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Authentication of Rhei Rhizoma

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Rhei Rhizoma (Dahuang in Chinese) is widely known as a purgative and antiinflammatory agent. In the Japanese Pharmacopoeia, Rhei Rhizoma is prescribed for 4 *Rheum* species, *Rheum palmatum, R. tanguticum, R. officinale,* and *R. coreanum,* while the first 3 species are prescribed for Dahuang in the Chinese Pharmacopoeia. Due to the morphologic similarity of the aerial parts, the taxonomy of this genus and the correct identification of *Rheum* species and their derivative drugs are very difficult. To resolve taxonomic problems of the genus *Rheum* and develop an ultimate identification method for plants and drugs, molecular analysis of the chloroplast *mat*K gene and nuclear 18S ribosomal RNA gene were performed on 9 species. The sequence comparison of the *mat*K gene revealed that most species had variable sequences not only inter- but also intraspecies. However, the specimens of the same species belonged to the same subclade in the phylogenetic tree constructed based on *mat*K gene sequences, except for *R. palmatum,* in which specimens belonged to 3 subclades related to their production areas. The nucleotide differences at positions 587, 707, and 838 distinguished official species from others, while specific nucleotides at positions 367 and 937 became identification markers for *R. palmatum, R. tanguticum,* and *R. officinale.* Moreover, three groups of *R. palmatum,* each belonging to 3 subclades, were characterized by the nucleotides at positions 619, 769, 883, and 1061. On the basis of the above marker nucleotides, a convenient and efficient identification method employing Polymerase Chain Reaction - Restriction Fragment Length Polymorphism and Amplification Refractory Mutation System analyses was further developed. The procedure enabled us to identify the botanic origins of 22 drug samples of Rhei Rhizoma. The *mat*K gene sequence was valuable in identifying *Rheum* species and Rhei Rhizoma and in predicting their production areas, and could be used as an index for quality evaluation of Rhei Rhizoma.

Key words *Rheum,* Rhei Rhizoma, phylogenetic relationship, *mat*K gene, PCR-RFLP analysis, ARMS analysis.

I. Introduction

Rhei Rhizoma, called "Dahuang" in Chinese, was first described in the medical classic, "Shen Nong Ben Cao Jing" in China, classified as low-level herbal drugs with some toxicity, but specific therapeutic effect.[1)] Since this era, Rhei Rhizoma has been widely used for the treatment of constipation and "Oketsu" (various syndromes caused by the obstruction of blood circulation such as dysmenorrhea, *etc.*) in traditional Chinese formulations and over-the-counter drugs.[2,3)] In the Chinese Pharmacopoeia,[4)] the official Dahuang is prescribed to be the dried rhizome and root of *Rheum palmatum* L., *R. tanguticum* Maxim. ex Balf., and *R. officinale* Baillon of the family Polygonaceae. On the other hand, the Japanese Pharmacopoeia[5)] prescribes not only the above 3 species but also *R. coreanum* Nakai, and their interspecific hybrids as the botanical origins of Rhei Rhizoma. Rhei Rhizoma is mainly produced in high altitude areas of China, especially in Qinghai, Sichuan and Gansu Provinces, and is available in the Japanese market under various commercial names such as "Gao (Ya-huang in Chinese)," "Kinmondaio (Jinwen-dahuang)," "Hakoo (Xiang-huang)," and "Rokuseikitsu (Liu-cheng-ji)" according to their geographical sources, morphological features, packing methods, quality grades, *etc.*[6,7)] Recently, a deterioration in the quality of commercial Rhei Rhizoma has been pointed out by Japanese Kampo doctors, in fact, our preliminary experiment suggested that some Dahuang from Gansu Province contained less sennoside A than that indicated in the Japanese Pharmacopoeia. Quality as Dahuang is related to various elements such as original plant, production area, environmental conditions and processing method. Since *Rheum* plants are self-incompatible in nature[8)] and have been hybridized at random since ancient times, morphologically intermediate forms (in the shape of the leaf blade, color of flowers, *etc.*) are frequently present. This situation made the taxonomic identification of *Rheum* plants very difficult. Accordingly, Dahuang derived from *Rheum* species is more difficult to be correctly identified and its quality has varied depending on differences in markets and production areas. Moreover, the underground parts of *R. franzenbachii* Münt., *R. rhaponticum* L., *R. undulatum* L., and *R. spicifome* Royle are sometimes confused with official Dahuang.

Namba *et al.* performed histological studies to identify Dahuang by comparing the microscopic characteristics of rhizomes of the above *Rheum* species, and resulted in Dahuang being identifiable by the line number of ray and

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presence or absence of mucilage cavities and their arrangement manner in secondary phloem, amount of vessels and presence or absence of fibers in xylem, presence or absence of star-spots (abnormal vascular bundles) and their shape and diameter in pith, and amount of mucilage cavities in the star-spots.[6,9] However, there has been some obscure points on histological variations depending on the difference of growing stage and production areas. As generally known, genotype rather than phenotype influenced neither by the physiologic stage of the plant nor by environmental conditions. Recently, DNA-based polymorphic assay were found to provide valuable information for assisting resolution of taxonomic problems.[10,11] Yang *et al.* described the nucleotide sequences of the chloroplast *trn*L/*trn*F gene region of 13 *Rheum* species to discriminate *R. palmatum, R. tanguticum*, and *R. officinale* from adulterated species.[12] However, their phylogenetic relationship and the identification markers for each species have not been clarified. The chloroplast *mat*K gene sequence has been widely employed as a powerful tool in examining inter- and intragenus phylogeny due to its high substitution rate.[13,14] The *mat*K gene is an open reading frame located in the intron region of chloroplast *trn*K gene encoded tRNALys^{UUU}.[15] As the chloroplast DNA shows maternal inheritance,[16] the information of nuclear DNA which is biparently inherited is necessary. The 18S ribosomal RNA gene, the small-subunit sequences of ribosomal RNA, is known to give essential information for constructing a phylogenetic relationship.[17] Accordingly, the determination of *mat*K gene and 18S rRNA gene sequences is thought to be suitable for phylogenetic analysis of *Rheum* species and authentication of Rhei Rhizoma.

For systematically pharmacognostic study, a wide collection of comparative plants, observation on morphology of plants and informative collection of medicinal uses in growing or producing areas are the primary steps for the subsequently laboratory research. Moreover, multiple samples of the same species from different localities are necessary to confirm intraspecies stability. In this review, field investigation of *Rheum* species growing in Qinghai and Sichuan Provinces was introduced, including morphological observation and information of their medicinal uses and quality. Subsequently, genetic polymorphism of *Rheum* species, especially *R. palmatum, R. tanguticum*, and *R. officinale* used as the official Dahuang in China and their phylogenetic relationship are elucidated. Using the sequence result, a correct identification of Rhei Rhizoma was performed. Moreover, a convenient and efficient method of identification for Rhei Rhizoma employing polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP)[18,19] and amplification refractory mutation system (ARMS)[18-20] analyses was developed.

II. Field investigation

1. Morphological characteristics of three *Rheum species* officially used as Rhei Rhizoma (Table 1)

Rheum palmatum: The specimens collected from Banma County, Qinghai Province were growing in the field at 3410 m alt. Their radical leaves were very large, often 70 cm in diameter, with semi-terete petiole. The leaf blades were ovate or roundish in shape, conspicuously palmatipartite, acute at the apex, cordate at the base, 5-veined with a prominent midrib, and with laciniate lobelets. Both sides of leaf blades were hairy. The inflorescence was compact bearing elongated leafy panicles that were crowded with yellowish-white flowers. The underground parts consisted of a strong vertical rhizome, irregular in shape, with fleshy, spreading roots; externally it was brown to grayish brown, internally yellow in color with a lot of mucilage oozing from the wound when freshly cut. The star-spots were arranged into 1-2 concentric rings (annulated network) in the outermost part of pith in the cross-section. The cross-section was, to some extent, coarse in texture. Specimens collected from Sichuan Province showed various morphological characteristics according to their geographical distributions. Those from the central to southern part of Sichuan such as Shimian, Kangding and Daofu differed from that of the Qinghai Province chiefly in that their leaf blades were palmatifid with triangular lobelets. The underground parts showed a deep-yellow color in cross-section. Specimens from the northwestern part of Sichuan such as Yulong closely resembled to those of the Qinghai Province except that their underground parts possessed less mucilage. Specimens cultivated in Dali, Yunnan Province had palmatifid leaf blade and divaricate in florescence with red flowers. In the specimens from Li, Tanchang and Min Counties, Gansu Province, the leaf blades were slightly or moderately palmate with platylobate or triangular lobelets, and glabrous or sparse on the upper side of leaf blades; the inflorescence was divaricate with red flowers; and mucilage was invisible or sparse in the cross-sections of underground parts. However, specimens collected from Zhouqu were similar to those from Qinghai Province except for the red colored flowers and triangular shaped lobelets.

R. tanguticum: Specimens growing wild in Qinghai Province at 3100-3650 m alt. had palmatipartite leaf blades. They could be distinguished from *R. palmatum* from the southeastern part of Qinghai Province by their lanceolate lobelets, red flowers and the margin paralleling to the midrib. Moreover, the cross-sections of *R. tanguticum* were compact in texture and exhibited a marbled network of abnormal vascular bundles.

R. officinale: Wild specimens were collected in Wanyuan County, eastern part of Sichuan Province at 1410 m alt. Their leaf blades were glabrous in upper side, hirtellous in lower side, and their margin was coarsely dentate or lobed. Flowers were yellow or yellowish-white.

2. Medicinal uses in local Tibetan people

The Rhizome of *Rheum* species (*R tanguticum* or *R. palmatum*) is cold in property and used for the treatment of inflammatory diseases with hot property, diarrhea, indigestion, food poisoning, constipation and obstruction of blood circulation. Tibetan doctors frequently use one formulation called Rokumishosekisan (Liuwei-xiaoji-san in Chinese, Zhi-byed 6 in Tibetan) including Rhei Rhizoma, Kieserite,

Table 1 Morphological characteristics of three official *Rheum* species collected in the field

Species	Producing area	Habitus / Altitude	Color of flowers	Leaf blade				Underground part				
				Palmate incision	Shape of lobelet	Margin paralleling to midrib	Hair Upper side / Lower side	Shape	Cross-section Color	Cross-section Texture	Network of star-spots	Mucilage
Rheum palmatum	Banma, Qinghai	cultivated / 3410m	yellowish-white	dissected	lacinule	−	++ / +++	irregular	yellow	coarse	annulated	++
	Shimian, Sichuan	cultivated / 1355m	yellowish-white	lacerated	triangular	−	+ / +++	conical	deep-yellow	coarse	annulated	++
	Kangding, Sichuan	wild / 3735m	yellowish-white	lacerated	triangular	−	++ / +++	irregular	deep-yellow	coarse	annulated	++
	Daofu, Ganzi, Sichuan	cultivated / 3305m	yellowish-white	lacerated	triangular	−	++ / +++					
	Yulong, Ganzi, Sichuan	wild / 3855m	yellowish-white	dissected	lacinule	−	++ / +++	conical	orange	coarse	annulated	+
	Shiqu, Sichuan	wild / 4200m	yellowish-white	dissected	lacinule	−	+ / +	irregular	yellow	coarse	annulated	+
	Dali, Yunnan	cultivated / 3115m	purplish-red	lobate	platylobate	−	− / ++	irregular	yellow	coarse	annulated	±
	Li, Gansu	cultivated / app. 2500m	purplish-red	lacerated	platylobate	−	+ / ++	irregular	pale-yellow	coarse	annulated	±
	Shuanshui, Li, Gansu	cultivated / app. 3000m	purplish-red	lobate	platylobate	−	− / ++	irregular	pale-yellow	coarse	annulated	±
	Tanchang, Gansu	cultivated / app. 2500m	purplish-red	lacerated	platylobate	−	− / ++	irregular	pale-yellow	coarse	annulated	±
	Min, Gansu	cultivated / app. 2500m	purplish-red	lacerated	triangular	−	− / ++	irregular	yellow	coarse	annulated	±
	Hanban, Zhouqu, Gansu	cultivated / app. 3000m	purplish-red	dissected	triangular	−	++ / +++					
	Baidian, Zhouqu, Gansu	wild / app. 3500m	purplish-red	dissected	triangular	−	+/ +++	conical	deep-yellow	coarse	annulated	++
R. tanguticum	Huangnan, Qinghai	wild / 3100m	purplish-red	dissected	lanceolate	+	++/ +++	conical	yellow	compact	marbled	+
	Dulan, Qinghai	wild / 3650m	purplish-red	dissected	lanceolate	+	++/ +++	conical	yellow	compact	marbled	+
R. officinale	Wanyuan, Sichuan	wild / 1410m	yellowish-white	lobate	dentate	−	−/ ++	conical	pale-yellow or yellow	coarse	annulated	±

−, absent; ±, not obvious; +, present or obvious; ++, many; +++, numerous

Chebulae Fructus, *etc.* for the treatment of stomach pain, constipation, indigestion and abdominal pain after childbirth. Tibetan doctors in Xining and pharmacists of pharmaceutical companies in Huangnan and Dulan Counties, Qinghai Province told the rhizome of *Rheum* species with red flowers (*R. tanguticum*) had good quality. On the other hand, according to the information of the pharmacists in Ganzi County, Sichuan Province, Dahuang producing in a boundary area between Sichuan and Qinghai Provinces (rhizome of *R. palmatum*) was best in quality, and from Wanyuan Conty, Sichuan Province, Dahuang in Wanyuan production (rhizome of *R. officinale*) was the real one used by the Chinese since ancient times.

III. Authentication of Rhei Rhizoma using molecular analysis

1. Genetic polymorphism of *Rheum species* *mat*K gene and 18S rRNA gene sequences

Partial *mat*K gene sequences of 56 plant specimens belonging to 7 *Rheum* species of 4 sections,[21] i.e. *R. palmatum, R. tanguticum, R. officinale*, and *R. coreanum*[22] of section Palmata, *R. franzenbachii, R. rhaponticum,* and *R. undulatum* of section Rhapontica, *R. kialense* Franch. of section Acuminata, and *R. przewalskyi* A. Los. of section Spiciformia were determined (Table 2, Fig. 1). Moreover, *trn*K gene sequences of 20 specimens of the first 5 species were also determined. The aligned *trn*K gene sequence of *R. palmatum*, a 5'-exon 37 bp in length, was found between positions 2 and 38, the 3'-exon (partial) between positions 2537 and 2546, and the *mat*K gene region was defined between positions 742 and 2259.[23] Partial *mat*K gene, which was easily amplified by PCR with the universal primers,[24] was defined between positions 796 and 2060. The complete or partial *mat*K gene was found to be 1518 bp or 1265 bp in length in 20 specimens of 5 *Rheum* species and showed sequence variation not only interspecies but also intraspecies. A total of 28 sites of nucleotide substitutions were observed in complete *mat*K gene region, of which 25 sites were included in partial 1265 bp region (Table 3). Therefore, the sequence comparison of all 56 samples of 9 *Rheum* species was performed on partial *mat*K gene region. The partial *mat* K gene of *R. kialense* and *R. przewalskyi* was found to be 1271 bp in length due to a 6-bp insertion, and that of the remnants was to be 1265 bp. A total of 51 sites of nucleotide substitutions were observed. Intraspecies sequence variation was found at 16 sites of nucleotide substitutions in *R. palmatum*, 2 sites in *R. tanguticum*, and 3 sites in *R. officinale*. However, common sequences within the same species were found at positions 367 and 937 (the nucleotide positions indicate the aligned position, starting from the 5' end of the *mat*K gene) in *R. palmatum* (possessing cytosine and cytosine), *R. tanguticum* (thymine and thymine), and *R. officinale* (cytosine and thymine), respectively. *R. coreanum* had a similar sequence to those of *R. officinale*. Although 23 sites of substitutions were observed in 4 species of the section Palmata, common sequences within these species existed at positions 587, 707, and 838. Based on the nucleotide differences at these positions, five other species belonging to different sections were separated from section Palmata species. The 37 specimens of *R. palmatum*, which varied in sequence, could be divided into groups, I, II, and III on the basis of nucleotide substitutions at positions 619, 769, 883, and 1061. The result of phylogenetic analysis revealed that each specimen of the 3 groups belonged to 3 subclades, RpI, RpII, and RpIII, respectively (Fig. 2). Group III was separated from groups I and II by the nucleotide differences at positions 883 and 1061, and group I and II were distinguished from each other by the nucleotides at positions 619 and 769.

The entire 18S rRNA gene sequences of 9 *Rheum* species were found to be 1811 bp in length. Eight sites of nucleotide substitutions were observed at positions 234, 283, 650, 671, 821, 1410, 1717, and 1731, and two indels at positions 669 and 676. There were no intraspecies substitutions and furthermore, no interspecies substitutions in the same section. *R. palmatum, R. tanguticum, R. officinale*, and *R. coreanum* of section Palmata were distinguished from the other species of different sections based on the nucleotide difference at position 234.

Phylogenetic analysis of *Rheum* species

The phylogenetic tree constructed on the basis of partial *mat*K gene sequences of 56 specimens of 9 *Rheum* species using unweighted pair group method with arithmetic mean (UPGMA method) with *Fagopyrum tataricum* (L.) Gaertn. of the family Polygonaceae as an outgroup showed that *R. kialense,* and subsequently *R. przewalskyi* was located at the basal position (Fig. 2: left). The other species were grouped into 2 clades. *R. rhaponticum, R. franzenbachii,* and *R. undulatum* of section Rhapontica formed one clade, whereas *R. officinale, R. coreanum, R. tanguticum*, and *R. palmatum* of section Palmata formed another clade. The latter large clade was divided into 2 clades, and then each clade into 2 subclades. The specimens of *R. palmatum* formed 3 subclades, RPI, RPII, and RPIII according to their distribution areas. Specimens from the southeastern part of Qinghai Province to the northwestern part of Sichuan Province were classified in subclade RPI, and those from the central to southern part of Sichuan were in subclade RPIII. Nearly 70% of specimens collected from Gansu Province were in RPII. However, some of the cultivated specimens obtained from Li, Tanchang, and Min in Gansu were in subclade RPIII and others under RPII, although both were growing in the same or neighboring field. In those from Dali in Yunnan, specimens with a cultivation history of more than 100 years were in subclade RPIII, while recently cultivated ones were classified in RPII. Specimens cultivated in Shimian in Sichuan belonged to all subclades, RpI, RPII, and RPIII. Such confusion was presumed to be a result of seed transportation and artificial fertilization for the purpose of propagation. However, in the case of wild plants, specimens from the northwestern part of Sichuan belonged to subclade RPI, those from the central part of Sichuan to RPIII, and those from Gansu to RPII. The specimens of *R. officinale* and *R. coreanum*, together with those of *R. tanguticum* formed one subclade, which had a closer

Table 2 Plant specimens used in this study

Species	Locality of voucher		Date of collection	Voucher no.	State-ment	Code no.	Clade[a)]	GenBank accession no.
Section Palmata A. Los.								
Rheum palmatum L.	Qinghai Prov., China	Banma (班瑪) Co.	2000.7.20	K. Komatsu et al. QS127-2*, QS129*	cult.	Pq1*, Pq2*	RPI	AB115669
		Dongqinghan (東傾溝)	2000.7.21	K. Komatsu et al. QS171-5*	cult.	Pq3*	RPI	AB115670
	Sichuan Prov., China	Heping (和坪), Shimian (石棉) Co.	2000.8.7	K. Komatsu et al. QS351	cult.	Ps4	RPII	AB115672
		Heping, Shimian Co.	2000.8.7	K. Komatsu et al. QS352-6	cult.	Ps5	RPI	AB115671
		Caoke (草科), Shimian Co.	2000.8.8	K. Komatsu et al. QS354-1	cult.	Ps6	RPIII	AB115676
		Caoke, Shimian Co.	2000.8.8	K. Komatsu et al. QS354-2	cult.	Ps7	RPIII	AB115677
		Zheduo (折多山), Kangding (康定) Co.	2000.8.8	K. Komatsu et al. QS365-2, -7, 365-4	wild	Ps8, 10, Ps9	RPIII	AB115678
		Geka (葛卡), Daofu (道孚) Co.	2000.8.9	K. Komatsu et al. QS405	cult.	Ps11	RPI	AB115670
		Geka, Daofu Co.	2000.8.12	K. Komatsu et al. QS479	cult.	Ps12	RPIII	AB115679
		Tagong (塔公), Kangding Co.	2000.8.12	K. Komatsu et al. QS480	cult.	Ps13	RPIII	AB115680
		Xinduqiao (新都橋), Kangding Co.	2000.8.12	K. Komatsu et al. QS481-1	cult.	Ps14	RPIII	AB115680
		Xinlong (新龍) Co.	2001.4.28	H. Cao QS0738	wild	Ps15	RPI	AB115671
		Yulong (玉隆), Dege (德格) Co.	2000.8.11	K. Komatsu et al. QS416-1*, -4*	wild	Ps16*, 17*	RPI	AB115670
		Shiqu (石渠) Co.	2000.8.14	Y. Sahashi QS2	cult.	Ps18	RPIII	AB115680
	Yunnan Prov., China	Heqing (鶴慶), Dali (大理) Co.	1999.7.30	K. Komatsu et al. Y229,Y231, Y232, Y233	cult.	Py19, 20, 21, 22	RPIII	AB115681
		Heqing, Dali Co.	1999.7.30	K. Komatsu et al. Y230	cult.	Py23	RPII	AB115673
		Heqing, Dali Co.	1999.7.30	X. Wang Y1*	cult.	Py24*	RPII	AB115673
	Gansu Prov., China	Li (礼) Co.	2001.7.30	D.Y. Yang G01	cult.	Pg25	RPII	AB115673
		Gezigou (格子溝), Li (礼) Co.	2001.7.30	D.Y. Yang G02-1	cult.	Pg26	RPII	AB115673
		Shuanshui (銓水), Li Co.	2001.7.31	D.Y. Yang G04-1	cult.	Pg27	RPIII	AB115677
		Shuanshui, Li Co.	2001.7.31	D.Y. Yang G04-6	cult.	Pg28	RPIII	AB115677
		Nanyang (南陽), Tanchang (宕昌) Co.	2001.7.31	D.Y. Yang G05-1	cult.	Pg29	RPII	AB115673
		Nanyang, Tanchang Co.	2001.7.31	D.Y. Yang G05-2	cult.	Pg30	RPIII	AB115677
		Chengjiao (城郊), Min (岷) Co.	2001.8.2	D.Y. Yang G06, 07	cult.	Pg31, 32	RPII	AB115673
		Chengjiao, Min Co.	2001.8.2	D.Y. Yang G08-1	cult.	Pg33	RPIII	AB115677
		Hanban (憨班), Zhouqu (舟曲) Co.	2001.8.4	D.Y. Yang G09	cult.	Pg34	RPII	AB115674
		Baidian (白点山), Zhouqu Co.	2001.8.4	D.Y. Yang G10-1, 2, 10-6	wild	Pg35, 36, Pg37	RPII	AB115675
R. tanguticum Maxim. ex Balf.	Qinghai Prov., China	Qunjia (群加), Huangzhong (湟中) Co.	2000.7.17	K. Komatsu et al. QS1	cult.	T1	RT	AB115682
		Mashidang (麻什当), Huangnan (黄南) Co.	2000.7.24	K. Komatsu et al. QS201-1*	wild	T2*	RT	AB115682
		Reshui (熱水), Dulan (都蘭) Co.	2000.8.2	K. Komatsu et al. QS307-1, 307-2*	wild	T3, T4*	RT	AB115683
R. officinale Baillon	Sichuan Prov., China	Fengtong (蜂桶), Wanyuan (万源) Co.	2000.8.19	K. Komatsu et al. QS500-3, QS501-1, 501-3*	cult.	O1, 2, O3*	ROC	AB115684
		Piwo (皮窩), Wanyuan Co.	2000.8.20	K. Komatsu et al. QS510	cult.	O4	ROC	AB115685
		Piwo, Wanyuan Co.	2000.8.20	K. Komatsu et al. QS514-2, -3	wild	O5, 6	ROC	AB115686
R. coreanum Nakai	Toyama Pref., Japan	Tateyama（立山）	2001.7.18	TMPU 82[b)]*	cult.	C*	ROC	AB115687
Section Rhapontica A. Los.								
R. rhaponticum L.	Qinghai Prov., China	Dulan (都蘭) Co.	2000.8.1	K. Komatsu et al. QS306*	cult.	R*	RR	AB115688
R. franzenbachii Münt.	Hebei Prov., China	Anguo (安国)	2002.9.16	K.Komatsu 1*	cult.	F*	RR	AB115689
R. undulatum L.	Ulaanbaatar, Mongolia	Hairthaan	2001.7.23	K. Komatsu et al. M24	wild	U1	RR	AB115690
	Ovorhangay, Mongolia	Gandyn Had	2001.7.29	K. Komatsu et al. M148	wild	U2	RR	AB115690
	Bayanhongor, Mongolia	Suuj Teeg Uul	2001.7.30	K. Komatsu et al. M194*	wild	U3*	RR	AB115691
Section Spiciformia A. Los.								
R. przewalskyi A. Los.	Qinghai Prov., China	Xidatan (西大灘), Geermu (格尔木)	2000.7.29	K. Komatsu et al. QS277-1*	wild	S1*	RS	AB115693
	Qinghai Prov., China	Reshui (熱水), Dulan (都蘭) Co.	2000.8.2	K.Komatsu et al. QS308-2*	wild	S2*	RS	AB115694
Section Acuminata C. Y.								
R. kialense Franch.	Sichuan Prov., China	Yulong (玉隆), Dege (德格) Co.	2000.8.11	K. Komatsu et al. QS417-6*	wild	K*	RK	AB115692

Specimens used for the analysis of 18S rRNA gene sequences are labeled with asterisks (*), whereas those used for the complete *mat*K gene are underlined. a) Each clade was determined on the basis of phylogenetic analysis of the *mat*K gene sequence. b) TMPU, Medicinal Plant Garden, Toyama Medical and Pharmaceutical University, Japan.

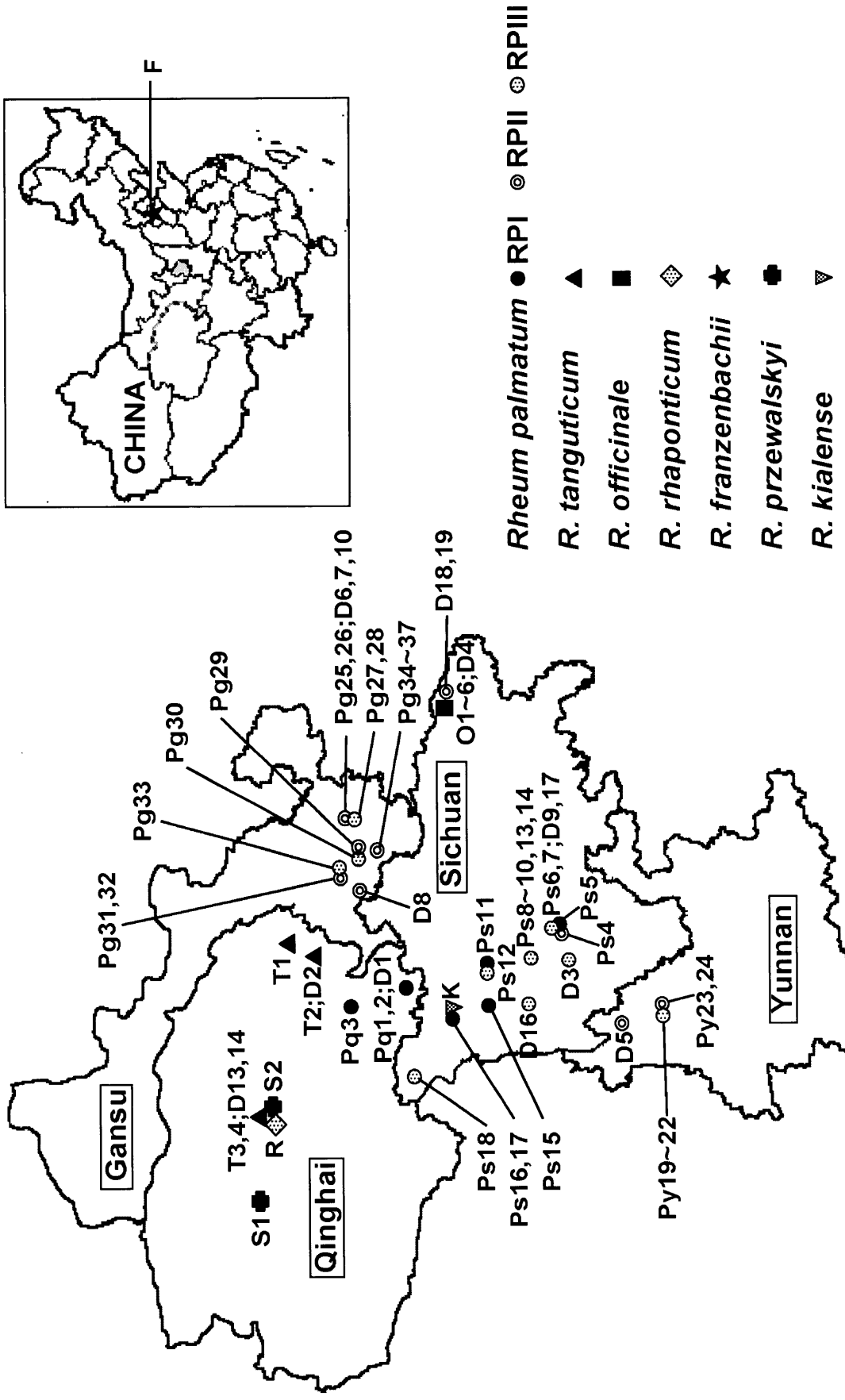

Fig. 1 Locations for Collecting Plant Specimens of *Rheum* Species and Production Areas of Drug Samples of Dahuang
The numerals indicate specimen code numbers shown in Table 2.

Table 3 Comparison of the *mat*K gene sequence among 56 specimens of 9 *Rheum* species and 18 Rhei Rhizoma

Species	Code no.	Nucleotide position																																																										Clade
		141	152	218	222	264	270	302	317	365	367	417	443	451	458	522	531	585	587	612	615	616	619	676	696	707	711	725	739	743	769	791	803	828	838	852	859	883	897	912	918	937	950	951	952	953	954	955	961	984	1032	1049	1061	1072	1112	1114	1246	1257	1354	1416	1497	
Rheum palmatum	Pq1,2; D1	T	G	T	T	G	G	G	C	G	C	A	T	G	T	C	G	G	A	A	A	A	A	G	C	C	A	C	G	C	T	C	T	G	C	A	A	C	G	C	C	C	-	-	-	-	-	-	T	G	C	C	C	G	T	C	C	C	G	T	T	RP
	Pq3; Ps11,16,17	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	C	*	*	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*	*	*	*	RPI
	Ps5,15; D21	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	C	*	*	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*				RPI
	Ps4; D18,19	*	A	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	A	G	*	*	*	*	*	C	*	*	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*	G	*	*	*				RPII
	Py23,Py24,Pg25,26,29,31,32; D5~7,10	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	A	*	*	*	*	*	C	*	*	*	*	*	*	A	G	*	*	*	*	*	C	*	*	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	T	A	*	*	RPII
	Pg34; D8	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	A	G	*	*	*	*	*	C	*	*	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*	*	*	*	RPII
	Pg35,36,37	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	A	G	*	*	*	*	*	C	*	*	A	T	*	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*	*	*	*	RPII
	Ps6	*	*	*	*	*	*	*	T	*	*	*	A	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	A	G	*	*	*	*	*	C	A	*	*	*	*	-	-	-	-	-	-	*	*	*	*	T	*	*	*	*	*				RPIII
	Ps7, Pg27,28,30,33; D9,17	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	A	G	*	*	*	*	*	C	A	*	*	*	*	-	-	-	-	-	-	*	*	*	*	T	*	*	*	*	*	*	*	C	RPIII
	Ps8,9,10; D3	*	*	*	*	A	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	A	G	*	*	*	*	*	C	A	T	*	*	*	-	-	-	-	-	-	*	*	*	*	T	*	*	*	*	*	*	*	*	RPIII
	Ps12	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	G	*	*	*	*	*	C	A	*	*	*	*	-	-	-	-	-	-	*	*	*	*	T	*	*	*	*	*	*	*	*	RPIII
	Ps13,14,18; D16	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	A	G	*	*	*	*	*	C	A	T	*	*	*	-	-	-	-	-	-	*	*	*	*	T	*	*	*	*	*	*	*	*	RPIII
	Py19~22	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	A	*	*	*	*	*	*	C	A	*	*	*	*	-	-	-	-	-	-	*	*	*	*	T	*	*	*	*	*				RPIII
R. tanguticum	T1,2; D2,13,22	*	*	*	*	*	*	*	*	*	T	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	G	*	A	*	*	*	C	*	*	*	*	T	-	-	-	-	-	-	*	*	*	*	*	*	*	T	*	*	*	G	*	RT
	T3,4; D14	*	*	*	*	*	*	*	*	*	T	C	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	G	*	*	*	*	*	C	*	*	*	*	T	-	-	-	-	-	-	*	*	*	*	*	*	*	T	*	*	*	G	*	RT
R. officinale	O1,2,3	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	A	G	*	*	*	*	*	C	*	*	*	*	T	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*	*	G	*	ROC
	O4; D4	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	G	*	*	*	*	*	C	*	*	*	*	T	-	-	-	-	-	-	*	*	*	*	*	*	*	T	*	*				ROC
	O5,6	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	A	G	*	*	T	*	*	C	*	*	*	*	T	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*				ROC
R. coreanum	C	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	G	*	*	*	*	*	C	*	*	*	*	T	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*	*	G	*	ROC
R. rhaponticum	R	*	*	*	*	*	*	*	*	*	*	*	A	A	*	*	*	T	G	*	*	*	C	*	*	G	*	*	*	A	G	*	*	*	A	*	C	*	*	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*				RR
R. franzenbachii	F	*	*	*	*	*	*	*	*	*	*	*	A	A	*	*	*	T	G	*	*	*	C	*	*	G	*	*	*	A	G	T	*	*	A	*	C	*	*	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*	*	*	*	RR
R. undulatum	U1,2	*	*	*	*	*	*	*	*	*	*	*	A	A	*	*	*	T	G	*	*	*	C	*	*	G	*	*	*	A	G	T	*	*	A	*	C	*	*	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*	*	*	T	*				RR
	U3	*	*	*	*	*	*	*	*	*	*	*	A	A	*	*	*	T	G	*	*	*	C	*	T	G	*	*	*	A	G	T	*	*	A	*	C	*	*	*	*	*	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*				RR
R. przewalskyi	S1	*	*	A	G	*	*	T	*	A	*	*	A	*	*	T	*	*	G	*	*	*	C	*	*	G	G	G	*	A	G	T	*	*	A	G	C	*	*	*	*	*	C	A	A	G	A	A	*	*	*	T	*	*	*	*	*	*				RS
	S2	*	*	A	G	*	*	T	*	A	*	*	A	*	*	T	*	*	G	*	*	*	C	*	*	G	G	G	*	A	G	T	*	*	A	G	C	*	*	*	*	*	C	A	A	G	A	A	*	T	*	T	*	*	*	*	*	*				RS
R. kialense	K	A	*	A	G	*	T	*	*	A	*	*	A	*	C	*	*	*	G	T	G	*	C	A	*	G	*	*	A	A	G	T	*	*	A	*	C	A	*	*	*	*	C	A	A	G	A	A	C	*	T	T	*	T	*	*	*	*				RK

Specimens used for the analysis of the complete *mat*K gene sequence are underlined. The enclosed square indicates the partial *mat*K gene region. Hyphens (-) denote alignment gaps. Asterisks (*) indicate sequence identity with *R. palmatum* from Banma Co., Qinghai Prov., China (Pq1). Nucleotide positions indicate the aligned position, starting from the 5' end of the *mat*K gene.

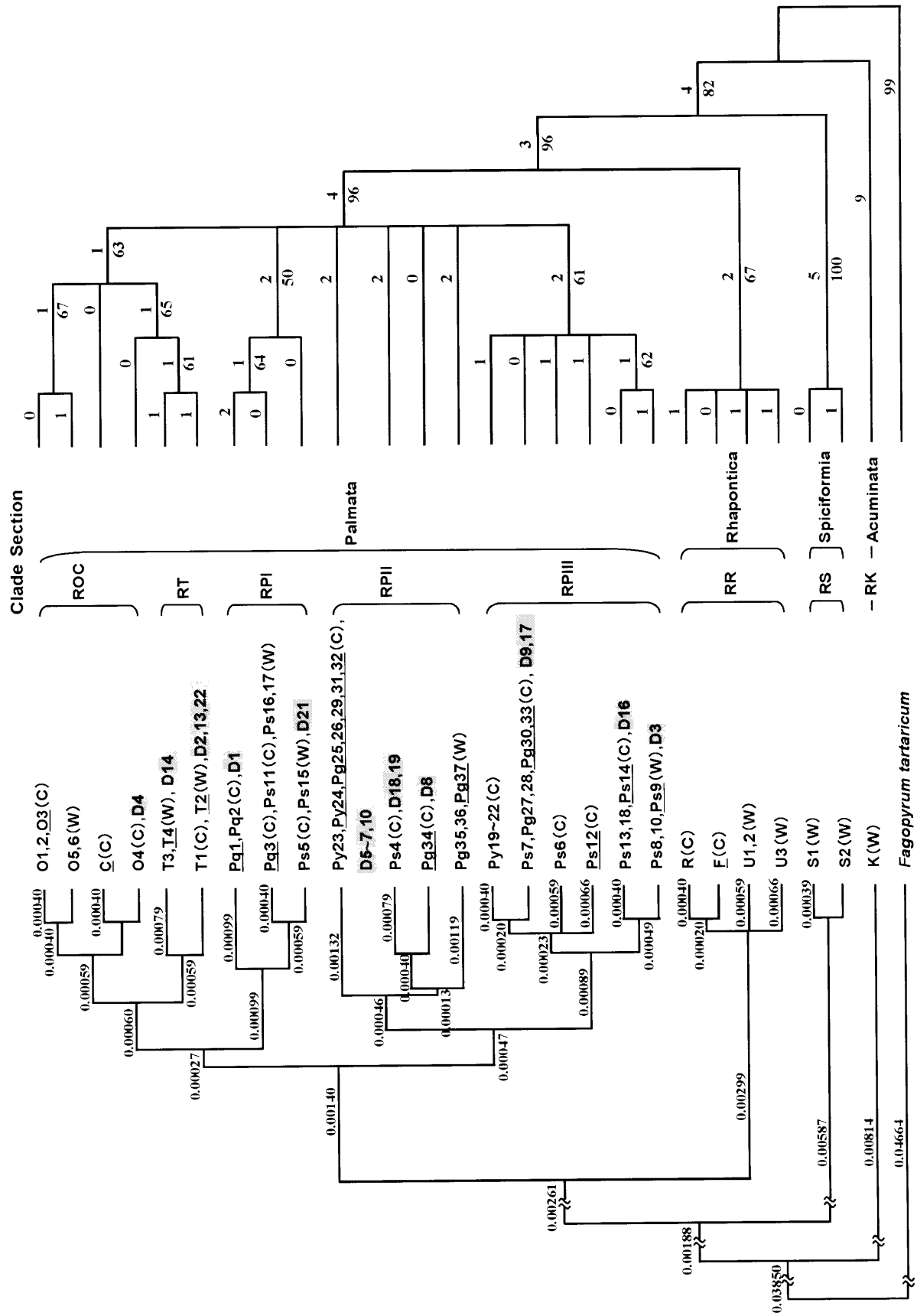

Fig. 2 Phylogenetic Trees of Nine *Rheum* Species Constructed on the Basis of Partial *mat*K Gene Sequences
Left: The phylogenetic tree constructed using the UPGMA method. Branch lengths were calculated using Kimura's two-parameter method and were mapped along each branch. Right: The strict consensus tree constructed on the basis of maximum parsimonious analysis. Tree length = 155, CI = 0.9484, RI = 0.9101. Numbers above the line indicate branch length, and numbers below the line are bootstrap values with 1000 replicates. C, cultivated; W, wild. Drug samples (Rhei Rhizoma) are indicated by shading; specimens used for the analysis of the complete *mat*K gene sequence are underlined.

relationship with the subclade RPI of *R. palmatum*. The strict consensus tree of the 6 most parsimonious trees (Fig. 2: right) showed similar topology to the tree yielded by the UPGMA method. Clade ROC/RT, subclade RPI, and subclade RPIII were supported by bootstrap values of 63%, 50%, and 61%, respectively. Subclade RPII was defined in the tree using the UPGMA method, but was not in the tree using the maximum parsimony method. The phylogenetic trees constructed using complete *mat*K gene sequences of 20 specimens of 5 *Rheum* species revealed similar topology to the trees constructed using the partial *mat*K gene sequences, except position of subclade RPI of *R. palmatum* belonging to the same clade as subclades RPII and RPIII.[23]

2. PCR-RFLP and ARMS analyses of *Rheum* species

The result of sequence comparison of the *mat*K gene revealed that the key nucleotides for identifying section Palmata, including official species, existed at positions 587, 707, and 838, and the marker nucleotides for identifying 3 official species such as *R. palmatum, R. tanguticum* and *R. officinale* were at 367 and 937, while those for distinguishing the 3 intraspecies groups of *R. palmatum* were at 619, 769, 883, and 1061. Based on the above marker nucleotides, PCR-RFLP and ARMS analyses were designed.[25] We found restriction enzyme sites detecting 3 marker nucleotides, one at position 587 which is useful for identifying section Palmata, and another two at positions 769 and 1061 for distinguishing the 3 groups of *R. palmatum* (Fig. 3). On the other hand, for the purpose of identification of *R. palmatum, R. tanguticum* and *R. officinale*, ARMS analysis using 3 sets of designed primers was performed.

Determination of *Rheum* species officially used as Rhei Rhizoma using PCR-RFLP analysis

The partial *mat*K gene region was amplified via PCR using a template DNA and a pair of primers, *mat*K191F and *mat*K8R (Table 4). The PCR products of 6 *Rheum* species were of 1129 bp in length, except for those of *R. przewalskyi* and *R. kialense* of 1135 bp. The restriction enzyme *Bgl* II was found to give diagnostic fragments among the 8 species. The *mat*K gene of 5 species, excluding the 3 official species, had a *Bgl* II restriction site at the nucleotide position 586-591 (Fig. 4A). The PCR products of these 5

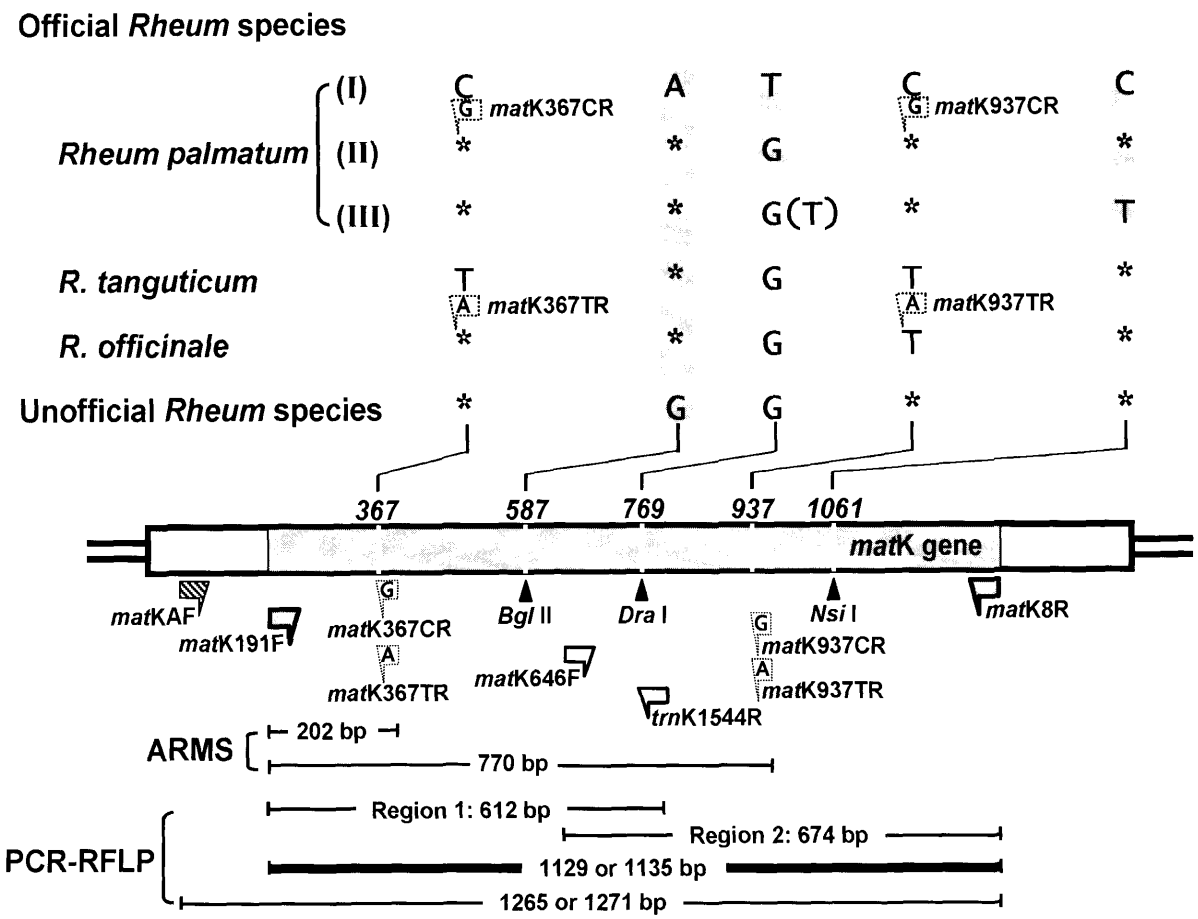

Fig. 3 Positions of Marker Nucleotides, PCR Primers for PCR-RFLP and ARMS Analyses, and Restriction Enzyme Sites on *mat*K Gene
Marker nucleotides used in this study are indicated by numerals in italics above the diagram; nucleotide substitutions with shadows at positions 587, 769 and 1061 were applied to PCR-RFLP analysis, and those at positions 367 and 937 to ARMS analysis. PCR primers for PCR-RFLP analysis are indicated by broad half-arrows, and those for ARMS analysis by dotted broad half-arrows, mapped below the diagram. The lengths of the resulting PCR products are shown at the bottom. For PCR-RFLP analysis, PCR products of 1129 bp (or 1135 bp) obtained from PCR or semi-nested PCR methods were used. The PCR product of 1265 bp was used as a template in semi-nested PCR for drug samples. The PCR products of regions 1 and 2 were used for PCR-RFLP analysis of one drug sample (D21). Important restriction enzyme sites of *Bgl* II, *Nsi* I and *Dra* I used for identifying section Palmata and the distinction of three group of *R. palmatum* are indicated by sharp arrows.

Table 4 Sequences of PCR primers for PCR-RFLP and ARMS analyses

Primer	Sequence (5' to 3')	Length (bp)	Application			
			PCR (plants)	Semi-nested PCR (drugs)	ARMS	PCR (drug D21) (Regions 1, 2)
matKAF	CTA TAT CCA CTT ATC TTT CAG GAG T	25		Step 1-F		
matK191F	TAG TTA TTC GAA TGT ATC AAC AG	23	F	Step 2-F	F	Region 1-F
matK367CR	GAT CGT AAA TTT TGA TAT TTT TTG AG	26			R1	
matK367TR	GAT CGT AAA TTT TGA TAT TTT TTG AA	26			R1'	
matK646F	TCC TAC GTG TGT GAA TGC G	19				Region 2-F
trnK1544R	GGA TAA CCC CAG AAT GCT TAG	21				Region 1-R
matK937CR	ATA TAG ATT CTT TGC AAC CAG AG	23			R2	
matK937TR	GAT ATA GAT TCT TTG CAA CCA GAA	24			R2'	
matK8R	AAA GTT CTA GCA CAA GAA AGT CGA	24	R	R		Region 2-R

F, forward primer; R, reverse primer. R1 and 1' indicate two kinds of reverse primers designed on the basis of nucleotide difference at position 367, whereas R2 and 2' are on the basis of nucleotide difference at position 937. Nucleotides with underlines indicate destabilizing mismatches.

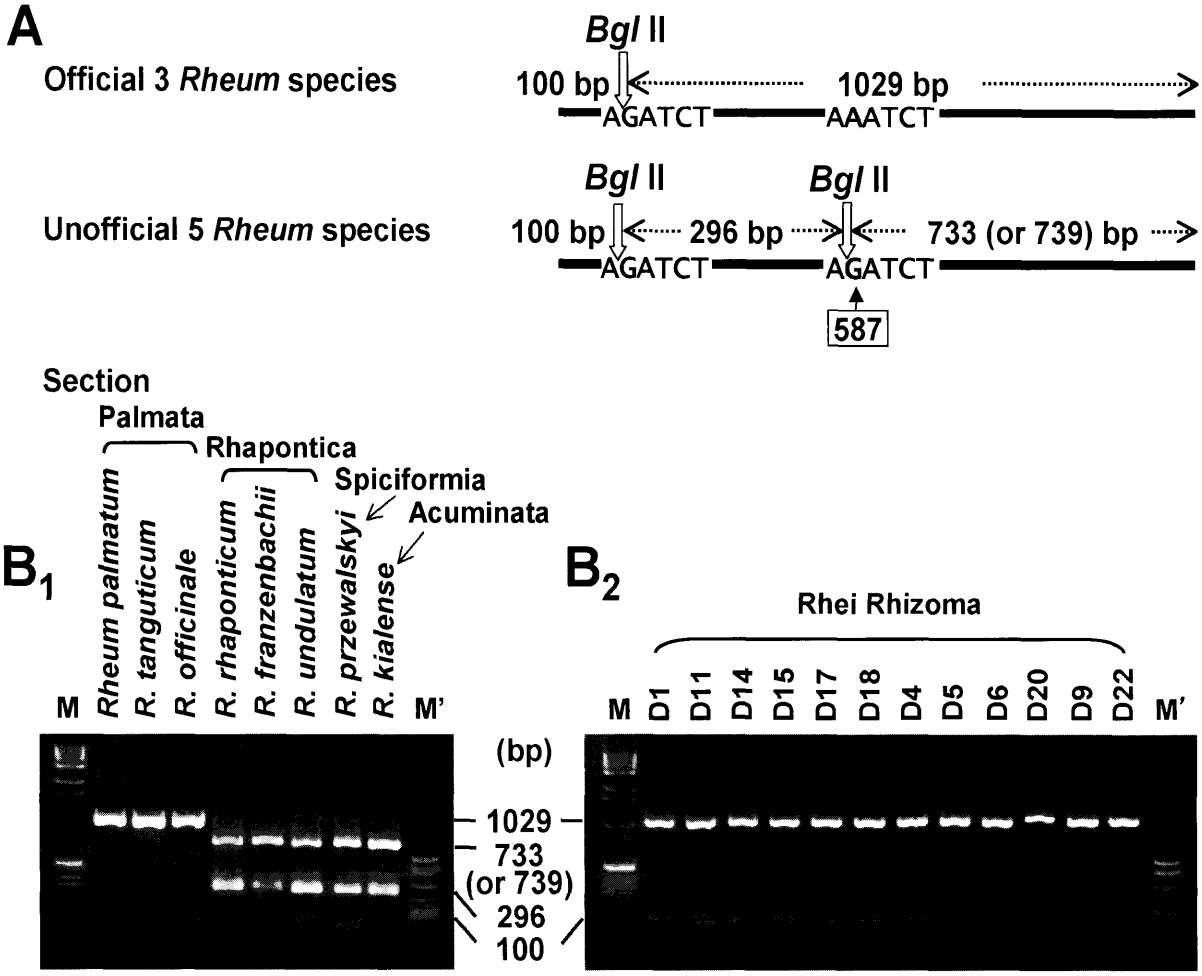

Fig. 4 PCR-RFLP Analysis Using the Restriction Enzyme *Bgl* II on Partial *mat*K Gene

A, *Bgl* II restriction sites in three official *Rheum* species and five other species; nucleotides with shadows indicate the defined identification marker at position 587. B, agarose gel electrophoretograms of PCR products digested with *Bgl* II; 1, eight *Rheum* species; 2, drug samples of Rhei Rhizoma; code numbers of drug samples are shown. Lane M, 1kb DNA ladder; M', pBR 322DNA-*Msp* I digestion.

species digested with *Bgl* II showed 3 fragments of 100, 296 and 733 or 739 bp, while in the 3 official species the products showed 2 fragments of 100 and 1029 bp in electrophoretogram (Fig. 4B$_1$).

Identification of 3 *Rheum* Species using ARMS Analysis

On the basis of the nucleotide substitutions at positions 367 and 937 among the 3 official species, at each position 2 kinds of reverse primers, matK367CR and matK367TR, and matK937CR and matK937TR with complementary 3'-terminal nucleotide were designed (Table 4, Fig. 3).[26] Upon PCR amplification using 3 sets of primers (A, B and C), each consisting of 2 kinds of reverse primers and one forward primer, matK191F (Fig. 5), and template DNA from each species, one or two fragments of 202 bp and/or 770 bp in length or no fragments were observed in the electrophoretogram. When the primer set A was used, one fragment of 202 bp was observed in *R. palmatum*, one fragment of 770 bp in *R. tanguticum*, and both fragments in *R. officinale* (Fig. 5A$_1$). When the primer set B was used, two fragments of 202 and 770 bp were observed in *R. palmatum*, one fragment of 202 bp in *R. officinale*, and no fragments in *R. tanguticum* (Fig. 5A$_2$). When primer set C was used, two fragments were observed in *R. tanguticum*, one fragment of 770 bp in *R. officinale*, and no fragments in *R. palmatum* (Fig. 5A$_3$). By observing 3 kinds of fragment profiles, the 3 official species were authenticated.

Distinction of the 3 groups of *Rheum palmatum*

The PCR products of *R. palmatum* amplified using a pair of primers, matK191F and matK8R were of 1129 bp in length. The restriction enzymes *Nsi* I and *Dra* I were found to give diagnostic fragments among the 3 groups of *R. palmatum* (I, II and III). The *mat*K gene of groups I and II had a *Nsi* I restriction site at the nucleotide position 1058-1063; moreover, that of group I had a *Dra* I restriction site at the position 768-773 (Fig. 6A, 6C). The PCR products of groups I and II digested with *Nsi* I showed two fragments of 263 and 866 bp, whereas that of group III showed one fragment of 1129 bp (Fig. 6B$_1$). On the other hand, the digestion of the PCR products with *Dra* I resulted in the formation of three fragments of 247, 333 and 549 bp in group I, and two fragments of 247 and 882 bp in group II (Fig. 6D$_1$).

3. Application for identification of Rhei Rhizoma

By comparing the sequences of 18 drug samples of Dahuang purchased from Chinese markets near the fields of *Rheum* species and Japanese market with the species-specific sequences of *Rheum* species, their botanic origins and genotypes were determined (Table 5, Fig. 2). Moreover, twenty-two drug samples including above samples were analyzed by using PCR-RFLP and ARMS methods. A semi-nested PCR method was applied using a pair of primers, matKAF and matK8R in the first PCR, and matK191F and matK8R in the second PCR amplifications (Table 4). The PCR products of 1129 bp in length were obtained except for one sample (D21) with no product. The digestion of the products with the restriction enzyme *Bgl* II resulted in the

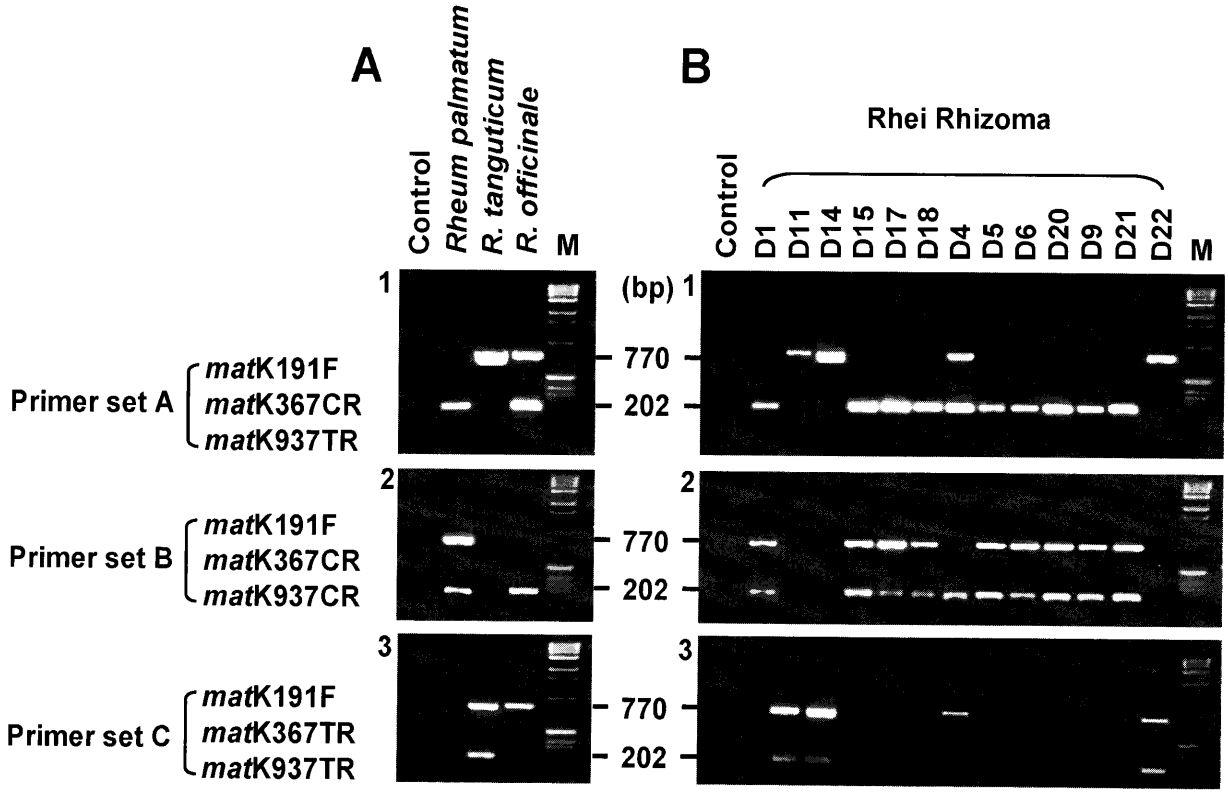

Fig. 5 ARMS Analysis Using Three Sets of Primers on Partial *mat*K Gene
PCR amplification with primer sets A, B and C was carried out using the DNA of each material as a template. A, three official *Rheum* species; B, drug samples of Rhei Rhizoma derived from official species; code numbers of drug samples are shown. 1, 2 and 3, fragment patterns when primer sets A, B and C were used, respectively. Control, blank without DNA templates; Lane M, 1kb DNA ladder.

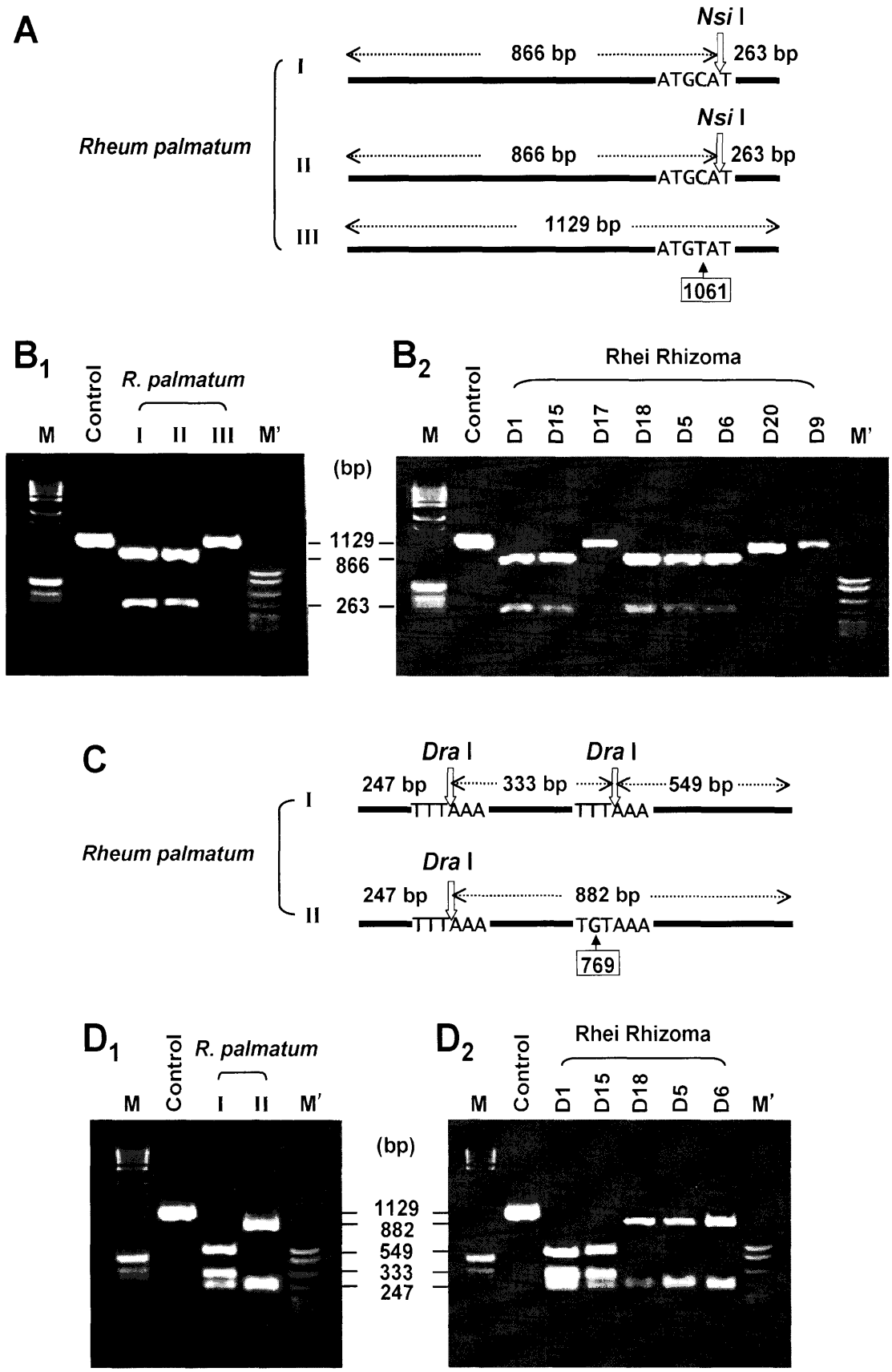

Fig. 6 PCR-RFLP Analysis Using the Restriction Enzyme *Nsi* I or *Dra* I on Partial *mat*K Gene

A, *Nsi* I restriction site in three groups of *Rheum palmatum*; nucleotides with shadows indicate the defined identification marker at position 1061. B, agarose gel electrophoretograms of PCR products digested with *Nsi* I; 1, three groups of *R. palmatum*; 2, drug samples of Rhei Rhizoma; code numbers of drug samples are shown. C, *Dra* I restriction sites in two groups of *R. palmatum*; nucleotides with shadows indicate the identification marker at position 769. D, agarose gel electrophoretograms of PCR products digested with *Dra* I; 1, two groups of *R. palmatum*; 2, drug samples of Rhei Rhizoma. Lane M, 1kb DNA ladder; M', pBR 322DNA-*Msp* I digestion.

Table 5 Rhei Rhizoma used in this study and their Botanic Origins

Herbal drug name	Market	Production area	Date of collection	TMPW no.[a)]	Code no.[b)]	Botanic origin (group)
Dahuang (大黄)	Banma (班瑪) County, Qinghai (青海), China	Banma County, Qinghai	2000. 7	20061	D1	*Rheum palmatum* (I)
Dahuang	Tongren (同仁) County, Qinghai, China	Huangnan (黄南) County, Qinghai	2000. 7	20065	D2	*R. tanguticum*
Dahuang	Tongren County, Qinghai, China	Huangnan County, Qinghai	2000. 7	20066	D11*	*R. tanguticum*
Dahuang	Dulan (都蘭) County, Qinghai, China	Dulan County, Qinghai	2000. 8	20107	D12*	*R. tanguticum*
Dahuang	Dulan County, Qinghai, China	Dulan County, Qinghai	2000. 8	20108	D13	*R. tanguticum*
Dahuang	Dulan County, Qinghai, China	Dulan County, Qinghai	2000. 8	20109	D14	*R. tanguticum*
Dahuang	Ganzi (甘孜) County, Sichuan (四川), China	Ganzi County, Sichuan	2000. 8	20218	D15*	*R. palmatum* (I)
Dahuang	Kangding (康定) County, Sichuan, China	Jiulong (九龍) County, Sichuan	2000. 8	20216	D3	*R. palmatum* (III)
Yin-huang (陰黄)	Yaan (雅安) County, Sichuan, China	Litang (理塘) County, Sichuan	2001. 2	20574	D16	*R. palmatum* (III)
Yin-kang-huang (陰康黄)	Yaan County, Sichuan, China	Shimian (石棉) County, Sichuan	2001. 2	20573	D17	*R. palmatum* (III)
Dahuang	Wanyuan (万源) County, Sichuan, China	Wanyuan County, Sichuan	2000. 8	20266	D18	*R. palmatum* (II)
Dahuang	Wanyuan County, Sichuan, China	Wanyuan County, Sichuan	2000. 8	20267	D4	*R. officinale*
Mati-dahuang (馬蹄大黄)	Wanyuan County, Sichuan, China	Wanyuan County, Sichuan	1987. 10	07422	D19	*R. palmatum* (II)
Dahuang	Zhongdian (中甸) County, Yunnan (雲南), China	Zhongdian County, Yunnan	1999. 7	19535	D5	*R. palmatum* (II)
Ba-cheng-ji (八成吉)	Li (礼) County, Gansu (甘粛), China	Li County, Gansu	2001. 7	20926	D6	*R. palmatum* (II)
Tong-huo (統貨)	Li County, Gansu, China	Li County, Gansu	2001. 7	20928	D7	*R. palmatum* (II)
Dahuang	Min (岷) County, Gansu, China	Diebu (迭部) County, Gansu	2001. 8	20931	D8	*R. palmatum* (II)
Dahuang	Zhouqu (舟曲) County, Gansu, China	Zhouqu County, Gansu	2001. 8	20932	D20*	*R. palmatum* (III)
Dahuang	Uchida Wakanyaku Co., Ltd., Tokyo, Japan	Shimian County, Sichuan	2000. 4	19928	D9	*R. palmatum* (III)
Liu-cheng-ji (六成吉)	Uchida Wakanyaku Co., Ltd., Tokyo, Japan	Li County, Gansu	2000. 3	19927	D10	*R. palmatum* (II)
Ya-huang (雅黄)	Tochimoto Tenkaido Co., Ltd., Osaka, Japan	Sichuan	2000. 5	19948	D21	*R. palmatum* (I)
Bao-huang (包黄)	Tochimoto Tenkaido Co., Ltd., Osaka, Japan	Qinghai	2000. 5	19949	D22	*R. tanguticum*

a) The specimen reference number of the Museum of Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University (TMPW).

b) Drug samples only analysed with PCR-RFLP and ARMS methods are labeled with asterisks (*).

formation of two fragments of 100 and 1029 bp (Fig. 4B$_2$), suggesting that the 21 samples were derived from official *Rheum* species of section Palmata. In the ARMS method using 3 sets of primers, six drug samples from Qinghai Province showed the same fragment patterns as those of *R. tanguticum* (Fig. 5), one sample from Wanyuan County, Sichuan Province, as those of *R. officinale*, and the remnants, as those of *R. palmatum*, respectively. As for the last 14 samples, their PCR products were digested with the restriction enzyme *Nsi* I or *Dra* I. The PCR products of 5 drug samples, mostly from Sichuan Province, digested with *Nsi* I showed one fragment (Fig. 6B$_2$), whereas those of the other 9 samples showed two fragments, which suggested that the former had originated from group III and the latter from groups I or II of *R. palmatum*, respectively. The digestion of the PCR products of the latter samples with *Dra* I resulted in the formation of three fragments in 2 samples from Banma County, Qinghai Province and Ganzi County, Sichuan Province (Fig. 6D$_2$), and two fragments in 7 samples from mostly Gansu and Yunnan Provinces, suggesting that the former had originated from group I and the latter from group II of *R. palmatum*, respectively. In the case of one sample (D21), the partial *mat*K gene region was divided into two regions, and PCR amplification of both regions using a pair of primers, matK191F and trnK1544R or matK646F and matK8R (Fig. 3) was performed to give the expected products of 612 and 674 bp, respectively. The PCR product of region 1 digested with *Bgl* II showed two fragments of 100 and 512 bp, which was the same as in the 3 official *Rheum* species (Fig. 7A). The result of ARMS analysis suggested that this sample was derived from *R. palmatum* (Fig. 5B). Then, the PCR product of region 2 was digested with *Nsi* I or *Dra* I. Two fragments of 263 and 411 bp and those of 125 and 549 bp were observed in the digestion with *Nsi* I and *Dra* I, respectively, which were the same as in group I of *R. palmatum* (Fig. 7B, 7C).

The botanic origins of all 22 drug samples were identified by using PCR-RFLP and ARMS methods. The results of 18 samples out of them were completely consistent with those demonstrated in the sequence analysis, suggesting the accuracy of a new convenient identification method. Although their botanic origins almost corresponded with those deduced from information about their production areas, two drug samples, Mati-dahuang and Dahuang (D18 and D19) from Wanyuan County, Sichuan Province and one sample, Ya-huang from Japanese market were found to be group II and group I of *R. palmatum*, not as expected from *R. officinale* and group III of *R. palmatum*, respectively.[27]

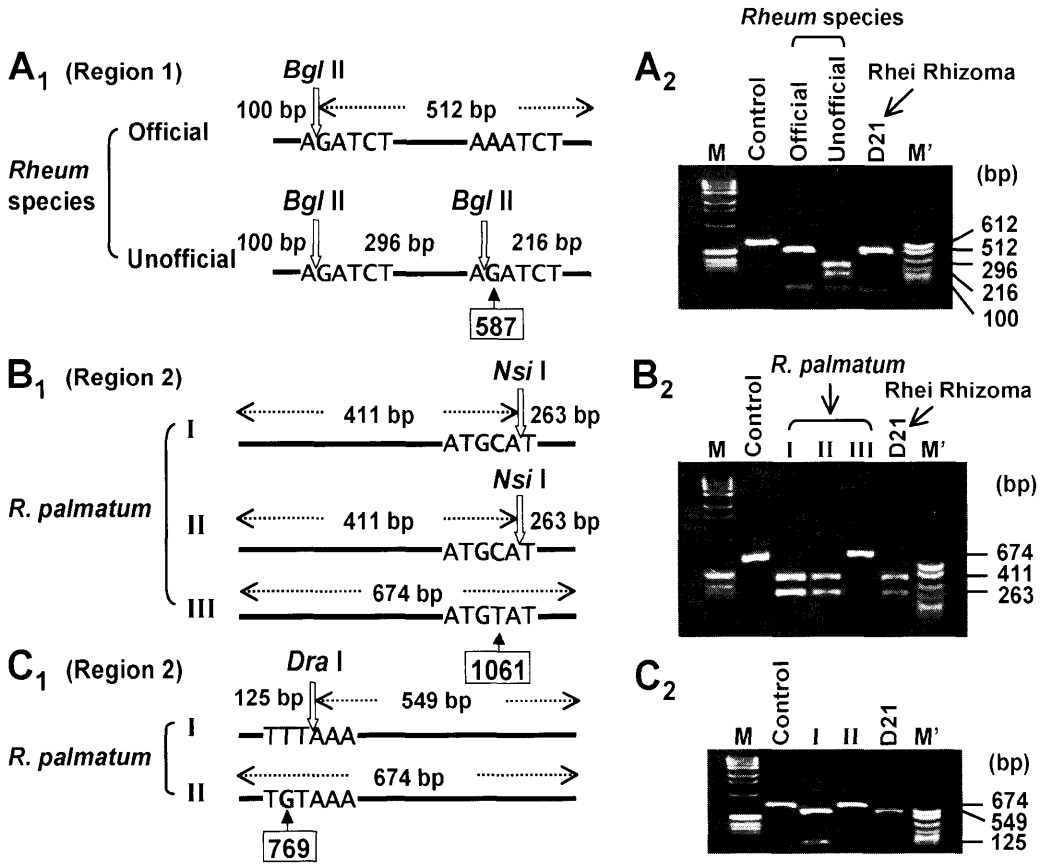

Fig. 7 PCR-RFLP Analysis of One Drug Sample (D21) Using the Restriction Enzyme *Bgl* II, *Nsi* I or *Dra* I on Partial *mat*K Gene

A, PCR products of region 1 (612 bp), amplified using DNAs from eight *Rheum* species (three official and five unofficial species) and one drug sample as a template, were digested with *Bgl* II. B and C, PCR products of region 2 (674 bp) were amplified using DNAs from three groups of *R. palmatum*, and one drug sample as template, were digested with *Nsi* I (B) or *Dra* I (C). 1, restriction sites of *Bgl* II, *Nsi* I or *Dra* I; 2, agarose gel electrophoretograms of the PCR products digested with three restriction enzymes; nucleotides with shadows indicate the defined identification markers at position 587 (A), 1061 (B) or 769 (C). Lane M, 1kb DNA ladder; M', pBR 322DNA-*Msp* I digestion.

The partial *mat*K gene sequence of the former was identical to that of a cultivated plant specimen of Heping, Shimian County (Ps4), while that of the latter to a wild plant specimen of Xinlong County (Ps15), both in Sichuan Province (Table 2). These might be the result of the migration of seeds or the movement of production area.

Conclusion

The *mat*K gene sequences of 9 *Rheum* species, especially of the 3 official species were elucidated. Accordingly, not only interspecies but also intraspecies nucleotide substitutions were observed. Since the *mat*K gene sequence of genus *Panax* of family Araliaceae was stable intraspecies,[17] the phenomena observed in *Rheum* species may be related to their self-incompatibility. However, plants growing in neighboring areas had the same or similar *mat*K gene sequences as usual, due to the maternal inheritance of the *mat*K gene. Nevertheless, the wide range of sequence variations existing in *R. palmatum* suggested that several types of *R. palmatum* may be distributed in China. Based on complete and partial *mat*K gene sequences, the specimens of *R. palmatum* were divided in the phylogenetic tree into 3 groups under the subclades RPI, RPII, and RPIII, which were thought to be demarcated by their distribution areas. Generally, specimens from the southeastern part of Qinghai to the northwestern part of Sichuan were classified in subclade RPI, those from Gansu were in subclade RPII, and those from the central to southern part of Sichuan were in subclade RPIII. The phylogenetic analysis demonstrated a closer relationship between *R. tanguticum* and *R. officinale*. The *mat*K gene sequence was valuable in grouping *R. palmatum* besides in identifying *Rheum* species, and could be used as an index for quality evaluation of Rhei Rhizoma.

By the comparison of *mat*K gene sequence, the key nucleotides for identifying section Palmata including the official species, for identifying 3 official species, and for distinguishing the 3 intraspecies groups of *R. palmatum* were clarified. On the basis of 5 marker nucleotides at positions 367, 587, 769, 937, and 1061 among them, PCR-RFLP and ARMS analyses were investigated to develop a convenient and efficient identification method. PCR-RFLP methods using restriction enzymes *Bgl* II, and *Nsi* I or *Dra* I were developed for identifying section Palmata and for identifying the 3 groups of *R. palmatum*, respectively. For the identification of 3 official species, ARMS method was developed using 3 sets of primers, each having two kinds of species-specific reverse primers designed on the basis of nucleotide differences at positions 367 and 937. The plant specimens were analyzed using the PCR-RFLP and ARMS methods to give the expected products in an electrophoretogram. These methods were also applied to drug samples of Rhei Rhizoma. The ARMS method was found to be valuable in identifying the species of all drug samples. On the other hand, in the PCR-RFLP method, a semi-nested PCR method was required to obtain PCR products before RFLP method. Moreover, it was understood that PCR products were easily obtained in dividing two regions of the *mat*K gene, that is, shorter region than 700 bp. As the result, the botanic origins of all 22 drug samples were identified to be official *Rheum* species, *R. tanguticum*, *R. officinale* or the 3 groups of *R. palmatum*. Moreover, the production areas of drug samples could be deduced.

Quality evaluation based on chemical constituents of the drug samples of Rhei Rhizoma unambiguously authenticated by molecular analysis has been performed, and resulted that each sample belonging to the same subclade showed a similar pattern of constituents. The relationship between genotypes and chemical constituents will be reported in the forthcoming article.

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References

1) Tao, H. J., Mori, T.: Ben Cao Jing Ji Zhu (revised ed.). Vol. 5, South Osaka Printing Center, Osaka, p. 1, 1973.
2) Namba, T.: The Encyclopedia of Wakan-Yaku (Traditional Sino-Japanese Medicines) with Color Pictures. Vol. I, Hoikusha Publishing Co., Ltd., Osaka, pp. 16-20, 1993.
3) Jiangsu New Medical College ed.: Chinese Materia Medica, Shanghai Sci. and Tech. Pub. House, Shanghai, pp. 102-108, 1977.
4) Chinese Pharmacopoeia Commission ed.: Pharmacopoeia of The People's Republic of China. Vol. 1, Chemical Industry Press, Beijing, pp. 18-19, 2000.
5) The Society of Japanese Pharmacopoeia ed.: The Japanese Pharmacopoeia (14th ed.). Part I, Hirokawa Publishing Co., Ltd., Tokyo, pp. 1026-1027, 2001.
6) Lou, Z. C., Wang, X., Mikage, M., Namba, T.: Pharmacognostical studies on the Chinese crude drug "Da-huang (Rhubarb)"(I) Botanical origin of the official Da-huang. *Shoyakugaku Zasshi,* **42**, 291-301, 1988.
7) Kariyone, T.: Pharmacognosy. Hirokawa Publishing Co., Ltd., Tokyo, pp. 79-86, 1988.
8) Goto, M., Kawanishi, F.: Production and resources of Rhubarb. *The Journal of Traditional Sino-Japanese Medicine,* **12**, 74-79, 1991.
9) Wang, X., Lou, Z. C., Mikage, M., Namba, T.: Pharmacognostical studies on the Chinese crude drug "Da-huang (Rhubarb)" (II) Botanical origin of three unofficial Da-huang. *Shoyakugaku Zasshi,* **42**, 302-309, 1988.
10) Mizukami, H., Okabe, Y., Kohda, H., Hiraoka, N.: Identification of the crude drug Atractylodes Rhizome (Byaku-jutsu) and Atractylodes Lancea Rhizome (So-jutsu) using chloroplast *trn*K sequence as a

molecular marker. *Biol. Pharm. Bull.,* **23**, 589-594, 2000.
11) Komatsu, K., Zhu, S., Fushimi, H., Qui, T. K., Cai, S. Q., Kadota, S.: Phylogenetic analysis based on 18S rRNA gene and *mat*K gene sequences of *Panax vietnamensis* and five related species. *Planta Med.,* **67**, 461-465, 2001.
12) Yang, M., Zhang, D., Liu, J., Zheng, J.: A molecular marker that is specific to medicinal Rhubarb based on chloroplast *trn*L/*trn*F sequences. *Planta Med.,* **67**, 784-786, 2001.
13) Hu, J. M., Lavin, M., Wojciechowski, M. F. and Sanderson, M. J.: Phylogenetic systematics of the tribe Millettieae (Leguminosae) based on chloroplast *trn*K/*mat*K sequences and ITS implications for evolutionary patterns in Papilionoideae. *Am. J. Bot.,* **87**, 418-430, 2000.
14) Ohsako, T. and Ohnishi, O.: Intra- and interspecific phylogeny of wild *Fagopyrum* (Polygonaceae) species based on nucleotide sequences of noncoding regions in chloroplast DNA. *Am. J. Bot.,* **87**, 573-582, 2000.
15) Sugita, M., Shinozaki, K. and Sugiura, M.: Tobacco chloroplast $tRNA^{LYS}$ (UUU) gene contains a 2.5-kilobase-pair intron: an open reading frame and a conserved boundary sequence in the intron. *Proc. Natl. Acad. Sci.,* **82**, 3557-61, 1985.
16) Hagemann, R., Schroder, M. B.: The cytological basis of the plastid inheritance in angiosperms. *Protoplasma,* **152**, 57-64, 1989.
17) Zhu, S., Fushimi, H., Cai, S. Q. and Komatsu, K.: Phylogenetic relationship in the genus *Panax*: inferred from chloroplast *trn*K gene and nuclear 18S rRNA gene sequences. *Planta Med.,* **69**, 647-653, 2003.
18) Sekiya, T., Fujinaga, S.: Protein, Nucleic Acid and Enzyme. Vol. 41, No.5, pp. 546-548, pp. 564-567, 1996.
19) Fushimi, H., Komatsu, K., Isobe, M. and Namba, T.: Application of PCR-RFLP and MASA analyses on 18S ribosomal RNA gene sequence for the identification of three Ginseng drugs. *Biol. Pharm. Bull.,* **20**, 765-769, 1997.
20) Sasaki, Y., Fushimi, H., Cao, H., Cai, S. Q. and Komatsu, K.: Sequence analysis of Chinese and Japanese *Curcuma* drugs on the 18S rRNA gene and *trn*K gene and the application of Amplification-Refractory Mutation System Analysis for their authentication. *Biol. Pharm. Bull.,* **25**, 1593-1599, 2002.
21) Li, A. R.: Flora Reipublicae Popularis Sinicae. Tomus 25, Science Press, Beijing, pp. 167-209, 1998.
22) Lee, T. B.: Illustrated Flora of Korea. Hyangmoonsa Publishing Co., Korea, p. 299, 1993.
23) Yang, D. Y., Fushimi, H., Cai, S. Q., Komatsu, K.: Molecular analysis of *Rheum* species used as Rhei Rhizoma based on the chloroplast *mat* K gene sequence and its application for identification. *Biol. Pharm. Bull.,* **27**, 375-383, 2004.
24) Ooi, K., Endo, Y., Yokoyama, J., Murakami, N.: Useful primer designs to amplify DNA fragments of the plastid gene *mat*K from angiosperm plants. *J. Jpn. Bot.,* **70**, 328-331, 1995.
25) Yang, D. Y., Fushimi, H., Cai, S. Q., Komatsu, K.: Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) and Amplification Refractory Mutation System (ARMS) analyses of medicinally used *Rheum* species and their application for identification of Rhei Rhizoma. *Biol. Pharm. Bull.,* **27**, 661-669, 2004.
26) Mcpherson, M. J., Hames, B. D., Taylor, G. R.: PCR 2 (A Practical Approach). Oxford University Press Inc., New York, pp. 224-227, 1995.
27) Department of Pharmacognosy, Nanjing College of Pharmacy ed.: Materia Medica. The People's Publishing House, Beijing, p. 388, 1960.