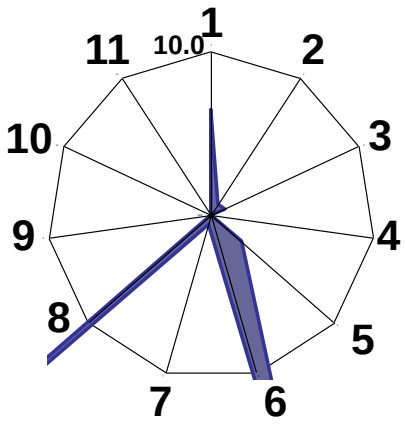
Chikusetuninjin
(*P. japonicus*, Japan)



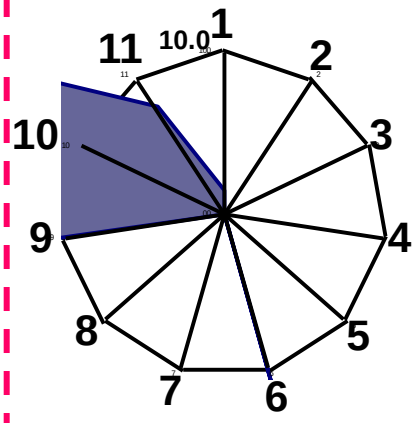
BaiSanchi
(*P. japonicus*, China)



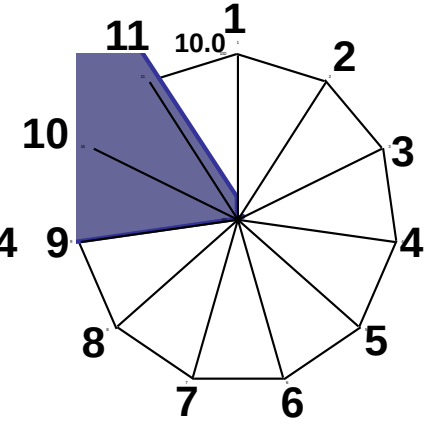
Vietnamense-ginseng
(*P. vietnamensis*)



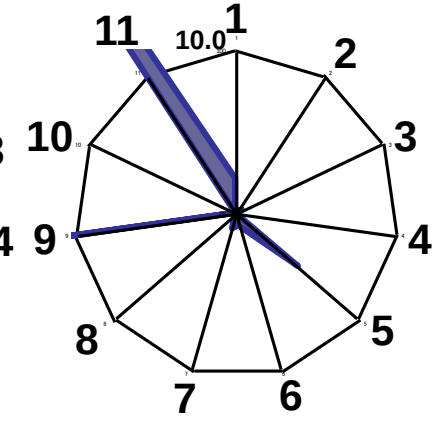
YeSanchi
(*P. vietnamensis*
var. *fuscidiscus*)



JiangSanchi
(*P. zingiberensis*)



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



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

1: G-Rb_1 2: G-Rc 3: G-Rd 4: C-III 5: G-Re 6: G-Rg_1 7: N-R_2 8: M-R_2 9: G-Ro 10: C-IV 11: C-IVa
G: ginsenoside; C: chikusetsusaponin; N: notoginsenoside; M: majonoside

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