Systematic pharmacognostical study on *Panax* drugs and *Curcuma* drugs - Phylogenetic analysis, molecular authentication and quality evaluation -

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We proposed pharmacognostical studies in the prime of molecular biology, citing the systematic studies of *Panax* drugs and *Curcuma* drugs. Each study was composed of three approaches, phylogenetic analysis of plants based on nuclear 18S rRNA and chloroplast *trn*K gene sequences, molecular authentication of herbal drugs, and quality evaluation on bioactive chemical constituents or pharmacological effect. Parsimony analysis of the combined *trn*K-18S rRNA gene sequence data yielded a well-resolved phylogeny within genus *Panax*. Based on species-specific sequences of the 2 genes, all the *Panax* drugs could be identified, furthermore, multiplex amplification refractory mutation system assay was developed for the authentication of 5 important drugs. Quantitative analysis on 11 saponins revealed that each taxon possessed its own characteristic pattern. The *trn*K/18S rRNA gene sequences could be used not only for an ultimate authentication but also for a speculation of the chemical constituent pattern that affects pharmacological effects. By the same molecular analysis as genus *Panax*, the potential method for identification of Chinese and Japanese *Curcuma* species was developed, making it possible to identify *Curcuma* drugs unambiguously. Using 5 drugs, we examined the effects on vasomotion in rat aortic rings as one index against "Oketsu." All methanol extracts exhibited intense NO-independent relaxation effects. All water extracts showed relaxation effects as the sum of the methanol-soluble compounds-induced relaxation and polysaccharides-induced contraction. Only the water extract of *C. zedoaria* showed NO-dependent relaxation besides NO-independent relaxation which is common to the other drugs, suggesting the drug derived from *C. zedoaria* has the potential to cure Oketsu with its various acting points. Such a series of studies will become necessary for standardization of herbal drugs and for their efficient uses.

Key words *Panax*, ginsenosides, *Curcuma*, effects on vasomotion, molecular authentication, quality evaluation.

I. Introduction

In Japan, as the society is aging, autoimmune diseases such as chronic rheumatoid arthritis, malignant tumors, degenerative cardiovascular or renal diseases, senile dementia and liver cirrhosis are becoming intractable diseases. For the treatment of such diseases appearing recently, the needs for Kampo or Chinese medical formulations are increasing gradually. The efficacy of a formulation changes depending on the quality of the herbal drugs included. Since the herbal drugs are derived from natural resources, the qualities estimated by botanical origins, chemical constituents, pharmacological effects, etc. are generally various, which causes instability in therapeutic effects. Therefore, the authentication and quality evaluation of herbal drugs play important roles in the guarantee of clinical efficacy and safety. The authentication has traditionally been carried out by comparative anatomical study and chemotaxonomic study, however, the characteristics of herbal drugs in morphology, anatomy and chemistry are often influenced by the growing stage of the original plant, its growing environment and processing method. As generally known, genotype rather than phenotype is influenced neither by the physiologic stage of the plant nor by environmental conditions. In the last three decades molecular biology has developed rapidly and influenced every scientific field including botany, also established a new category - molecular phylogeny of plants. Accordingly, we try to apply the molecular biological method to authentication of herbal drugs as well as phylogeny of medicinal plants. Several methods have been performed for the plant phylogeny using fresh leaves as materials, such as restriction fragment length polymorphism analysis (RFLP),[1)] randomly amplified polymorphic DNA analysis (RAPD),[2)] and direct determination of nucleotide sequences in certain gene regions. In the field of pharmacognosy, however, many difficulties are faced to apply RFLP and RAPD analyses because herbal drugs are dried and stored for a long period in which the DNA is expected to be cleaved, and the contamination of microorganisms leads to the fears of whether amplified products via the polymerase chain reaction (PCR) come from plant DNA or microorganism DNA. On the other hand, many studies showed that the small-subunit sequences of ribosomal RNA

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as 18S rRNA contain variable and conserved regions, including several evolutionarily conserved "functional domains,[3,4]" which suggested the 18S rRNA gene sequence could give more essential information for phylogenetic relationship. Recently, chloroplast *trn*K gene sequence as well as its containing *mat*K gene sequence has been widely employed as a powerful tool in examining inter- and intragenus phylogeny due to its high substitution rate.[5,6] The exon of chloroplast *trn*K gene, encoded tRNALys^{UUU}, consists of only 70 base nucleotides which is divided into two parts by a long intron of about 2500 bp in length.[7] An open reading frame, *mat*K gene is located in the intron region (Fig. 1). Accordingly, we decided to determine 18S rRNA gene and *trn*K gene sequences for phylogenetic analysis of medicinal plants and authentication of herbal drugs.

In spite of many descriptions on obvious therapeutic effects and biological activities of *Panax*-derived drugs (Ginseng drugs) and *Curcuma*-derived drugs (Turmeric related drugs), their botanical sources, *Panax* spp. and *Curcuma* spp. have still now some taxonomic problems, due to the morphological similarities of plants and the frequent occurrence of intermediate forms. Moreover, the scientific names are divergent according to different taxonomists. The taxonomical controversy of original plants results in great difficulty in identification of the derived drugs. In addition, the names of drugs may sometimes be unhelpful for identification, for example, there are drugs with the same name of different botanical sources and also different names of the same botanical origin.

In this review, we proposed the pharmacognostical study in the prime of molecular biology, citing the systematic studies on *Panax* drugs and *Curcuma* drugs. Each study was composed of three approaches: the first, phylogenetic analysis based on 18S rRNA gene and *trn*K gene sequences were performed to clarify the phylogenetic relationship among each taxon and to assist the taxonomic assignment; the second, on the basis of species-specific gene sequences, a convenient and efficient method for authentication of herbal drugs was developed; and the third, quality evaluation on bioactive chemical constituents in *Panax* drugs, and that on pharmacological effect in *Curcuma* drugs were described.

Ⅱ. Genus *Panax* and related drugs

Genus *Panax* (Araliaceae) consists of more than 10 species, which are distributed mainly over the Sino-Japanese floristic region. Two species grow in the eastern part of North America. The roots and/or rhizomes of almost all taxa in this genus are available as medicinal resource in traditional Chinese medicine as well as in folk medicine, such as Ginseng, American Ginseng, Notoginseng, Chikusetsu-ninjin (Japanese Ginseng) and Vietnamese Ginseng, *etc.* Three of them are highly esteemed medicines, therefore have been widely cultivated for more than 200 years, that is, Ginseng which has been used as a tonic and adaptogenic agent for more than 2000 years,[8,9] American Ginseng which has been applied as similar usage to Ginseng, but is considered to have a cold property,[8,10] and Notoginseng which is famous for its hemorrhage, disperse extravasated blood effect[11] and potential to cure hepatitis and cardiac diseases.[12] In recent years, a new species, *P. vietnamensis*[13] and its main constituent, majonoside R_2, have attracted much attention due to the high pharmacological potentials.[14-16]

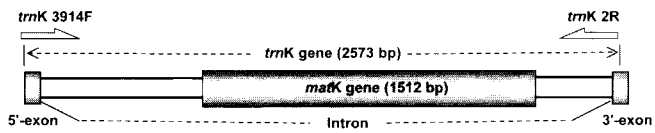


Fig. 1 Structure of *trn*K gene
A pair of primers, *trn*K-3914F and *trn*K-2R flanking *trn*K gene region have been used for PCR amplification. The lengths of *trn*K gene and *mat*K gene are shown in the case of *Panax ginseng*.

1. Phylogenetic relationship in genus *Panax*[17]

We determined the entire *trn*K gene and 18S rRNA gene of 53 samples belonging to 13 *Panax* taxa,[18] i.e. *P. ginseng* C. A. Meyer (PG), *P. japonicus* C. A. Meyer (PJJ from Japan, PJC from China), *P. japonicus* C. A. Meyer var. *major* C. Y. Wu et Feng (PJMH from Hubei, PJMY from Yunnan), *P. japonicus* C. A. Meyer var. *angustifolius* Cheng et Chu (PJA), *P. japonicus* C. A. Meyer var. *bipinnatifidus* C. Y. Wu et Feng (PJB), *P. quinquefolius* L. (PQ), *P. notoginseng* F. H. Chen (PN), *P. zingiberensis* C. Y. Wu et Feng (PZ), *P. stipuleanatus* H. T. Tsai et K. M. Feng (PS), *P. vietnamensis* Ha et Grushv. (PV), *P. vietnamensis* Ha et Grushv. var. *fuscidiscus* K. Komatsu, S. Zhu et S. Q. Cai (PVF),[19] *P. pseudoginseng* Wall. (PP), and *P. pseudoginseng* Wall. subsp. *himalaicus* Hara (PPH1-4),[20] which were widely collected from Northeast China, Japan to Southwest China, Himalayan region and southward extending to Central Vietnam. Total DNA was extracted from dried leaves of the plant specimens using the DNeasy™ plant Mini Kit, and from the herbal drug samples by CTAB method.[21] The 18S rRNA gene and *trn*K gene regions were amplified via PCR for all samples and the products were purified for the subsequent determination. Through sequencing reaction using a set of fluorescent-labeled sequencing primers,[22] each sequence was determined directly by a 4000L DNA sequencer and analyzed using the BaseImagIR program.

The 18S rRNA gene sequences were found to be of 1808 or 1809 bp in length, and only 10 types of sequences were observed among 13 taxa. Three sites around position 500 (positions 497, 499 and 501) revealed high variability. The length of *trn*K gene sequence varied from 2537 bp to 2573 bp according to the taxa, whereas the *mat*K gene sequence, embedded in the intron of *trn*K gene, were of 1512 bp in all taxa (including terminal codon). The nucleotide substitutions were found to be scattered in the whole *trn*K intron region, whereas the insertion and deletion (indels) were observed outside the *mat*K gene region. Through sequencing multiple samples of each taxon from different locations, the intraspecies-stability of *trn*K gene sequence has been demonstrated for most of *Panax* taxa, except for PJM and

PPH. PP and PS showed remarkable nucleotide differences, amounting to 30-43 sites of substitutions from other taxa. Species-specific *trn*K/*mat*K sequence provided much insight into phylogeny and taxonomy of this genus (Table 1). Parsimony and neighbor-joining analyses of the combined data sets of *trn*K-18S rRNA gene sequences yielded a well-resolved phylogeny within the genus *Panax* (Fig. 2), where three main clades were indicated. PP and PS formed a sister group, located at a basal position in the phylogenetic tree, which suggested the relative primitiveness of these two species. Monophyly of PG, PJJ and PQ, which are distributed in the northern parts of Asia or America, was well supported (Northern Clade). The remaining taxa distributed in southern parts of Asia formed a relatively large clade (Southern Clade). PN was located at a basal position within the Southern Clade, suggesting its distinction and primitiveness comparing to the other southern taxa. PV and a new taxon discovered during our botanical expedition in Yunnan prov., China, named as *P. vietnamensis* var. *fuscidiscus* (PVF) based on morphological and molecular evidences[19] were sister to each other, and together with PZ formed one cluster with high bootstrap value. The taxonomical debated taxa formerly treated as subspecies or varieties of *P. pseudoginseng* or *P. japonicus*,[18,20] distributed from Central to Southwest China, and extend to the Himalayan region, fell into one cluster with low sequence divergence (enclosed in the broken-line frame in Fig. 2), but being far from their original species, PP and PJJ. The result obtained so far

Table 1 Nucleotide differences of *Panax* taxa in *trn*K gene region

	5' intron region (39th-761st)																					3' intron region (2274th-2563rd)							
	112	176	210	215	220–229	237–241	310	320	327	459	542	578	604	621	631	656	713	724	731	752	753	2286	2361–2375	2391–2408	2413	2435	2462	2481	Length (bp)
Panax ginseng (PG)	C	G	T	T	TTTTGAAACG	AAAT	G	A	C	C	T	C	A	A	C	G	A	C	C	A	T	C	ATCCTTTTTTTATAT	TAGGATGTAGGATGTAGT	G	C	G	G	2573
P. japonicus (Japan) (PJJ)	*	*	*	*	**********	*****	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	***********T***	********** *	*	*	*	*	2566
P. quinquefolius (PQ)	*	*	*	*	**********	*****	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	***************	******************	*	*	*	*	2573
P. notoginseng (PN)	*	*	*	*	**********	*****	*	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*********** ****	********** *	*	*	*	*	2564
P. vietnamensis (PV)	*	*	*	*	**********	*****	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	***********T***	********** *	*	*	*	*	2566
PVF*	*	*	*	*	**********	*****	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	***************	********** *	*	*	*	*	2566
P. zingiberensis (PZ)	T	*	*	*	**********	*****	*	*	*	*	*	A	*	*	*	*	*	*	*	T	*	*	***************	********** *	*	T	*	*	2566
P. japonicus (China) (PJC)	*	*	*	*	**********	*****	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	***************	******A***********	*	*	*	*	2573
PJA*	*	*	*	*	**********	*****	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	***************	******A***********	*	*	*	*	2573
PJMH* (Hubei)	*	*	*	*	**********	*****	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	***************	******A***********	*	*	*	*	2573
PJMY* (Yunnan)	*	*	*	*	**********	*****	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	***************	********** *	*	*	*	*	2566
PJB*	*	*	C	*	**********	*****	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	***************	********** *	*	*	*	*	2566
PPH1* (India)	*	*	*	*	**********	*****	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	***************	********** *	*	*	*	*	2566
PPH2* (Chame, Nepal)	*	T	*	*	**********	*****	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	***************	********** *	*	*	*	*	2566
PPH3* (Langtang, Nepal)	*	T	*	*	**********	*****	*	*	*	A	*	*	*	G	*	*	*	*	*	*	*	*	***************	******************	*	*	*	*	2573
PPH4* (Gokyo, Nepal)	*	*	*	*	**********	*****	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	***************	******************	*	*	*	*	2573
P. pseudoginseng (PP)	*	*	*	*	* *	* *	T	*	*	*	G	*	T	*	T	A	*	*	T	*	G	*	** **	*** *	A	*	A	A	2537
P. stipuleanatus (PS)	*	*	*	A	* *	* *	*	G	*	A	G	*	T	*	T	*	*	*	T	*	*	*	** **	*** *	*	*	A	*	2537

	*mat*K gene region (762nd-2273rd)																																														
	790	865	887	917	959	977	1008/9	1097	1117/8	1127	1157	1186	1201	1259	1280	1330	1332	1337	1363	1367	1374	1379	1380	1462	1536	1551	1604	1613	1629	1634	1731	1743	1804	1815	1845	1873	1889	1925	1938	1966	1975	1989	2024	2102	2177	2194	2252
Panax ginseng (PG)	T	A	C	T	G	G	AG	A	AA	C	A	C	G	C	T	C	C	T	G	T	C	C	T	C	G	C	A	T	T	G	C	A	A	C	G	G	G	G	G	A	G	C	G	T	C	C	T
P. japonicus (Japan) (PJJ)	*	*	*	*	*	*	**	*	**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
P. quinquefolius (PQ)	*	*	*	A	*	*	**	*	**	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
P. notoginseng (PN)	*	*	*	*	*	T	**	*	**	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	C	*	*	*	A	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*
P. vietnamensis (PV)	*	G	*	*	*	*	**	*	GG	*	G	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	G	*	*	*	*	T	*	C
PVF*	*	*	*	*	*	*	**	*	GG	*	G	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	T	*	C
P. zingiberensis (PZ)	*	*	*	*	*	*	**	*	GG	*	G	*	*	*	*	*	*	*	*	C	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	T	*	C
P. japonicus (China) (PJC)	*	*	*	*	*	*	**	*	*G	A	G	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	G	A	A	*	*	T	*	*
PJA*	*	*	*	*	*	*	**	*	*G	A	G	T	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	A	*	*	*	T	*	*
PJMH* (Hubei)	*	*	*	*	*	*	**	*	*G	A	G	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	A	*	*	*	T	*	*
PJMY* (Yunnan)	*	*	*	*	*	*	**	*	*G	*	G	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	T	*	*
PJB*	*	*	*	*	*	*	**	*	*G	*	G	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	G	*	*	T	*	T	*	*
PPH1* (India)	*	*	*	*	*	*	**	*	*G	*	G	*	*	G	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	T	*	*
PPH2* (Chame, Nepal)	*	*	*	*	A	*	**	*	*G	*	G	*	*	*	*	*	*	*	*	C	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	T	*	*
PPH3* (Langtang, Nepal)	*	*	*	*	A	*	**	*	*G	*	G	*	*	*	*	*	*	*	*	C	*	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	T	*	*
PPH4* (Gokyo, Nepal)	*	*	*	*	*	*	**	*	*G	A	G	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	G	*	*	*	*	T	*	*
P. pseudoginseng (PP)	C	*	T	*	*	*	CT	T	**	A	*	*	T	*	G	G	A	A	*	*	A	*	*	*	T	T	*	A	*	*	*	C	*	A	A	*	T	*	*	G	*	*	T	C	*	A	*
P. stipuleanatus (PS)	C	*	T	*	*	*	CT	T	**	A	*	*	T	*	*	*	A	*	T	*	*	*	*	T	T	*	*	A	C	T	*	C	*	A	A	*	*	T	T	G	*	A	*	C	*	*	*

* PVF: *P. vietnamensis* var. *fuscidiscus*; PJA: *P. japonicus* var. *angustifolius*; PJMH: *P. japonicus* var. *major* from Hubei prov. of China; PJMY: *P. japonicus* var. *major* from Yunnan prov. of China; PJB: *P. japonicus* var. *bipinnatifidus*; PPH: P. *pseudoginseng* subsp. *himalaicus*
The numbers above sequence are aligned nucleotide positions. Asterisks indicate the identical nucleotides with those of *P. ginseng* in the first line, and hypens represent aligned gaps.

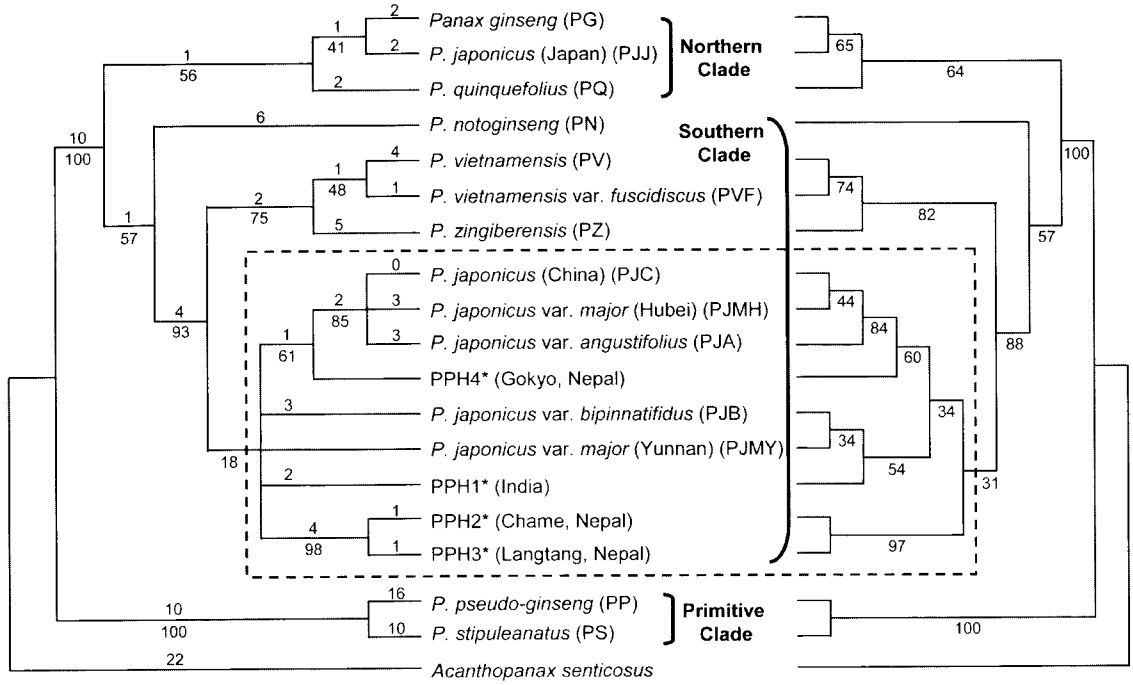


Fig. 2 Phylogenetic tree based on combined data set of *trn*K gene and 18S rRNA gene sequences
Left: The semistrict consensus tree reconstructed on the basis of maximum parsimonious analysis. Tree length=120, CI=0.9083, RI=0.8642, RC=0.7850. Number above line is branch length, and number below line is the bootstrap value with 1000 replicates. Right: The topology obtained by using Neighbor-joining method. Bootstrap values were obtained from 1000 replications. The taxa enclosed in broken-line frame are traditionally taxonomic debate taxa. *PPH: *P. pseudoginseng* subsp. *himalaicus*.

suggested that it might be inappropriate to treat these taxa as subspecies or varieties of PP or PJJ and that they might be differentiated from a common ancestor and are in a period of high variation. Moreover, although PJJ and PJC have been treated as the same species, *P. japonicus* for a long time, we strongly recommended to re-denominate the Chinese taxa beyond the consideration that PJJ is endemic to Japan, because PJJ and PJC revealed distinct characteristics on genetic evidence, pollen morphology,[23] anatomical features,[24] chemical constituents[25] and chromosome numbers.[26]

In conclusion, the *trn*K/*mat*K gene sequence showed high potential in resolving phylogeny and assisting taxonomic delimitation in this genus.

2. Identification of *Panax* drugs[27]

The species-specific sequences of 18S rRNA gene and *trn*K gene gave valuable information for identification of *Panax* drugs. When the nucleotide sequences of drugs in the two gene regions are determined and compared with those of plants, their botanical origins could be completely clarified. Practically, the accurate identification of drugs is the basis and prerequisite of clinical application. In order to develop an objective and convenient method for identification, the multiplex amplification refractory mutation system (MARMS)[28] assay was investigated.

The amplification refractory mutation system (ARMS) assay is based on the fact that primer with a mismatched 3'-terminal is refractory to extension under appropriate condition and hence no PCR product is obtained,[28,29] whereas, the multiplex ARMS assay shows merit of allowing detection of many sites of nucleotide difference at one time. We applied the MARMS assay to authenticate plants and herbal drugs derived from 5 important *Panax* species (PG, PJJ, PQ, PN and PV). According to the comparison among nucleotide sequences of the 5 species, two pairs of primers with specific 3'-terminal to detect the species-specific nucleotides were carefully designed for each species, one pair placed on *trn*K gene (Fig. 3) and another on 18S rRNA gene (Fig. 4). For instance, two pairs of primers, PntK951F - PntK1731R and PnS172F - PnS1718R were designed to detect the specific nucleotides for *P. notoginseng* at positions 977 and 1731 from upstream in *trn*K gene (Fig. 3) and positions 191 and 1718 in 18S rRNA gene (Fig. 4), respectively. This primer set was refractory to extension when using DNAs of other four species as a template. In the same manner, five kinds of primer sets specific for each species were designed and synthesized (Table 2). PCR amplifications with 2 pairs of primers (one set) per tube were performed using total DNA of each species as template, then the resulting products were detected by 2.5% agarose gel electrophoresis.

As shown in Fig. 5A, PCR amplification with primer set of *P. ginseng,* consisting of primer pairs PgjqtK1341F - PgjqtK1966R and PgS481F - P-S712R, generated 2 fragments of 649 bp and 249 bp in length only when DNA of *P. ginseng* or Ginseng were used (Lane 1, 2). Whereas, only a single fragment of 649 bp was detected when DNAs of

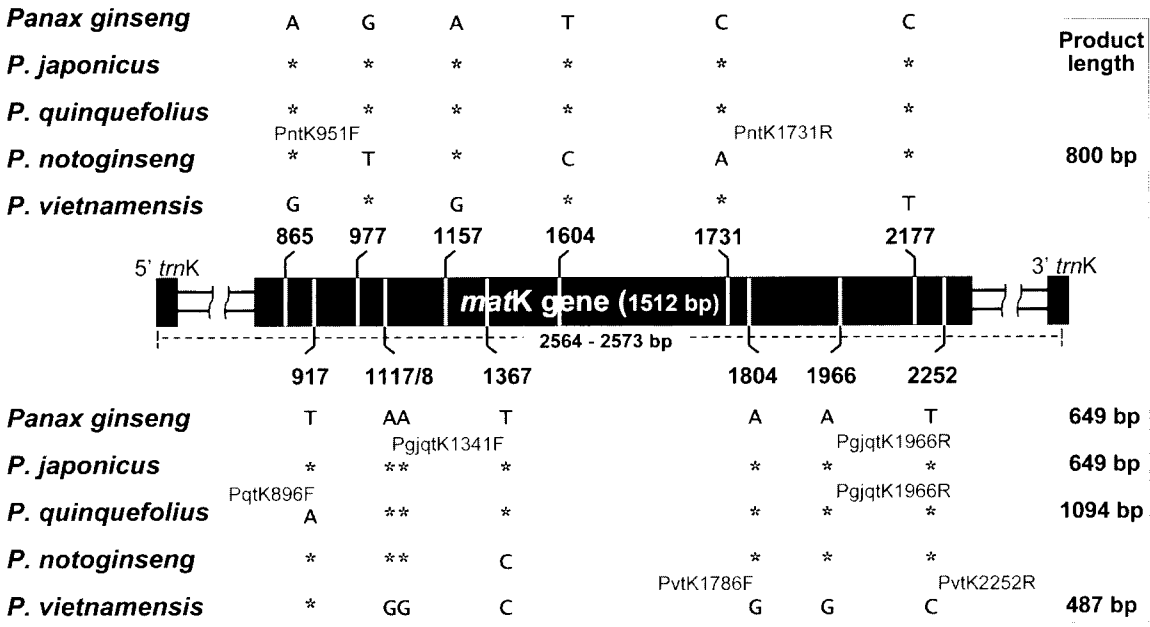


Fig. 3 Species-specific primer designed on the basis of sequence differences of *mat*K gene among five *Panax* species
The primer is denoted by an arrow, and its name is labeled beside it. An asterisk means the same sequence as that of *P. ginseng* in the 1st line. The number below or above a sequence indicates the aligned position of nucleotide differences.

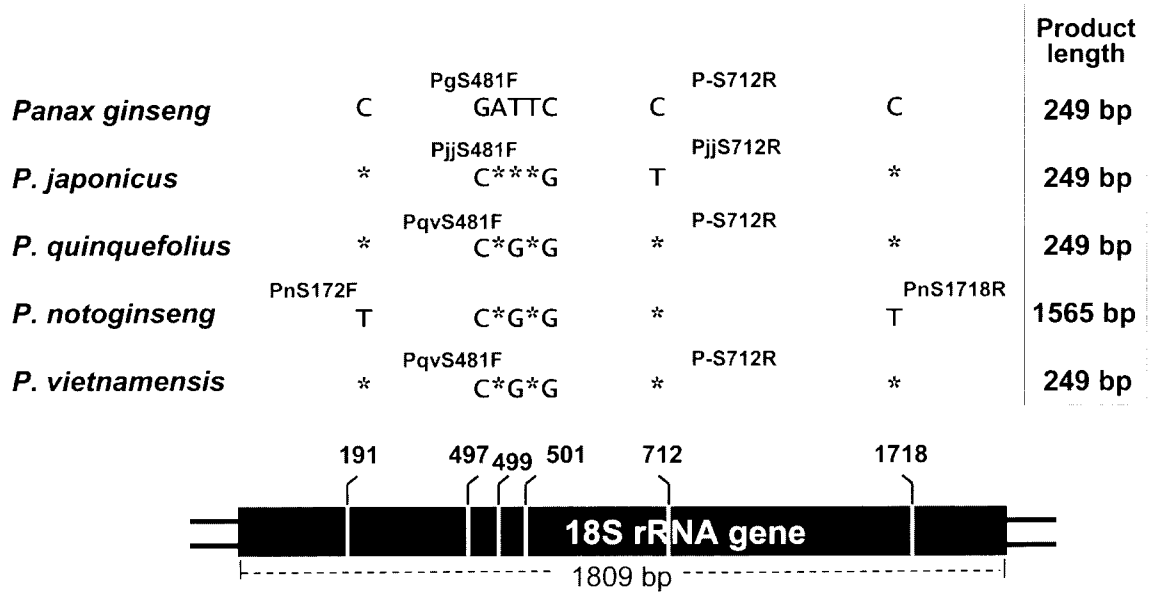


Fig. 4 Species-specific primer designed on the basis of sequence differences of 18S rRNA gene among five *Panax* species
The primer is denoted by an arrow, and its name is labeled beside it. An asterisk means the same sequence as that of *P. ginseng* in the 1st line. The number below a sequence indicates the aligned position of nucleotide differences.

P. japonicus, Chikusetsu-ninjin, *P. quinquefolius* and American Ginseng were used (Lane 3-6), since the former two possess completely identical *mat*K gene sequence with *P. ginseng*, and the latter two show 1 bp difference at nucleotide position 917. No fragment was detected when using DNAs of *P. notoginseng*, Notoginseng, and *P. vietnamensis* as template (Lane 7-9). The same results were observed in the cases of using the primer-set of *P. japonicus*, *P. quinquefolius*, *P. notoginseng* and *P. vietnamensis* (Fig. 5B, C, D, E), respectively. Thus, the two expected fragments, one from *trn*K gene and another from 18S rRNA gene were observed simultaneously only when the set of species-specific primers encountered template DNA of corresponding species. Such specific amplification profiles were observed under the established optimal PCR conditions, including annealing temperature and time, as well as Mg^{2+}

Table 2 Sequence of designed species-specific primer

	Primer	Sequence (5' to 3')
trnK gene region	PgjqtK1341F	GCC GGT TCT TCT TTT TCA AAA AGA AAT
	PgjqtK1966R	GAT TTC TGC ATA TAC GCC CAA ATT
	PqtK896F	GGA AAA TGC GGG TTA TGA CAA A
	PntK951F	GCT CGA ATG TAT CAA CAG AAT CAT TTT
	PntK1731R	CCC AGA ATG TCG AGG GAA TT
	Pvtk1786F	TAC GCG GTC AAA TGC TAG G
	PvtK2252R	CAT TCA TGA TTG GCC AAA TCG
18S rRNA gene region	PgS481F	ATA ACA ATA CCG GGC TGA TTC
	PjjS481F	ATA ACA ATA CCG GGC TCA TTG
	PqvS481F	ATA ACA ATA CCG GGC TCA G
	P-S712R	GCC AGT TAA GGA CAG GAG
	PjjS712R	GCC AGT TAA GGA CAG GAA
	PnS172F	GTG CAA CAA ACC CCG ACT TT
	PnS1718R	GAC TTC TTT CGA CGT CGC A

concentration. Four Ginseng drugs were identified by the same fragment patterns as their original plants, even for a sample purchased ten years ago (Fig. 5C; Lane 6). This assay could give more reliable results for identification of not only 5 *Panax* species but also corresponding Ginseng drugs by simultaneous detection of 4-site nucleotide differences on two completely different genes.

3. Comparative study on triterpene saponins of *Panax* drugs[30,31]

The main bioactive constituents of *Panax* drugs are considered to be triterpene saponins, generally referred to as ginsenosides. Until now, more than 80 ginsenosides have been isolated from these drugs and most of them possess four types of aglycone moieties.[32,33] Pharmacological studies showed that bioactivities of ginsenosides varied depending on different types of aglycones and various sugar moieties.[34,35] Although comparative studies of the protopanaxadiol- and protopanaxatriol-types in Ginseng, American Ginseng and Notoginseng have been reported,[36,37] a systematic comparison of the 4-type ginsenosides, including ocotillol- and oleanolic acid types, were not investigated so far. Furthermore, the herbal drugs were confined on a narrow range of *Panax* species and those derived from PJC and its varieties, PV, PVF, PZ, *etc.* were not compared despite the fact that they have been widely used in China, Vietnam and the Himalayan regions as hemostatic, expectorant and tonic, *etc.* Therefore, eleven ginsenosides (ginsenoside Rb_1 (**1**), Rc (**2**), Rd (**3**), chikusetsusaponin III (**4**) of protopanaxadiol-type, ginsenoside Re (**5**), Rg_1 (**6**), notoginsenoside R_2 (**7**) of protopanaxatriol-type, majonoside R_2 (**8**) of ocotillol-type and ginsenoside Ro (**9**), chikusetsusaponin IV (**10**), IVa (**11**) of oleanolic acid type, Fig. 6) were chosen for quality evaluation based on a consideration of extensive existence with relatively high concentration in different *Panax* drugs, also with biological potentials and phytotaxonomic significance. First a convenient HPLC method for simultaneous determination of eleven

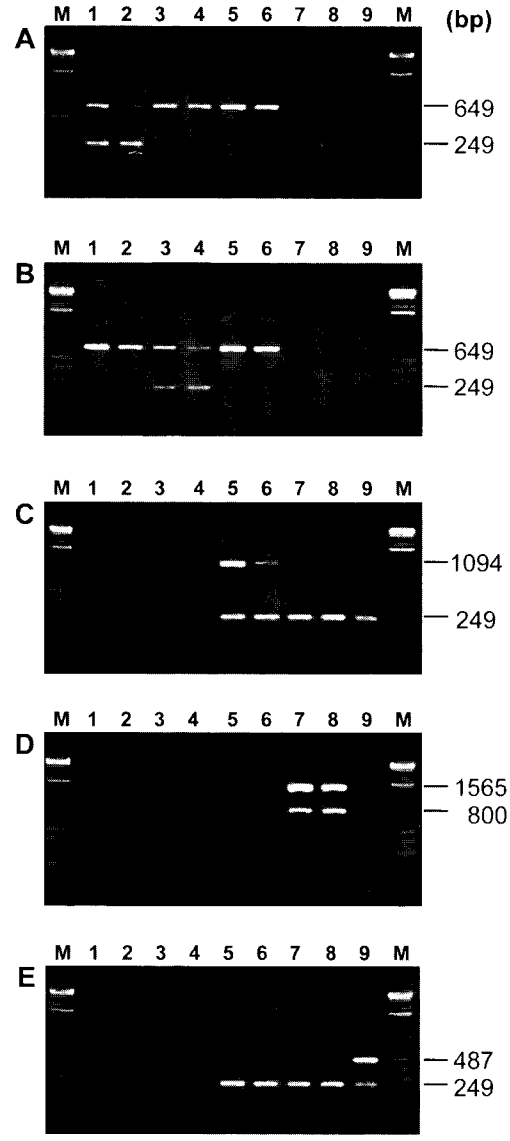
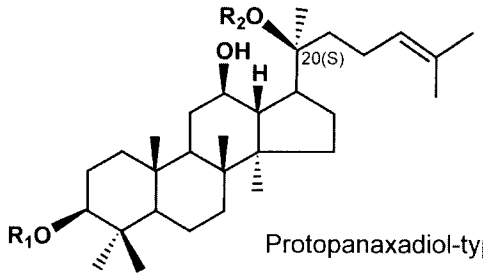


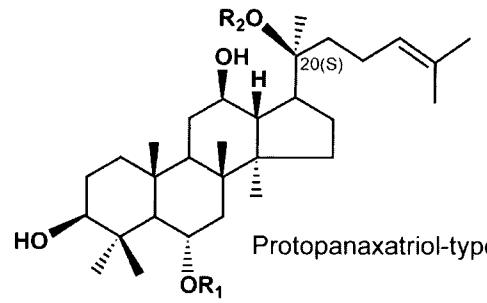
Fig. 5 MARMS assay using species-specific primer sets of *Panax ginseng* (A), *P. japonicus* (B), *P. quinquefolius* (C), *P. notoginseng* (D), and *P. vietnamensis* (E)
Two bands are detected simultaneously only when the specific primer set of target species encountered the total DNA of the corresponding species as template. The optimal PCR condition for each primer-set is shown as follows: hot start at 94°C for 5 min, 40 cycles of denaturation at 94 °C for 30 sec and combined annealing and extension at 66°C for 60 sec (**A**, **B**, **D**) or 66°C for 80 sec (**C**, **E**), final extension at 72°C for 5 min. M: 1 Kb plus ladder, Lane 1: *Panax ginseng*, Lane 2: Ginseng, Lane 3: *P. japonicus*, Lane 4: Chikusetsu-Ninjin, Lane 5: *P. quinquefolius*, Lane 6: American Ginseng, Lane 7: *P. notoginseng*, Lane 8: Notoginseng, Lane 9: *P. vietnamensis*.

ginsenosides was investigated and validated.[30] By using a gradient of acetonitrile and 10 mM K-phosphate buffer (pH 5.80) as mobile phase, and UV detection at 196 nm, more than 18 ginsenosides with different aglycones were separated satisfactorily within 60 minutes. Then, a comparative study was performed on the saponin constituents of 47 drug samples derived from 12 *Panax* taxa which were identified by morphology and nucleotide sequence analysis, in order to characterize the chemical constituent pattern of each



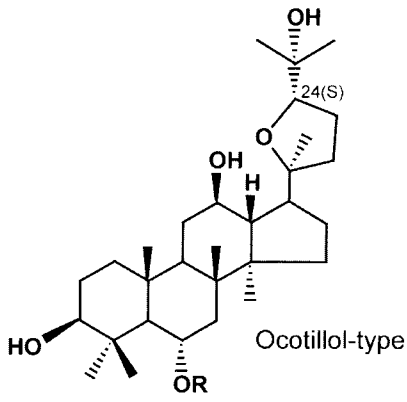
Protopanaxadiol-type

	R_1	R_2
1 Ginsenoside Rb_1	glc-2-glc	glc-6-glc
2 Ginsenoside Rc	glc-2-glc	glc-6-ara(f)
3 Ginsenoside Rd	glc-2-glc	glc
4 Chikusetsusaponin III	glc-2-glc | 6-xyl	H



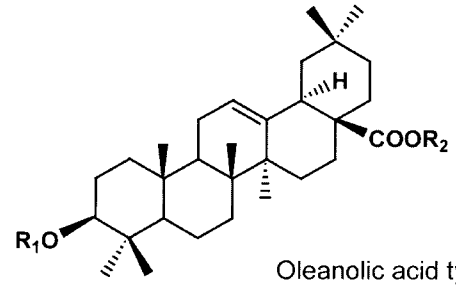
Protopanaxatriol-type

	R_1	R_2
5 Ginsenoside Re	glc-2-rha	glc
6 Ginsenoside Rg_1	glc	glc
7 Notoginsenoside R_2	glc-2-xyl	H



Ocotillol-type

	R
8 Majonoside R_2	glc-2-xyl



Oleanolic acid type

	R_1	R_2
9 Ginsenoside Ro	gluA-2-glc	glc
10 Chikusetsusaponin IV	gluA-4-ara(f)	glc
11 Chikusetsusaponin IVa	gluA	glc

Fig. 6 Chemical structures of 11 standard saponins used in quality evaluation

Panax drug and investigate the relationship among the genetic varieties and the chemical constituent patterns.[31]

The result showed that the ginsenoside compositions in *Panax* drugs of different botanical origins were of considerable variability (Table 3). Total saponin contents varied by 10-fold from the highest drug to the lowest one. Chikusetsuninjin derived from PJJ was found to have the highest content (192.80-296.18 mg/g) and Ginseng from PG to be the lowest (5.78-15.63 mg/g). The two main groups (I and II) proposed by Zhou and Yang *et al.*[33,38] on the basis of phytochemical data were also clearly observed; group I, mainly containing dammarane saponins, consisted of PG, PQ, PN, PV and PVF; and group II, containing a large amount of oleanolic acid saponins, was composed of PJJ, PZ, PJC, PJA, PJM, PJB and PS. The ratio of subtotal of dammarane saponins to that of oleanolic acid saponins (D/O) was found to be >1.9 and <0.25 for groups I and II, respectively. The drug samples derived from the same botanical origin revealed a similar constituent pattern, in other words, each *Panax* taxon showed its own characteristic chromatogram (Fig. 7). On the basis of the results of the quantitative analysis, an eleven-direction radar graph was constructed for a visually-apparent discrimination. Each taxon could be distinguished clearly from others by its distinctive shape (Fig. 8). The typical HPLC chromatograms of White Ginseng and Red Ginseng were shown in Figs. 7A and 7B. Peaks between 23-32 minutes in chromatogram of White Ginseng, assignable to malonyl-ginsenosides Rb_1, Rc, Rd, *etc.* disappeared and peak areas of **1**, **2** and **3** increased significantly in that of Red Ginseng, because thermo-unstable malonyl-ginsenosides were conversed into their parent saponins as **1**, **2** and **3**, *etc.*[36,37] However, similar

Table 3 Contents of 11 triterpene saponins in Ginseng drugs (mg/g)

Original plant / Crude drug		*Panax ginseng*										*P. quinquefolius*					*P. japonicus (Japan)*					*P. stipuleanatus*			
		White Ginseng							Red Ginseng			American Ginseng				Wild	Chikusetsu-ninjin				Satsuma-ninjin	Pingbian-Sanchi		Tam That Hoang	
Aglycone type		WPG1	WPG2	WPG3	WPG4	WPG5	WPG6	WPG7	RPG1	RPG2	RPG3	PQ1	PQ2	PQ3	PQ4	PQ5	PJJ1	PJJ2	PJJ3	PJJ4	PJJ5	PS1	PS2	PS3	PS4
Protopana-xadiol	1	2.03	1.13	1.88	1.39	1.91	1.35	1.38	2.08	3.79	5.22	12.44	21.29	18.66	12.55	37.14	trace	trace	trace	1.00	3.38	N.D.	N.D.	N.D.	N.D.
	2	1.20	0.48	0.81	0.66	1.31	0.42	1.00	0.90	1.40	2.42	1.48	1.05	1.62	1.78	3.02	N.D.	N.D.	N.D.	trace	1.01	N.D.	N.D.	N.D.	N.D.
	3	0.22	0.07	0.14	0.15	0.47	0.14	0.22	0.29	0.45	0.47	1.82	1.89	1.90	1.36	1.64	N.D.	N.D.	N.D.	N.D.	trace	N.D.	N.D.	N.D.	N.D.
	4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	30.41	31.00	44.73	27.59	2.38	N.D.	N.D.	N.D.	N.D.
Protopana-xatriol	5	0.74	0.85	1.04	1.10	1.29	0.70	1.32	0.56	0.87	1.13	9.59	6.27	10.59	6.57	2.86	0.24	trace	0.69	1.09	2.44	N.D.	N.D.	N.D.	N.D.
	6	3.15	2.47	2.71	2.02	1.99	2.90	1.21	1.45	2.50	4.46	0.68	1.58	0.43	0.32	21.52	trace	trace	trace	0.24	4.97	N.D.	N.D.	N.D.	N.D.
	7	trace	0.18	0.15	0.44	trace	0.23	trace	trace	trace	trace	trace	N.D.	trace	N.D.	trace	0.59	0.82	1.08	0.75	1.97	N.D.	N.D.	N.D.	N.D.
Ocotillol	8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Dammarane subtotal		**7.33**	**5.18**	**6.73**	**5.75**	**6.97**	**5.75**	**5.14**	**5.28**	**9.00**	**13.70**	**26.01**	**32.08**	**33.20**	**22.58**	**66.18**	**31.24**	**31.81**	**46.50**	**30.66**	**16.16**	--	--	--	--
Oleanolic acid	9	1.77	2.45	1.67	3.03	1.22	1.17	0.64	1.12	2.31	1.93	2.99	3.57	3.35	3.10	9.24	107.88	144.25	162.99	111.74	37.94	N.D.	N.D.	N.D.	N.D.
	10	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	51.80	79.82	82.38	44.85	38.19	trace	4.01	0.76	trace
	11	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	6.10	6.73	4.30	5.55	2.29	trace	trace	trace	trace
Oleanolic acid subtotal		**1.77**	**2.45**	**1.67**	**3.03**	**1.22**	**1.17**	**0.64**	**1.12**	**2.31**	**1.93**	**2.99**	**3.57**	**3.35**	**3.10**	**9.24**	**165.78**	**230.80**	**249.68**	**162.14**	**78.41**	--	**4.01**	**0.76**	--
	D/O*	4.15	2.11	4.03	1.90	5.69	4.90	8.06	4.71	3.89	7.12	8.71	8.98	9.92	7.28	7.16	0.19	0.14	0.19	0.19	0.21	--	--	--	--
	total	**9.10**	**7.63**	**8.40**	**8.78**	**8.19**	**6.92**	**5.78**	**6.40**	**11.31**	**15.63**	**29.00**	**35.65**	**36.55**	**25.68**	**75.42**	**197.03**	**262.61**	**296.18**	**192.80**	**94.57**	--	**4.01**	**0.76**	--

Original plant / Crude drug		*P. notoginseng*								*P. vietnamensis*	*P. vietnamensis* var. *fuscidiscus*			*P. zingiberensis*		*P. japonicus* (China)**									
		Sanchi (Sanchi Ginseng)								Vietnamese Ginseng	Ye-Sanchi			Jiangzhuan-Sanchi				var. angustifolius		var. *major*				var. *bipinnatifidus*
		200 Tou	160 Tou	80 Tou	40 Tou	20 Tou	60 Tou													Hubei		Yunnan		
Aglycone type		PNY1	PNY2	PNY3	PNY4	PNY5	PNG1	PNG2	PNG3	PV	PVF1	PVF2	PVF3	PZ1	PZ2	PJC1	PJC2	PJA1	PJA2	PJMH1	PJMH2	PJMY1	PJMY2	PJB
Protopana-xadiol	1	17.95	23.37	27.24	22.63	21.72	22.50	24.46	20.63	8.75	6.44	15.59	6.15	1.38	3.43	3.29	1.20	1.35	2.36	2.05	4.65	4.66	2.08	2.88
	2	trace	trace	trace	trace	trace	trace	trace	trace	0.69	0.83	1.41	2.25	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	trace	0.21	0.26	trace	N.D.
	3	4.86	5.69	6.31	5.54	5.15	4.57	5.58	4.88	12.37	0.95	5.97	0.18	0.27	0.86	0.73	0.25	0.42	0.75	0.34	0.63	11.62	10.34	0.75
	4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Protopana-xatriol	5	2.68	2.07	3.61	3.15	2.44	1.72	3.55	2.15	1.35	2.42	0.96	3.67	N.D.	N.D.	2.20	5.34	N.D.	N.D.	4.92	2.86	trace	trace	2.49
	6	28.84	30.74	27.85	37.96	31.09	28.90	31.75	30.78	30.99	23.05	56.70	31.71	20.50	23.75	3.57	4.09	trace	trace	0.81	4.51	trace	trace	trace
	7	0.72	0.99	0.84	1.07	0.97	0.73	0.92	0.95	0.20	0.66	1.72	1.55	trace	0.24	trace	0.43	N.D.	N.D.	0.90	trace	0.99	0.26	0.32
Ocotillol	8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	13.72	57.80	84.77	65.32	N.D.	N.D.	trace	6.70	N.D.	N.D.	N.D.	N.D.	trace	trace	trace
Dammarane subtotal		**55.04**	**62.85**	**65.84**	**70.35**	**61.37**	**58.41**	**66.26**	**59.40**	**68.07**	**92.14**	**167.12**	**110.84**	**22.15**	**28.29**	**9.78**	**18.02**	**1.77**	**3.11**	**9.02**	**12.85**	**17.53**	**12.68**	**6.44**
Oleanolic acid	9	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	trace	trace	trace	trace	68.32	93.91	55.94	79.92	55.00	81.76	120.37	165.69	102.68	74.13	92.01
	10	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	27.32	38.46	16.47	33.15	46.96	29.36	trace	trace	1.40	trace	0.83
	11	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	7.76	8.04	10.93	19.95	40.18	45.65	60.22	84.18	43.28	22.20	9.66
Oleanolic acid subtotal		--	--	--	--	--	--	--	--	--	--	--	--	**103.40**	**140.40**	**83.33**	**133.01**	**142.14**	**156.77**	**180.59**	**249.87**	**147.37**	**96.33**	**102.49**
	D/O*	--	--	--	--	--	--	--	--	--	--	--	--	0.21	0.20	0.12	0.14	0.01	0.02	0.05	0.05	0.12	0.13	0.06
	total	**55.04**	**62.85**	**65.84**	**70.35**	**61.37**	**58.41**	**66.26**	**59.40**	**68.07**	**92.14**	**167.12**	**110.84**	**125.55**	**168.69**	**93.11**	**151.03**	**143.91**	**159.88**	**189.62**	**262.72**	**164.90**	**109.01**	**108.93**

* D/O: the ratio of subtotal of dammarane type saponins and oleanolic acid saponins

** Crude drugs drived from these taxa are named as Zhu Gen Qi, Bai-Sanchi, Zhu Jie Shen, Zhu Zi Shen, Kou Zi Qi, *etc.* in central to southwestern China.

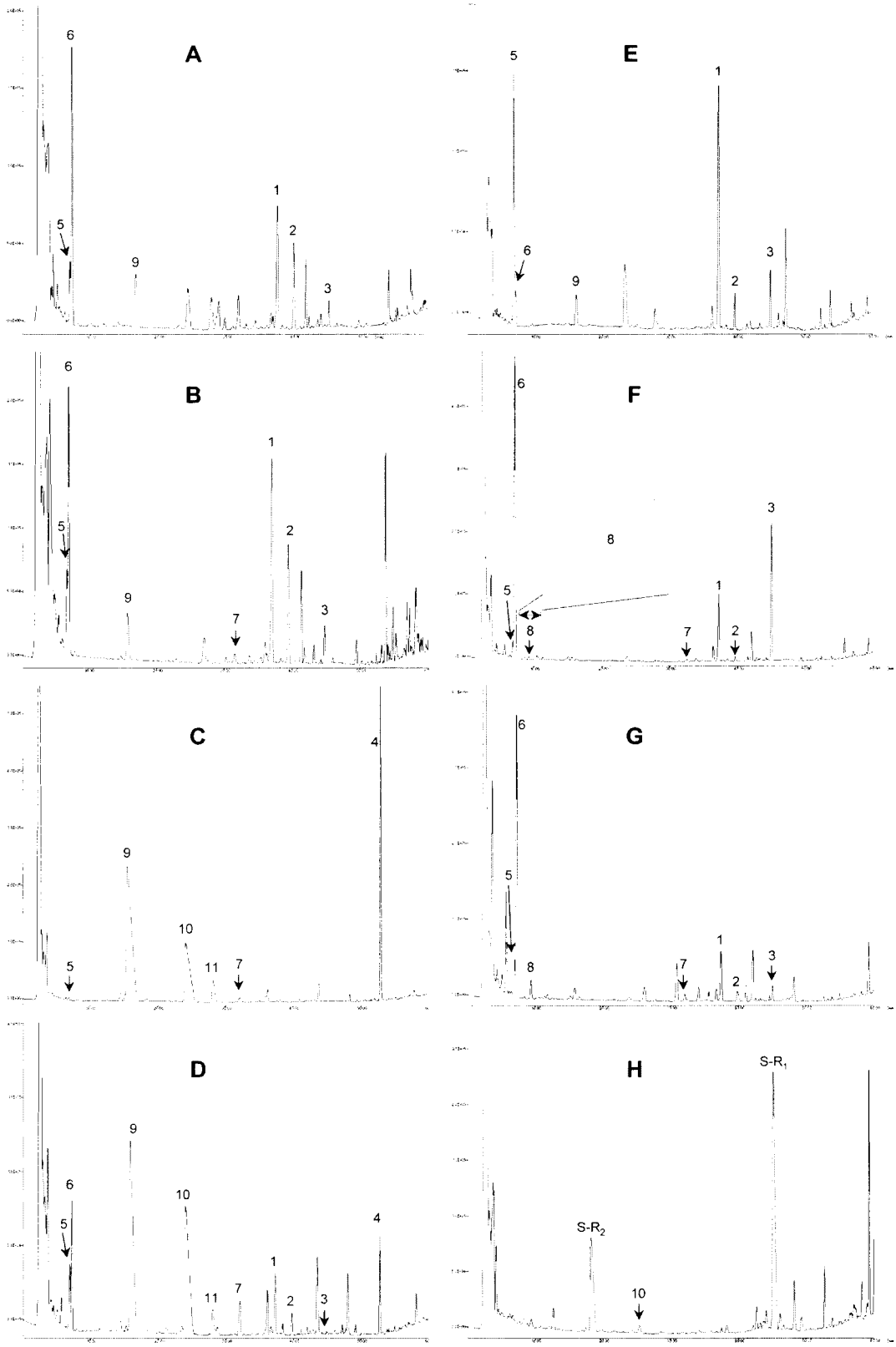


Fig. 7 HPLC chromatogram of various *Panax* drugs derived from 7 taxa
A: WPG1 (*P. ginseng*); **B**: RPG3 (*P. ginseng*); **C**: PJJ1 (*P. japonicus,* Japan); **D**: PJJ5 (*P. japonicus*, Satsuma-ninjin); **E**: PQ1 (*P. quinquefolius*); **F**: PV (*P. vietnamensis*); **G**: PVF1 (*P. vietnamensis* var. *fuscidiscus*); **H**: PS1 (*P. stipuleanatus*), S-R1: stipuleanoside R_1, S-R2: stipuleanoside R_2.

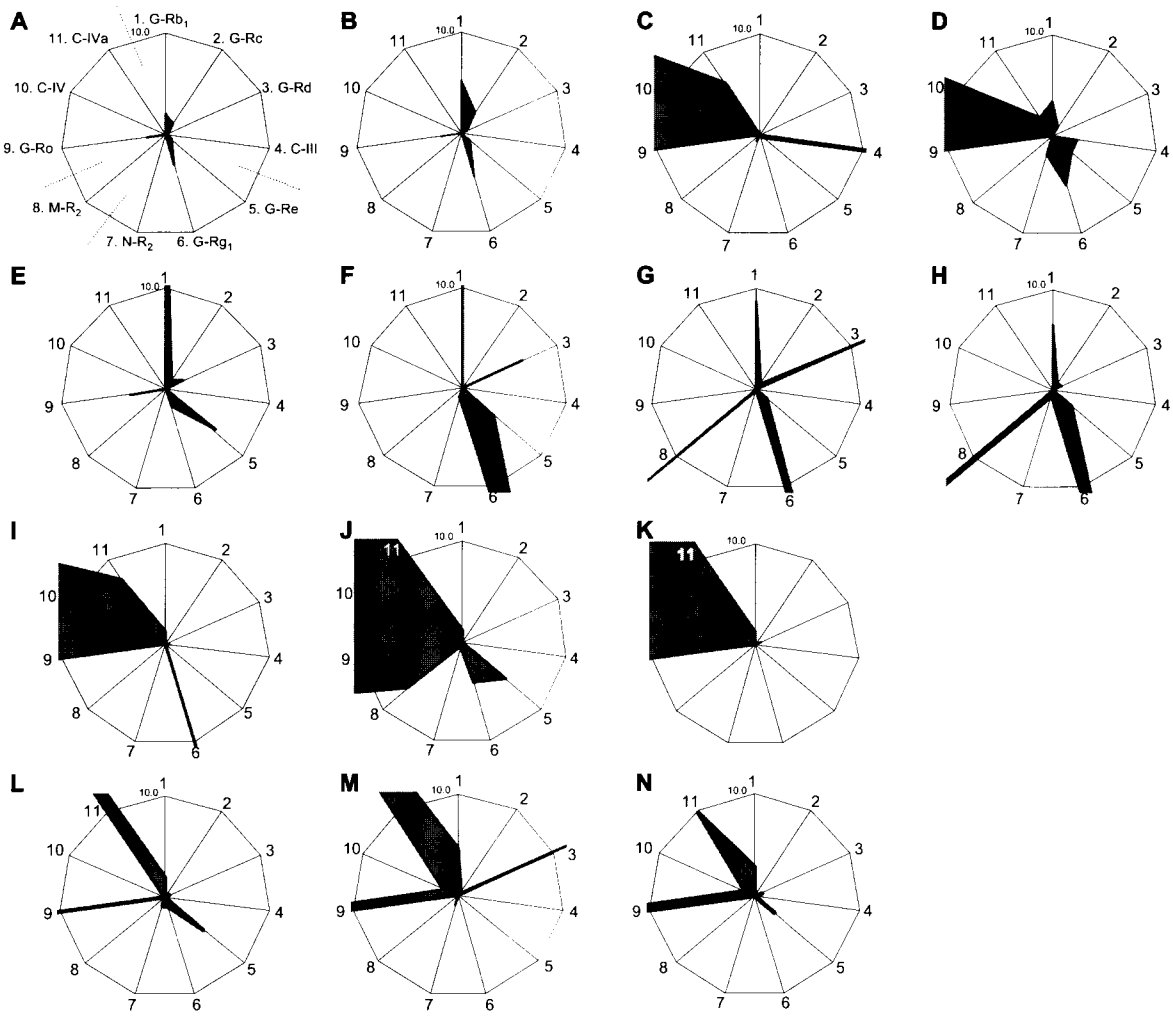


Fig. 8 Eleven-direction radar graph of various *Panax* drugs derived from 12 taxa
A: WPG1 (*P. ginseng*); **B**: RPG3 (*P. ginseng*); **C**: PJJ1 (*P. japonicus*, Japan); **D**: PJJ5 (*P. japonicus*, Satsuma-ninjin); **E**: PQ1 (*P. quinquefolius*); **F**: PNY3 (*P. notoginseng*); **G**: PV (*P. vietnamensis*); **H**: PVF1 (*P. vietnamensis* var. *fuscidiscus*); **I**: PZ1 (*P. zingiberensis*); **J**: PJC2 (*P. japonicus*, China); **K**: PJA1 (*P. japonicus* var. *angustifolius*); **L**: PJMH1 (*P. japonicus* var. *major*, Hubei); **M**: PJMY1 (*P. japonicus* var. *major*, Yunnan); **N**: PJB (*P. japonicus* var. *bipinnatifidus*). Each direction indicates the content of a determined compound (mg/g).

shapes were observed in the radar graphs for White Ginseng and Red Ginseng (Figs. 8A, B). American Ginseng derived from PQ was reported to possess very similar composition of saponins to that of Ginseng,[39] however, the quantitative analysis in our study distinguished them clearly (Figs. 7E, 8E). Chikusetsu-ninjin showed a characteristic HPLC chromatogram, in which 3 main peaks assigned to **9**, **10** and **4** were observed (Fig. 7C). However, one sample was rich in dammarane saponins **1**, **2**, **5** and **6** (Fig. 7D), which might be Satsuma-ninjin, a special population of PJJ distributed in southern Kyushu, Japan.[40] Notoginseng derived from PN has been graded into different ranks according to dried weight now (called "Tou" in Chinese and expressed as the number of roots in 500 grams). It is believed that the bigger the size, the higher the quality. The results of 8 samples from Yunnan and Guangxi, China with different sizes showed that there was no significant difference in the composition and quantity of saponins, however, a small increase in saponin content was observed with the increase of size (Table 3). Higher contents of **1**, **3** and **6** and the absence of oleanolic acid saponins distinguished this taxon from other kinds of Ginseng drugs (Fig. 8E). A high content of **8** was the differentiation marker for PV and PVF, but a high ratio of ginsenoside Rd/Rb_1 in the former and a complicated peaks pattern in 25-50 minutes in the latter distinguished them (Figs. 7F, G). The underground part of PZ revealed a unique constituent profile in contrast to the other group II taxa: besides the high content of oleanolic acid saponins **9**, **10** and **11**, a notable amount of dammarane saponin **6** was observed, whereas **5** and **2** were not detected (Fig. 8I). *P. japonicus* in China, including PJC, PJA, PJM and PJB, showed very similar constituent patterns, in which more than 85% of total saponins was composed of oleanolic acid saponins. Regarding the content of three oleanolic acid saponins **9**, **10** and **11**, two patterns were recognized: one, represented by PJC and PJA, showed a high content of **9**, **10**

and **11** (Figs. 8J, K); the other, represented by PJM and PJB, was characterized by the lack or only a small amount of 10, but large amounts of **9** and **11** (Figs. 8L-N). Furthermore, PJC and PJA were distinguished by the presence of **8** in the former and only trace of protopanaxatriol-type saponins in the latter (Table 3). PJM collected from Hubei (PJMH) and Yunnan (PJMY) showed different patterns: the former contained 8 (Fig. 8L) and the latter showed a high content of **3** and only trace of **5** and **6** (Fig. 8M). Although PS belonged to group II, its main constituents were different from those of the other group II taxa. Two main peaks in its chromatogram (Fig. 7H) were assigned to be stipuleanosides R_2 and R_1.[41]

As described above, chemical constituent similarities among the closely related taxa inferred from genetic data[17] were demonstrated, including PG and PQ, PV and PVF, and PJC and its varieties. PS of primitive clade showed a specific constituent pattern. On the other hand, although PJJ and PG, PZ and PV/PVF formed sister groups, respectively, in the phylogenetic trees reconstructed by *trn*K-18S rRNA gene sequences, their saponin constituent patterns were quite different from each other. The observed relationship between *trn*K/18S rRNA gene sequences and saponin constituents of *Panax* taxa suggested that the determination of the two gene sequences could be used not only for an ultimate authentication but also for a speculation of the chemical constituent pattern.

Ⅲ. Genus *Curcuma* and related drugs[42,43]

Genus *Curcuma* (Zingiberaceae) consists of about 70 species in the world, of which more than 10 are distributed or cultivated in China[44] and Japan. There are many reports on the pharmacological effects of *Curcuma* drugs such as antitumor,[45] anti-inflammatory,[46] and immunological activities.[47] Traditionally, *Curcuma* drugs have been used for treatment of "Oketsu" (various syndromes due to obstruction of blood circulation such as arthralgia, psychataxia and dysmenorrhea) in the system of Chinese Medicine. In "Ben Cao Gang Mu," a famous work of Chinese herbal literature[48] written in the 16th Century, it was described that "Yujin" enters the heart to control blood circulation, "Jianghuang" enters the spleen to share with the stomach function and to promote "Qi," and "Ezhu" enters the liver to eliminate blood stasis. Although these drugs used to be thought to have different effects, it is doubtful whether the botanical origins of drugs with these names correspond to those of present day drugs with the same names. Nowadays, four kinds of *Curcuma* drugs are prescribed in Chinese Pharmacopoeia[49]; Yujin (the tubers of *C. wenyujin* Y. H. Chen et C. Ling (CW), *C. longa* L. (CL), *C. kwangsiensis* S. G. Lee et C. F. Liang (CK), or *C. phaeocaulis* Val. (CP)), Jianghuang (the rhizome of CL), Pian-Jianghuang (the rhizome of CW) and Ezhu (the rhizomes of CP, CK or CW). Among them, Jianghuang and Ezhu have been imported from China to Japan, called as "Ukon" and "Gajutsu," respectively. Furthermore, Japanese productions, Gajutsu derived from the rhizome of *C. zedoaria* Rosc. (CZ) and Ukon from the same botanical origin as Chinese Ukon are also used as medicine, prescribed in Japanese Pharmacopoeia[50] and in the Standards of Crude Drugs Outside the Japanese Pharmacopoeia,[51] respectively. These two drugs, together with "Haruukon" derived from the rhizome of *C. aromatica* Salisb. (CA) are used as health foods and are great demand in Japan. Thus, although *Curcuma* drugs derived from several species have been available in Japanese and Chinese markets, they have not been able to be classified by botanical origins, and differences of effects among *Curcuma* drugs of different botanical origins are not obvious.

1. Phylogenetic relationship in genus *Curcuma*

Besides the medicinally-used 6 species described above, five new species, i.e. *C. sichuanensis* X. X. Chen (CS), *C. chuanezhu* Z. Y. Zhu (CCZ), *C. chuanyujin* C. K. Hsieh et H. Zhang (CCY), *C. chuanhuangjiang* Z. Y. Zhu (CCH) and *C. yunnanensis* N. Liu et Senjen (CY) have been recently recorded in Chinese botanical papers[52,53] based on minute morphological differences from the existing species. In order to adjust the controversies in taxonomy of genus *Curcuma* in China and Japan and to reconstruct phylogenetic relationship among these species, a molecular systematic study was performed.

The 18S rRNA gene sequences of medicinally-used 6 species were found to be of 1810 bp in length. Compared with the common sequence of CL, CP, CW and CA, only one base substitution was observed at nucleotide position 234 in CK, and the same base difference was observed between the Chinese and the Japanese populations of CZ [CZ(CN) and CZ(JP)]. The entire *trn*K gene (Fig. 9) was found to vary in length from 2698 bp to 2705 bp according to the species. Moreover, even in the same species, one base indel resulted in length variety. CZ(CN) and CZ(JP) were characterized by different length and sequence. Although the *trn*K gene was highly conserved among the 6 species, there were base substitutions and indels within the intron region except embedded *mat*K gene. Comparing the sequences of 5 species except CA, four base substitutions were observed at nucleotide positions 177, 200, 531 and 2575. The number of poly thymine observed from nucleotide position 501 varied from 10 to 14, according to species or specimens. The sequence of CP and CZ(CN) possessed a 4-bp insertion repeat (from 728 to 731). In CA, a 9-bp deletion (from 714 to 722) and a 14-bp insertion repeat (from 750 to 763) besides 5 base substitutions (146, 147, 645, 2511 and 2602) were observed. The specimens of CK were divided into two groups on the basis of nucleotide differences (177, 200, 531 and 2575). Morphologically, one group had leaf blades with a purple-colored band along the midrib and lateral spikes (purple-cloud type: pl), whereas another group had pubescent leaf blades without a purple band and central spikes (pubescent type: gl). The sequence of CK(pl) was identical to that of CZ(JP), and the sequence of CK(gl) to that of CW. As for the five new species, the 18S rRNA gene and *trn*K gene sequences of CS corresponded completely to those of CL and both sequences of CCZ to those of CP. CCY and CCH had the same sequences

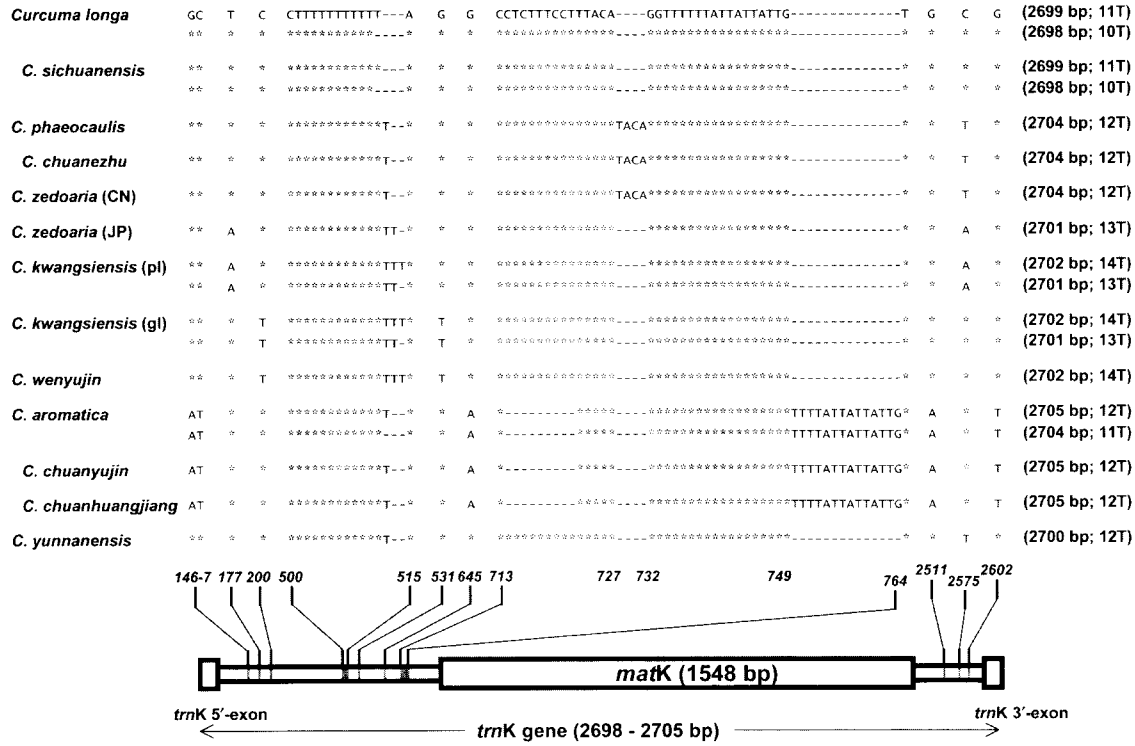


Fig. 9 Comparison of *trn*K gene sequences of eleven *Curcuma* species
Hyphens (-) denote alignment gaps; asterisks (*) indicate sequence identity with *C. longa*. The numerals in italics under the sequence indicate the aligned nucleotide position from the 5' end of the forward primer, *trn*K-3914F. The numerals in the parentheses indicate the whole length of *trn*K gene sequence between *trn*K-3914F and *trn*K-2R, and the number of poly thymine between the nucleotide position 501 and 514 from upstream. CN, The Chinese population; JP, The Japanese population; pl, purple-cloud type; gl, pubescent type.

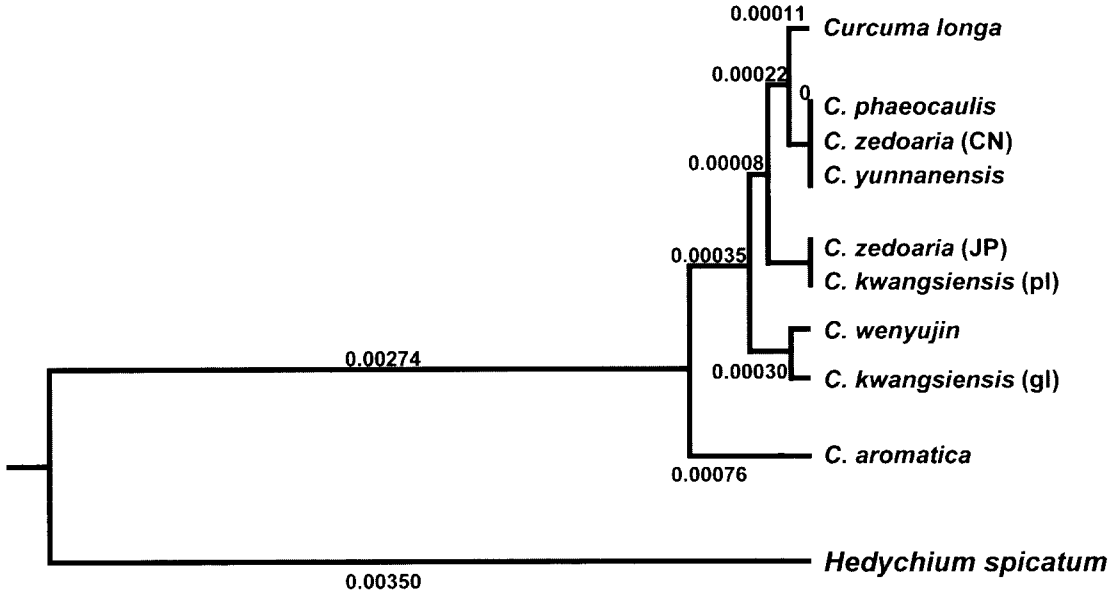


Fig. 10 The phylogenetic trees using the UPGMA method among seven *Curcuma* species
The tree was reconstructed based on combined 18S rRNA-*trn*K gene sequence data. The trees were outgroup-rooted using the sequence data of *Hedychium spicatum*. Branch lengths were calculated by Kimura's two-parameter method and mapped along each branch. CN, The Chinese population; JP, The Japanese population; pl, purple-cloud type; gl, pubescent type.

as CA. CY only possessed a specific *trn*K gene sequence of 2700 bp, which was similar to that of CL except for the 12 successive thymines from nucleotide position 501 and thymine at position 2575, and similar to that of CP except for the absence of the 4-bp insertion. Morphologically, CY is characterized by the presence of a central terminal spike, and of a leaf blade with a purple-colored band as in CP. A series of sequence analysis suggested that 11 species of the genus *Curcuma* distributed in China and Japan could be converged in 7 typical groups. The phylogenetic tree was reconstructed based on the combined 18S rRNA-*trn*K gene sequence data of seven species by UPGMA method using the computer program PAUP* (Fig. 10). The tree showed that CA was separated from the other species with a bootstrap value of 100%, and subsequently CW and CK(gl) were separated from the others. CP and CZ(CN), both having an identical sequence, formed one cluster together with CL, separating from another cluster of CZ(JP) and CK(pl). CY belonged to the same cluster as CP.

2. Identification of *Curcuma* drugs[43]

The 18S rRNA gene was amplified successfully via PCR using DNAs of drugs as templates. Whereas, since the direct amplification of the *trn*K gene did not produce the expected product, the gene region was divided into three overlapping sections, and PCR amplifications of these three parts were performed using the DNA or first PCR product of whole *trn*K gene as template (nested PCR). Comparing the sequences of drugs with those of the 7 *Curcuma* species, the botanical origins of 25 drug samples were determined. In the case of drugs derived from CK(gl) or CW and CZ(JP) or CK(pl), information of the producing area was necessary to determine the botanical origin. Subsequently, to develop a more convenient and effective method for identification of *Curcuma* plants and drugs, ARMS analysis of both gene regions was investigated. Based on the nucleotide differences at position 234 of 18S rRNA gene, two kinds of forward primers, CS234CF and CS234TF were designed (Fig. 11). Using each primer, together with a common reverse primer (ZR1108) and template DNAs from plants or drugs, PCR amplification was carried out and analyzed by 1% agarose gel electrophoresis. As expected, PCR amplification with CS234CF primer generated a fragment when the DNAs of CL, CP, CZ(CN) and CA were used as templates, those

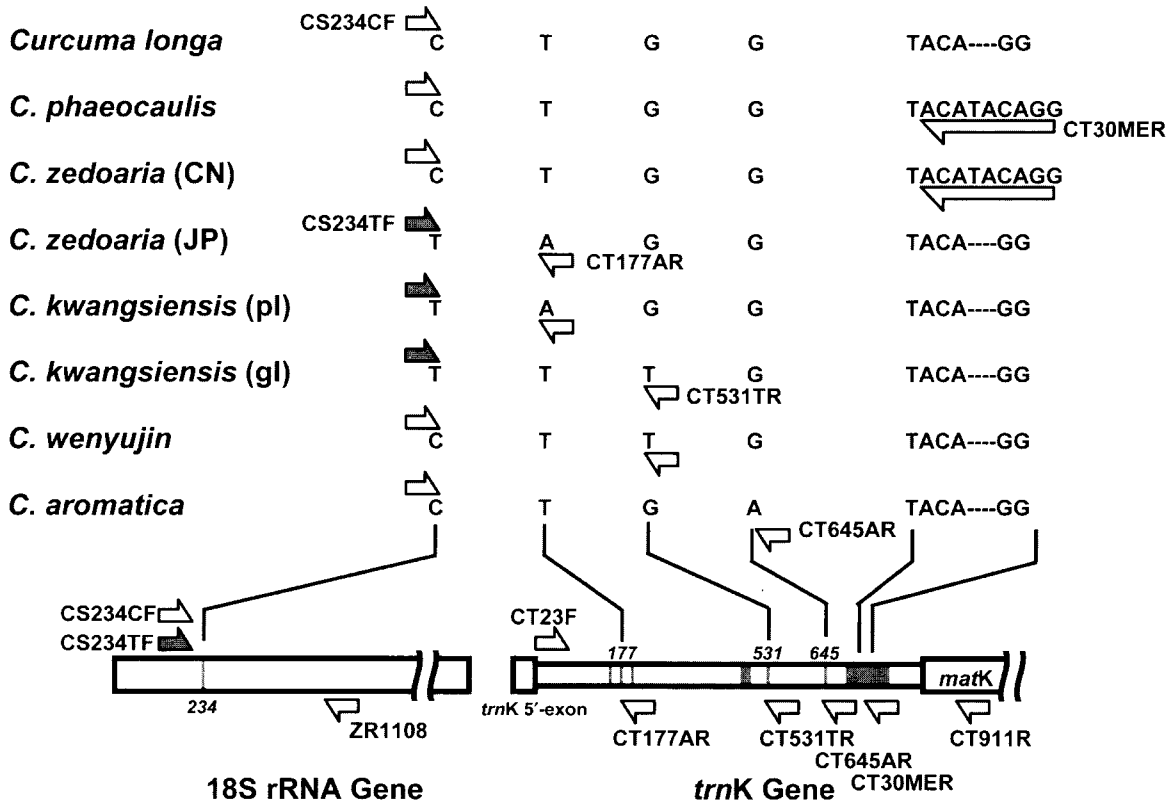


Fig. 11. Locations of primers for ARMS methods

A primer set of either CS234CF and ZR1108 or CS234TF and ZR1108 was used in the ARMS method for the 18S rRNA gene. A primer mixture of CT23F, CT177AR, CT531TR, CT645AR, CT30MER, and CT911R was used in the ARMS method for the *trn*K gene. The numerals in italics along the diagrams indicate the aligned nucleotide position from the 5' end of the forward primers: 18S-5'F (18S rRNA gene) and *trn*K-3914F (*trn*K gene). The broad half-arrows indicate primers used in the ARMS method: CS234CF, 5'-TAAAAGGTCGATGCGGGCTC-3'; CS234TF, 5'-TAAAAGGTCGATGCGGGCTT-3'; ZR1108, 5'-CCATACTCCCCCCGGAACC-3'; CT23F, 5'-AGTACTCGGCTTTTAAGTGC-3'; CT911R, 5'-TATAGAAACTGTTGTTGCCG-3'; CT177AR, 5'-TTTCAATGAAAATAAAATAATAAATCTATTT-3'; CT531TR, 5'-GTCTCTAACCCATAATAATA-3'; CT645AR, 5'-GGTTTATCTTCGTTATAATTAT-3'; CT30MER, 5'-ATATACAATAATAATAAAAAACCTGTATGT-3'. CN, Chinese population; JP, Japanese population; pl, purple-cloud type; gl, pubescent type.

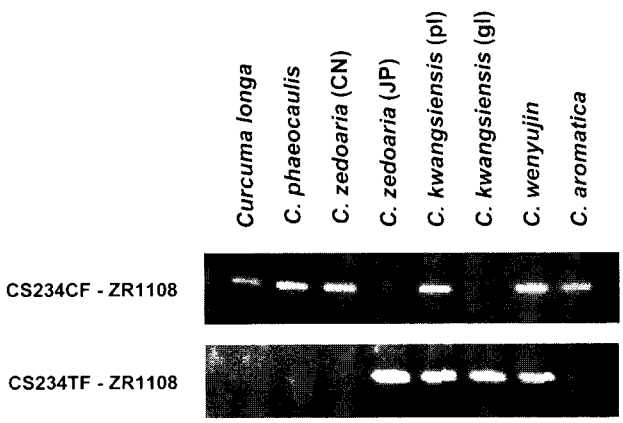


Fig. 12 Agarose gel electrophoresis of PCR products in the ARMS method for the 18S rRNA gene
PCR amplification with a primer set of either CS234CF and ZR1108 or CS234TF and ZR1108 was carried out using the DNA of each species as a template. CN, Chinese population; JP, Japanese population; pl, purple-cloud type; gl, pubescent type.

have cytosine at position 234. On the contrary, the PCR amplification with CS234TF primer generated a fragment of the same length when the DNAs of CZ(JP) and CK(gl) were used. However, in the case of CK(pl) and CW, the fragment was detected using both 2 primers, suggesting that these 2 species were heterozygotes (Fig. 12). For *trn*K gene, four reverse primers specific for 4 *Curcuma* groups, namely, CT30MER for CP and CZ(CN), CT177AR for CZ(JP) and CK(pl), CT531TR for CK(gl) and CW, and CT645AR for CA were designed, based on their own sequences (Fig. 11). At the same time, common forward and reverse primers (CT23F and CT911R) were prepared. PCR amplification was carried out using a mixture of 6 primers and DNAs from plants or drugs, then analyzed by 5% polyacrylamide gel electrophoresis. A fragment of nearly 900 bp was detected in every species. Moreover, CP and CZ(CN) had a fragment of 730 bp, CZ(JP) and CK(pl) had a fragment of 185 bp, CK(gl) and CW, of 527 or 528 bp, and CA, of 641 or 642 bp. This ARMS method was applied to *Curcuma* drugs by using the PCR products of *trn*K gene as template

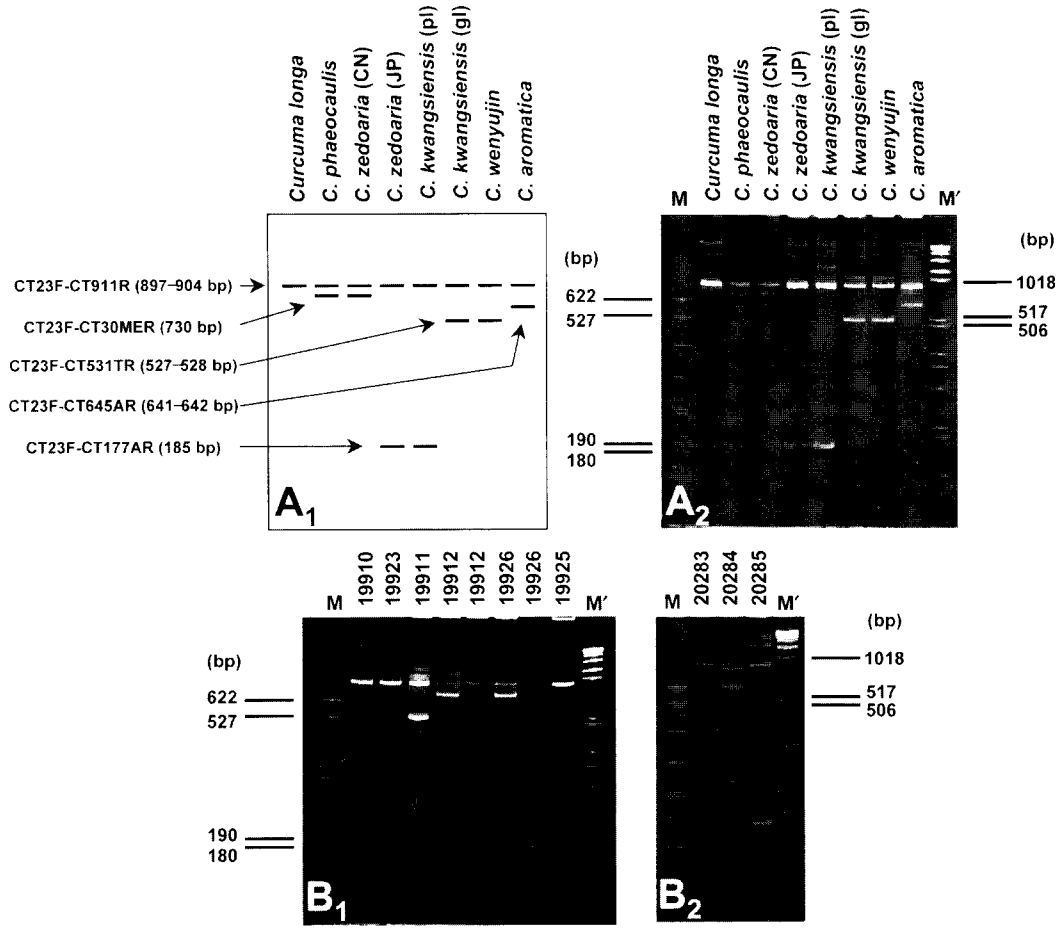


Fig. 13 Polyacrylamide gel electrophoresis of PCR products in the ARMS method for the *trn*K gene
A_1, a diagram of the expected fragment pattern of each species; A_2, fragments generated in PCR amplification when the DNA of each species was used as a template; B_1 and B_2, fragments when the DNA of each drug was used (1, drugs imported from China to Japan; 2, drugs produced in Japan). The numerals over the electrophoretograms indicate specimen reference numbers of Museum of Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University (TMPW). Lane M, pBR322DNA-*Msp* I Digest; M', 1Kb DNA ladder; CN, Chinese population; JP, Japanese population; pl, purple-cloud type; gl, pubescent type.

instead of total DNAs. Ukon (TMPW No.19910, 19923, 19925 and 20283) only generated a fragment of nearly 900 bp, namely, this result corresponded to that of CL. Haruukon (No.20284) generated two fragments of nearly 900 bp and 641 bp, suggesting this drug was derived from CA. Gajutsu (No.19912) and Kei-Gajutsu (No.19926) showed two fragment patterns, suggesting the mixture of CP and CK(pl). Thus, the established ARMS method was demonstrated to be applicable for identification of *Curcuma* drugs as well as plants (Fig. 13).

3. Effect of *Curcuma* drugs on vasomotion in isolated rat aorta[54]

The effectiveness of *Curcuma* drugs against "Oketsu" and the differences in their efficacy were evaluated by examining the effects on vasomotion,[55] especially on endothelial-dependent relaxation of the blood vessel as a key pathway for anti-"Oketsu" as one index. Five *Curcuma* drugs used were correctly identified as CL, a mixture of CK and CP (CKP), CP, CW and CZ(JP) by molecular biological analysis. After preparing rings from the thoracic aorta of Wistar rats, the rings were mounted in an organ bath, filled with Krebs solution. For relaxation experiments, each aortic strip was contracted by treatment with 6×10^{-6} M $PGF_{2\alpha}$. When the contraction reached a plateau, the test sample was cumulatively added at concentrations ranging from 10^{-6} to 10^{-3} g/ml for water extracts, methanol extracts and polysaccharides, or from 10^{-7} to 10^{-4} M for compounds isolated from methanol extracts (Fig. 14). Relaxation was expressed as percentage of the decrease in maximal tension obtained by $PGF_{2\alpha}$-induced contraction. To investigate the involvement of nitric oxide (NO), ring preparations with the endothelium were exposed to 10^{-4} M L-NAME for 60 min before precontraction. For contraction experiments, polysaccharides were cumulatively added to rings at concentrations ranging from 10^{-6} to 10^{-3} g/ml without precontraction. Contraction was expressed as a percentage of the increase from baseline when the maximal tension by 60 mM KCl was regarded as 100%. The involvement of NO was determined by exposing to 10^{-4} M L-NAME for 60 min before treatment with samples.

Methanol extracts of 5 *Curcuma* drugs exhibited intense relaxation effects in the rings precontracted by $PGF_{2\alpha}$ despite pretreatment with and without L-NAME as an inhibitor of NO synthesis. The maximal activities were approximate 80% at 10^{-3} g/ml (Fig. 15). The relaxation effects of water extracts were lower than those of methanol extracts, and the intensities at 10^{-3} g/ml were different, that is, 65.3% in CL, 54.4% in CW, 47.7% in CP, 38.3% in CZ(JP), and 22.6% in CKP. The greater the content of methanol-soluble compounds in water extracts, the stronger could be the effects of relaxation expected. Polysaccharides, the main constituents in methanol-insoluble fractions of water extracts showed contraction effects: the contraction activities were 21.9% in CL, 11.3% in CKP, 47.8% in CP, 45.3% in CW, and 36.3% in CZ(JP) at a concentration of

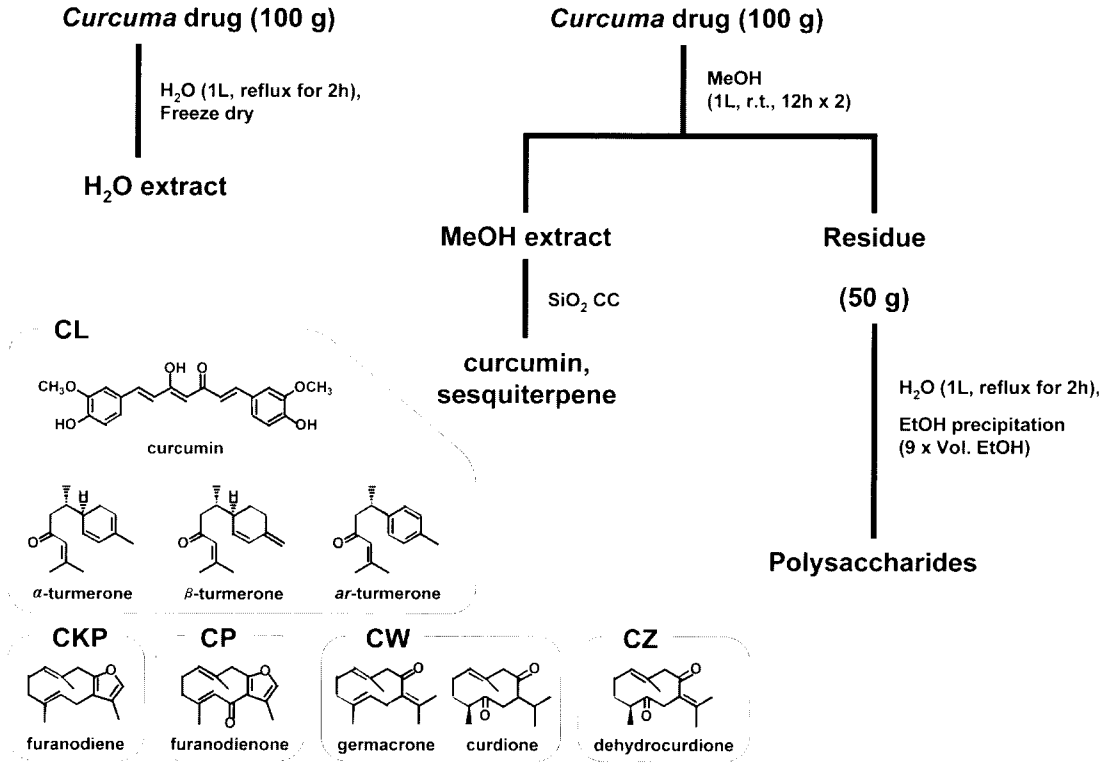


Fig. 14 Sample preparations and compounds isolated from *Curcuma* drugs
Abbreviations are as follows: CL, *C. longa*; CKP, *C. kwangsiensis*, *C. phaeocaulis*; CP, *C. phaeocaulis*; CW, *C. wenyujin*; CZ, *C. zedoaria*.

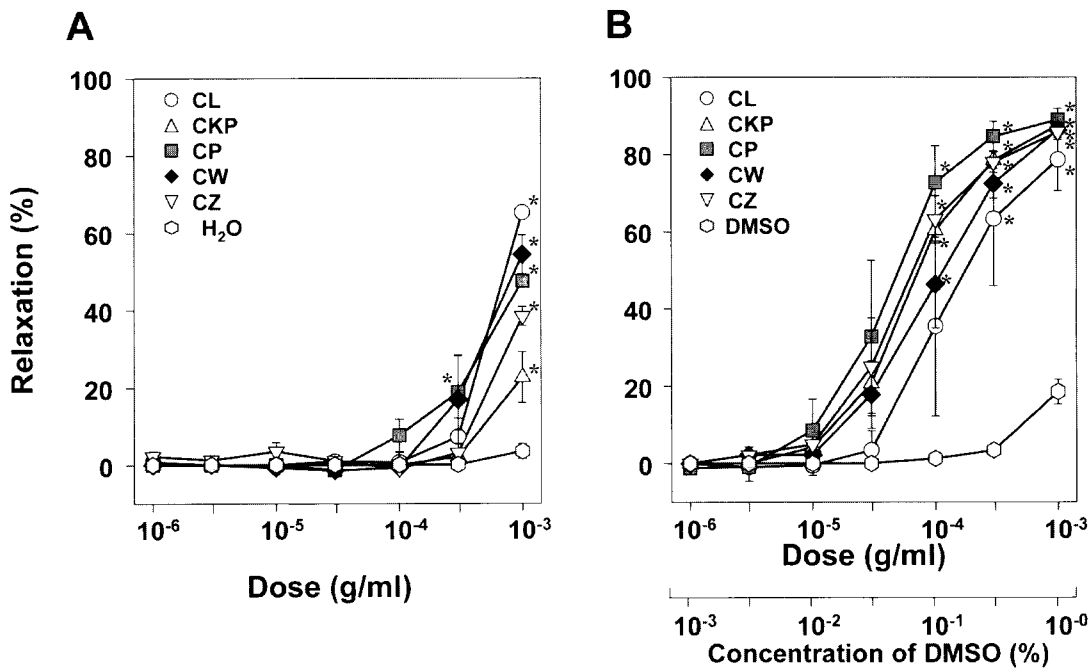


Fig. 15 Concentration-response curves for (A) water extracts or (B) methanol extracts-induced relaxation in rat aortic rings precontracted with $PGF_{2\alpha}$

Vehicle solutions were water (H_2O) and DMSO for water and methanol extracts, respectively. Values are expressed as percentage of decrease in the maximal tension contracted with $PGF_{2\alpha}$ (6×10^{-6} M). Results are shown as the mean ± S.E. of 4 samples. *$p<0.05$ vs. vehicle. Abbreviations same as in Fig. 14.

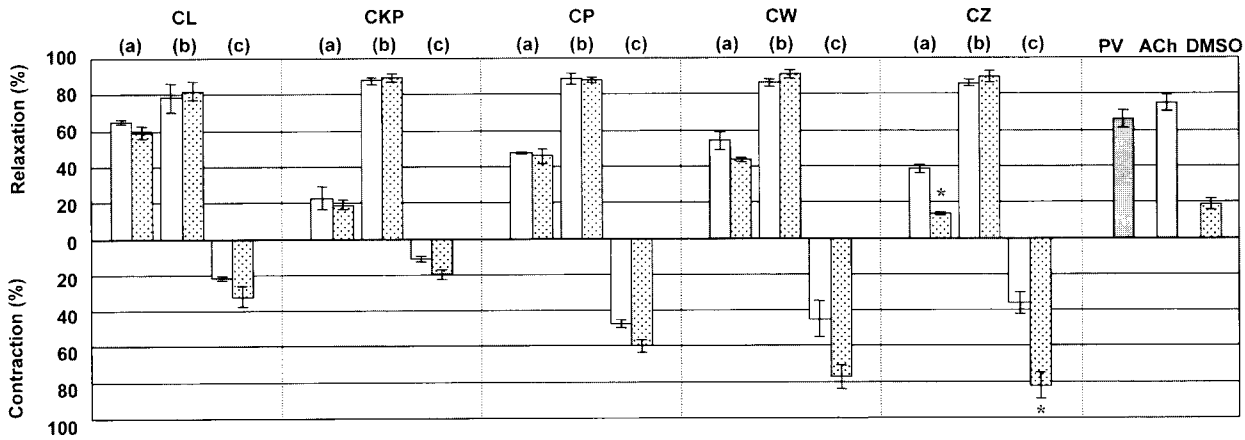


Fig. 16 Relaxation (upper) and contraction (lower) responses to (a) water extracts, (b) methanol extracts, and (c) polysaccharides on isolated rat aorta

Concentrations of extracts and polysaccharides were 10^{-3} g/ml. Rings were pretreated with (dotted columns) or without (open columns) L-NAME (10^{-4} M). For relaxation experiments, rings were precontracted with $PGF_{2\alpha}$ (6×10^{-6} M). Papaverine (10^{-5} M) and acetylcholine (10^{-6} M) were used as a positive control, and vehicle solution was water (water extract and polysaccharides) or DMSO (1.0%, methanol extract). Results are shown as the mean ± S.E. of 4 samples. *$p<0.05$ vs. without L-NAME. Abbreviations: PV, papaverine and ACh, acetylcholine; others same as in Fig. 14.

10^{-3} g/ml. Relaxation effects of water extracts were due to the sum of methanol-soluble compounds-induced relaxation and polysaccharides-induced contraction in all 5 *Curcuma* drugs. The water extract of CZ(JP) showed significant decrease in relaxation activity by pretreatment of L-NAME (38.3% → 13.2%), while the other four water extracts showed no differences with such pretreatment (Fig. 16). On the other hand, polysaccharides from CZ(JP) showed significant increase in contraction effect (36.4 → 82.4%) by L-NAME pretreatment. These facts indicate that CZ(JP) contains some polysaccharides which induce relaxation by promoting NO production. Although CZ(JP) seems to contain polysaccharides with an opposite effect on the vasomotion, it is unknown yet what they are.

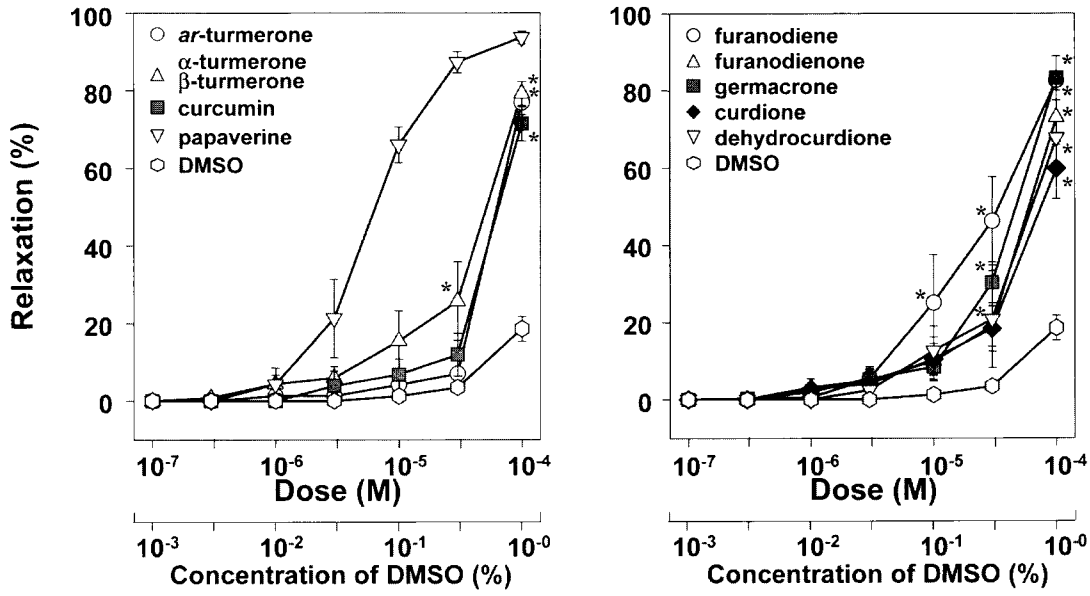


Fig. 17 Concentration-response curves for isolated compound-induced relaxation of rat aortic rings precontracted with $PGF_{2\alpha}$
The rings were pretreated with $PGF_{2\alpha}$ (6 x 10^{-6} M) and L-NAME (10^{-4} M). Vehicle solutions were DMSO. Papaverine was used as a positive control. Values are expressed as percentage of decrease in the maximal tension contracted with $PGF_{2\alpha}$. Results are the mean ± S.E. of 3 samples. *$p<0.05$ vs. vehicle.

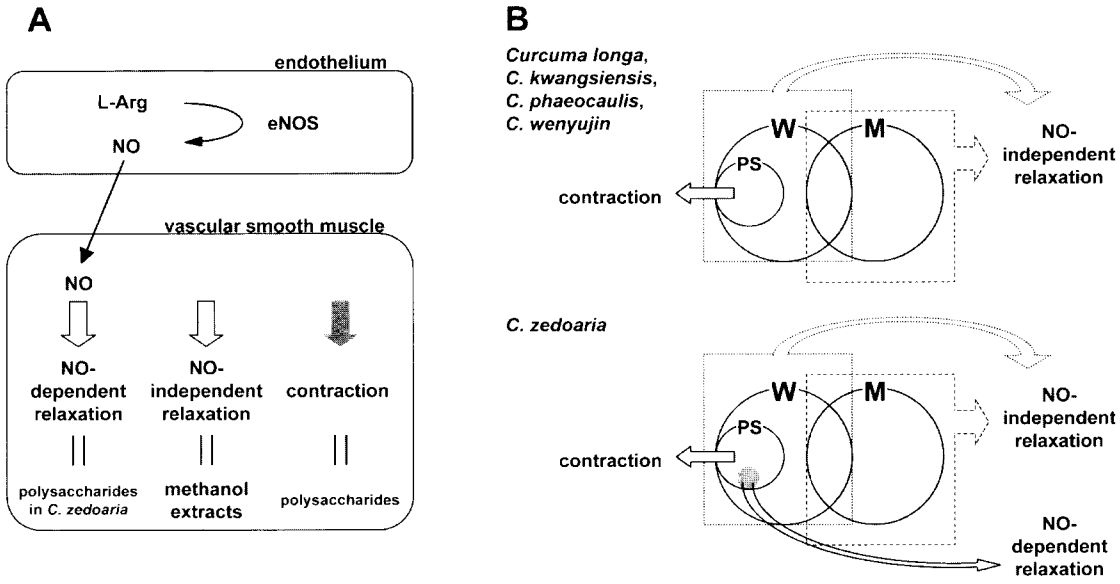


Fig. 18 Schematic diagrams of the effects on vasomotion (A) and inclusive relations of water and methanol extracts, and polysaccharides (B)
Abbreviations: L-Arg, L-arginine; eNOS, endothelial NO synthase; PS, polysaccharides; W, water extract; M, methanol extract.

From the methanol extracts, curcumin and 8 kinds of sesquiterpenes were isolated (Fig. 14). Only furanodiene showed significant effects of relaxation from 10^{-5} M, and the mixture of α-turmerone and β-turmerone, germacrone, and dehydrocurdione showed significant relaxation from 3×10^{-4} M. At 10^{-4} M, neither significant differences in relaxation activity nor a clear structure-activity relationship was seen among compounds (Fig. 17). Intensions of relaxation activities of isolated compounds without L-NAME treatment did not differ from those with L-NAME treatment.

CL contained a rich amount of curcumin (ca. 1.8%) and CP also contained a small amount (ca. 0.1%). Since there are many reports about pharmacological activities of curcumin like anti-inflammatory,[56] free-radical scavenging activity,[57] or antihepatotoxic activity[58] in addition to these vasodilator effects, CL may be highly potential for Oketsu

supervened with an inflammatory disease.

Oketsu is defined as "the state in which the blood is, for some reason, static and does not flow smoothly."[59] These states are seen in various diseases like hypertension, arteriosclerosis, or menopausal syndrome. It is reported that NO production in endothelium is decreased in arteriosclerosis,[60] and that endothelium dependent vasodilation is effective for hypertension.[61] These reports showed that NO production in blood vessels, especially in endothelium, is effective to improve blood flow. That is, the relaxation through the NO-dependent pathway is induced by the increase of NO in endothelium. As CZ(JP) was the only drug to show NO-dependent relaxation among the 5 *Curcuma* drugs, CZ(JP) may be expected to cure Oketsu from various acting points. Although we proved the anti-Oketsu effects of *Curcuma* drugs by the effects on vasomotion in isolated rat aorta, *in vivo* experiments such as vasomotion of capillary and blood rheology should be studied further to confirm usefulness of the drugs.

Although several *Curcuma* drugs are used with discrimination as anti-Oketsu substances, the pharmacological differences among them have been obscure. In our study, the differences of relaxation effects among 5 *Curcuma* drugs, CL called "Ukon" and CW, CP, CKP and CZ(JP) called "Gajutsu" in Japanese, were demonstrated. Methanol extracts and methanol-soluble compounds showed intense relaxation effects in rings of rat aorta pretreated by $PGF_{2\alpha}$. The vasodilator abilities of *Curcuma* drugs can be estimated from the total amounts of methanol-soluble compounds, curcumin and essential oil. Moreover, since the water extracts of only CZ(JP) showed NO-dependent relaxation besides NO-independent relaxation which were common to the other 4 drugs, this drug is thought to be more effective against "Oketsu."

We proposed pharmacognostical studies in the prime of molecular biology. Through the systematic study of *Panax* drugs, we found that the *trn*K/18S rRNA gene sequences could be used not only for an ultimate authentication but also for a speculation of the chemical constituent pattern which affects the pharmacological and clinical effects. Pharmacologically, we focused on the genus *Panax* to find out effective antidementia drugs which could activate and recover the function of degenerated brain by reorganizing synaptic connections. Among ginsenosides, protopanaxadiol (ppd)-type compounds were shown to have neurite outgrowth activity.[62] Moreover, it was demonstrated that 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol (M1), into which ppd-type saponins were transformed when metabolized by human intestinal bacteria, had axonal extension activity and improve memory disorder and synaptic loss in Aβ(25-35)-induced neuronal deficient mice.[63] M1 was shown to be effective *in vitro* and *in vivo*, indicating that *Panax* drugs containing ppd-type saponins may reactivate neuronal function in Alzheimer's disease by p.o. administration. On the other hand, the development of a potential method for identification of *Curcuma* drugs led to the clarification that the effects on vasomotion as one index against "Oketsu" were different according to botanical origins of drugs. Such a series of pharmacognostical study will become more and more necessary for standardization of herbal drugs and for their efficient uses.

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Japanese abstract

Panax 属植物及び *Curcuma* 属植物に由来する人参類生薬及び鬱金類生薬に関する研究を例にして，分子生物学全盛期の現代における生薬学的研究を提唱した。研究は，核 18S rRNA 遺伝子及び葉緑体 *trn*K 遺伝子の塩基配列に基づく植物の分子系統学的解析，遺伝子多型に基づく生薬の同定，薬理活性成分または薬理活性に基づく生薬の品質評価から構成される。2 遺伝子の塩基配列に基づいて構築した系統樹から *Panax* 属植物の系統関係が明確に整理され，一方各分類群に固有な塩基配列からすべての同属由来の生薬が同定された。重要な 5 種類の生薬を簡便に同定する方法として MARMS 法を開発した。*Panax* 属 12 分類群に由来する生薬について，11 サポニン成分の定量分析を行った結果，分類群固有の含有パターンが見出された。したがって，遺伝子の塩基配列を決定すれば，生薬の同定のみならず，各種活性を有する成分の組成をも推定できることが示唆された。同様に中国及び日本産 *Curcuma* 属植物の遺伝子解析を行い，鬱金類生薬の正確な同定を可能にした。5 種類の生薬の駆瘀血作用を比較する目的で，血管リング標本を用いて血管作動性を検討した結果，全生薬のメタノールエキスに強い NO 非依存性の血管弛緩作用，熱水エキスにそれより弱い弛緩作用が認められた。これは，熱水エキスにはメタノール可溶性画分に起因する血管弛緩作用の他，多糖類に起因する血管収縮作用が認められることによるものである。この弛緩作用は基源種により異なり，また日本産ガジュツのみ他種と同様の NO 非依存性の血管弛緩作用に加えて，NO 依存性の血管弛緩作用が認められた。ここで概説した一連の研究は，生薬の標準化とそれらの効率的な利用の点から重要になるものと考えられる。

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