—Original— Natural Medicines 55 (1), 17-22 (2001)

Evaluation of *Valeriana* Plants and Their Improvement for Crude Drug Production

1. Phylogenetic Relationship in The Genus *Valeriana* as Revealed by RAPD Markers

Miwa Takeuchi [*,a], Shoichi Suzuki [b], Hiroharu Fujino [c], Munehisa Arisawa [c], Hiroshi Kohda [a]

[a] *The Experimental Station of Medicinal Plant, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan,* [b] *Department of Agricultural Science, Ishikawa Agricultural College, 1-308 Suematsu, Nonoichi-machi, Ishikawa-gun, Ishikawa 921-8836, Japan,* [c] *Herbal Garden, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan*

(Received September 21, 2000)

Genomic DNAs from fresh leaves of *Valeriana fauriei* Briquet ecotypes and the relating species were studied by random amplified polymorphic DNA (RAPD) analysis.

Species- or ecotype-specific RAPD markers were obtained from 13 of the 16 species and ecotypes used in this study. Quite a large number of markers were observed in *V. wallichii* and *V. fauriei* ecotype Akkado.

The dendrogram revealed that the ecotypes of *V. fauriei* (except Akkado) were clearly distinguishable from *V. officinalis* and *V. wallichii*. Interestingly, Akkado was found to be a unique ecotype ; *V. officinalis* was more closely related to the other ecotypes of *V. fauriei* than Akkado.

It was found that RAPD markers can be used to study the genetic relationship of *Valeriana*.

Keywords : *Valeriana fauriei* ; *Valeriana officinalis* ; *Valeriana wallchii* ; *Valerianaceae* ; RAPD ; Polymerase Chain Reaction (PCR) ; Unweighted Paired Group Method with Arithmetic Average (UPGMA)

The crude drug Japanese Valerian "Kanokoso" is defined as the root and rhizome of *Valeriana fauriei* Briquet (*Valerianaceae*) or the relating plants in the Pharmacopoeia of Japan[1]. It has been used for sedative and antispasmodic purposes[1].

V. fauriei is a perennial plant, distributed in Northeastern district of China, the Korean Peninsula, Sakhalin, the Southern Kurile, Japan and Taiwan[1]. In Japan, this species grows in the moist glass land at the foot of mountains from Hokkaido to Kyushu[1]. The crude drug "Japanese Valerian" on the market is originated from the ecotype Hokkaikisso (*V. fauriei* forma *yezoensis* Hara). Two cultivars, "Botan" and "Tako" are known in this ecotype[1]. In Europe, the rhizomes and roots[1] of *V. officinalis* or its ecotypes are used as a crude drug "Valeriana root" and in India, some relating plants including *V. officinalis* and *V. wallichii* are cultivated and used for the same purpose[1]. Interestingly, in *V. fauriei*, *V. officinalis* and *V. wallichii*, different constituents are considered to be responsible for the sedative action[2, 3]. The characteristic terpenes in *V. fauriei*, such as kessyl glycol diacetate (KGD), kessyl glycol-2-acetate (KG2A), kessyl glycol-8-acetate (KG8A) and α

-kessyl acetate (KAC), are not detected in *V. officinalis* and *V. wallichii*[2, 3] and the sedative action of *V. officinalis* and *V. wallichii* is reported to be due to an iridoid mixture, valepotriates[4, 5, 6]. Intraspecific variations were found among the *V. fauriei* plants in the morphological characters, response for environmental condition, yields of essential oil[7], and the amounts of constituents. Especially, marked differences were noted in the KG2A, KG8A and KGD contents[8-16]. To obtain reproducible medicinal effects of this drug, the uniformity of its active constituents is essential. Phylogenetic systematics of *V. fauriei* has been studied on the basis of the morphological and chemical characteristics[7]. These approaches are expensive and time-consuming, since cultivation and chemical analysis are involved.

In this study, we obtained information on the genetic diversity among *Valeriana* plants using RAPD analysis. We also searched the specific RAPD markers on each ecotype or species of *Valeriana* plants for the breeding program.

MATERIALS AND METHODS

Plant materials Sixteen species and ecotypes of *Valeriana* plants were used in this study. Ten of them were collected in various habitats and six were from botanical institutes, as listed in Table 1. All these plants were then grown in the field of Herbal garden, Toyama Medical and Pharmaceutical University.

Preparation of genomic DNA Genomic DNA was extracted from fresh leaves (about 0.5g) by the modified cetyltrimetylammonium bromide (CTAB) method[17]. The followings are the modified points to improve the extraction efficiency. ① Increase of the amount of CTAB solution (2×CTAB extraction buffer 1 ml/gFW → 10 ml/gFW). ② Extension of incubation period (2×CTAB extraction buffer, 65 ℃, 3 min. → 15 min. CTAB precipitation buffer, 65 ℃, 1 min. → 10 min.). ③ Increase of the amount of 98 % ethanol (100 μl → 500 μl). ④ Finally, RNase treatment was performed (37 ℃, 1hr).

The quality of extracted DNA was assessed by visual inspection on 0.8 % agarose gel, and the concentration of DNA was measured by absorbance at 260 nm (A_{260}). The purity of extracted DNA was determined by the ratio, A_{260}/A_{280}. The DNA solution for the template of PCR was adjusted to 12.5 ng/μl with 1/2 TE buffer (5 mM Tris-HCl, 0.5 mM ethylenediamine-N, N, N', N'-tetraacetic acid, disodium salt, dihydrate (Na_2EDTA) (pH 8.0)).

RAPD analysis PCR was performed according to the modified method of Williams *et al*[18]. The reaction mixture (25 μl) for PCR was composed of 10 mM

Table 1. Origin of 16 samples of *Valeriana*

Species or ecotypes	Origin
Valeriana fauriei	
Ezokanokoso	Cultivated in Hokkaido Experimental Stations for Medicinal Plants.
Hokkaikisso	Cultivated in Hokkaido Experimental Stations for Medicinal Plants.
Akkado	Collected in Akkado, Iwaizumi, Iwate Prefecture.
Tobishima	Collected in Tobishima, Sakata, Yamagata Prefecture and cultivated in Herbal Garden, Tohoku University.
Ohiwa	Collected in Kamaike, Kamiichi, Toyama Prefecture.
Tateyamaonsen	Collected in Tateyamaonsen, Ohyama, Toyama Prefecture.
Midagahara	Cultivated in Midagahara, Tateyama, Toyama Prefecture.
Tengudaira	Collected in Tengudaira, Tateyama, Toyama Prefecture.
Ibukiyama	Collected in Mt. Ibuki, Shiga Prefecture.
Himehara	Collected in Himehara, Niimi, Okayama Prefecture.
Ishidateyama	Collected in Mt. Ishidate, Monobe, Kohchi Prefecture.
Hiraodai	Collected in Hiraodai, Kitakyushu, Fukuoka Prefecture.
Wakasugiyama	Collected in Mt. Wakasugi, Sasaguri, Fukuoka Prefecture.
Kumamura	Collected in Sakaguchi, Kuma, Kumamoto Prefecture.
V. officinalis	Cultivated in Tsukuba Experimental Stations for Medicinal Plants.
V. wallichii	Cultivated in Royal Botanical Garden, Godawari, Nepal.

Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.001 % gelatin (TAKARA), 100 μM dNTPs (TAKARA), 0.4 μM primer (OPERON), 50 ng of genomic DNA and 0.5 units of Ampli *Taq* DNA polymerase (TAKARA). The thermal cycling was as follows ; 2 minutes at 94 ℃ for initial denaturation, 40 cycles of 1 minute at 94 ℃, 2 minutes at 45 ℃ and 2 minutes at 72 ℃, 5 minutes at 72 ℃ for final extension. Forty decamer primers (OPERON) were used for PCR amplification (Table 2.). The amplified products were subjected to electrophoresis at 50 V in 1.6 % agarose gels with 1× Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM sodium acetate, 2.0 mM Na_2 EDTA (pH 8.0)). After staining with ethidium bromide (1 μg/ml), DNA bands were visualized under UV light (320 nm). Amplification was repeated 3 to 10 times for each primer to confirm the reproduction.

Table 2. List of primers produced RAPD

	Number of bands		
Primer*	Total	Monomorphic	Polymorphic
OPA-01	18	1	17
OPA-02	10	0	10
OPA-03	11	1	10
OPA-04	12	0	12
OPA-05	9	1	8
OPA-06	20	0	20
OPA-07	11	0	11
OPA-08	13	0	13
OPA-09	7	2	5
OPA-10	14	1	13
OPA-11	12	0	12
OPA-12	10	0	10
OPA-13	11	2	9
OPA-14	10	0	10
OPA-15	11	1	10
OPA-16	16	1	15
OPA-17	16	1	15
OPA-18	11	2	9
OPA-19	11	1	10
OPA-20	8	1	7
OPO-01	17	2	15
OPO-02	16	0	16
OPO-03	13	1	12
OPO-04	12	2	10
OPO-05	11	2	9
OPO-06	13	0	13
OPO-07	15	1	14
OPO-08	11	0	11
OPO-09	6	0	6
OPO-10	3	1	2
OPO-12	13	0	13
OPO-13	6	2	4
OPO-14	10	1	9
OPO-15	11	1	10
OPO-16	8	1	7
OPO-18	14	0	14
OPO-19	11	0	11
OPO-20	13	0	13
Total	444	29	415

* : OPA and OPO, Operon Technologies, Alameda, Calif., USA

We chose 38 primers out of the 40 primers used. The band patterns were visually scored for the presence/absence matrix. Only the distinct bands with small differences in the staining intensities were counted, excepting the bands appeared in the negative control (genomic DNA free). Similarity values used for the UPGMA clustering analysis were calculated on the basis of the proportion of common bands, and a dendrogram was prepared[19, 20].

RESULTS AND DISCUSSION

High quality DNAs were obtained in good yield, which had a clear absorption peak at UV 260 nm with an admissible value of A_{260}/A_{280} (about 1.8 ～ 2.1).

Of the 40 primers used in this experiment, 38 primers gave interpretable and reproducible results. OPO-11 gave unsatisfactory results, and OPO-17 did not amplify the DNA products. Examples of the RAPD pattern (primer : OPO-07, OPO-18, OPA-01) are indicated in Fig. 1. Three to twenty bands per primer were suitable for the data analysis. The numbers of DNA bands from each primer, and then polymorphic and monomorphic bands are given in Table 2. A total of 444 bands was produced from these primers, ranging in size from 220 to 3450 base pairs (bp). Most of the obtained bands (415 ; 93 %) were polymorphic. Fig. 1. (1) (primer : OPO-07) shows that the bands at 1650 bp and 690 bp are "present" in the RAPD pattern of *V. wallichii* and that the band at 1180 bp observed in 15 species or ecotypes is specifically "absent" in Ishidateyama. Fig. 1. (2) (primer : OPO-18) shows that the band at 1940 bp is "present" in Midagahara and that the band at 1030 bp observed in 15 species or ecotypes is

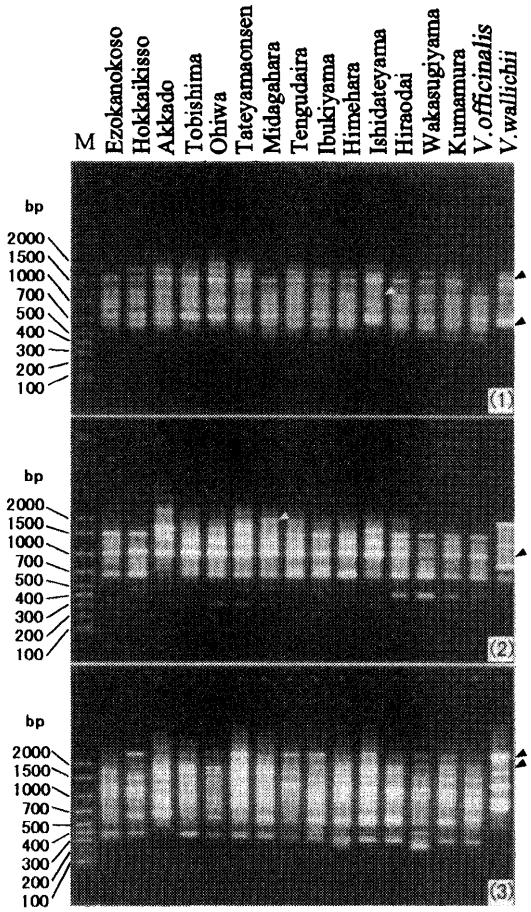

Fig.1. RAPD patterns in 16 samples of *Valeriana*
M:molecular weight size marker (50-2000 bp Ladder). Photos (1), (2) and (3) indicate the DNA products generated by the primers OPO-07, OPO-18 and OPA-01, respectively.

"absent" in *V. wallichii*. Fig. 1. (3) (primer : OPA-01) shows that in *V. wallichii* the band at 2450 bp was observed but the band at 1930 bp was not. (These bands are indicated with arrowheads in Fig. 1.). Forty-four percent (181 bands) of polymorphic bands was present or absent in only one species or ecotype (Table 3.). These bands, which were considered specific markers of the species or ecotypes and useful for identification of *Valeriana*, were found in 13 species and ecotypes but not in 3 ecotypes, Tateyamaonsen, Himehara, Kumamura. Many specific markers were observed in Akkado and *V. wallichii*. Therefore, apparently these two were more specific than other *Valeriana*.

The dendrogram prepared from the similarity values reveals that the ecotypes of *V. fauriei* (except Akkado) are clearly distinguishable from *V. officinalis* and *V. wallichii* at similarity value of 0.775 (Fig. 2.). Most of the ecotypes of *V. fauriei* do not cluster according to the geographic locations. Of the four ecotypes collected in Toyama prefecture, only three, Ohiwa, Tateyamaonsen and Tengudaira, showed a close relation between the genetic clustering and the geographic location. On the other hand, the dendrogram prepared on the basis of the RAPD assay in this study partially agreed with that prepared according to the morphological characters[7]. In both dendrograms, Kumamura and Hiraodai, Tateyamaonsen

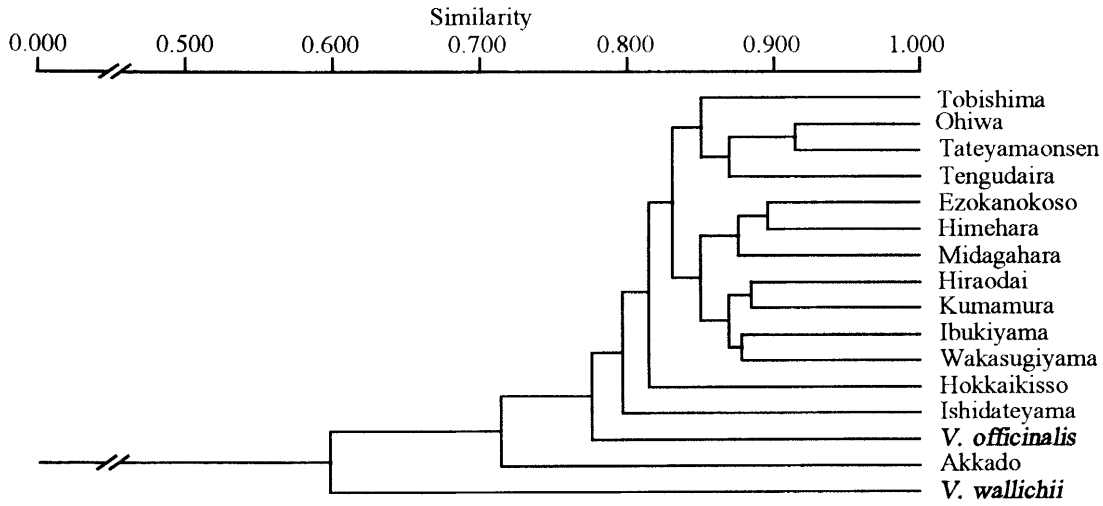

Fig. 2. Dendrogram of 16 samples of *Valeriana* according to the UPGMA cluster analysis

Table 3. Species or ecotype-specific RAPD markers

Species or ecotypes	Markers (Figures following the primers represent the number of base pairs)					
	Present			Absent		
Valeriana fauriei						
Ezokanokoso	OPA-10 1230	OPO-03 760				
Hokkaikisso	OPA-05 700	OPA-06 1740	OPA-06 1620	OPO-14 570		
	OPA-15 1600	OPO-01 840	OPO-02 760			
	OPO-06 620					
Akkado	OPA-03 1290	OPA-03 1110	OPA-05 1440	OPA-05 880	OPA-17 510	OPO-06 1000
	OPA-05 640	OPA-10 1480	OPA-11 610			
	OPA-12 1720	OPA-14 1790	OPA-15 920			
	OPA-15 650	OPA-16 1560	OPA-17 930			
	OPA-17 610	OPA-17 560	OPA-20 1290			
	OPA-20 1200	OPO-02 790	OPO-02 710			
	OPO-02 610	OPO-03 1970	OPO-05 1870			
	OPO-05 1600	OPO-06 1070	OPO-06 830			
	OPO-07 670	OPO-08 1160	OPO-12 810			
	OPO-14 1700	OPO-15 520	OPO-16 1930			
	OPO-20 630					
Tobishima	OPA-12 1100	OPA-12 980	OPA-12 810			
	OPA-12 700	OPA-16 1870	OPO-02 1870			
	OPO-18 760					
Ohiwa	OPA-10 1080	OPO-04 1580	OPO-07 1030			
	OPO-20 1220					
Midagahara	OPA-01 550	OPO-18 1940				
Tengudaira	OPA-02 1190	OPA-06 520	OPA-10 960			
Ibukiyama	OPO-02 810					
Ishidateyama	OPA-06 1240	OPA-16 2830	OPO-03 750	OPO-07 1180	OPO-15 1100	
	OPO-12 760					
Hiraodai	OPA-11 840	OPO-15 530				
Wakasugiyama	OPO-02 1350					
V. officinalis	OPO-02 580	OPO-06 850	OPO-12 780			
V. wallichii	OPA-01 2450	OPA-02 1210	OPA-02 990	OPA-01 1930	OPA-02 950	OPA-02 250
	OPA-02 850	OPA-02 780	OPA-02 440	OPA-03 980	OPA-03 780	OPA-03 400
	OPA-02 280	OPA-03 620	OPA-04 1540	OPA-03 300	OPA-04 1790	OPA-04 580
	OPA-04 1160	OPA-04 1080	OPA-04 980	OPA-07 440	OPA-13 1030	OPA-18 1630
	OPA-04 910	OPA-07 2460	OPA-07 1730	OPA-18 860	OPA-19 1660	OPA-20 1870
	OPA-07 1220	OPA-07 950	OPA-07 560	OPO-04 580	OPO-07 1550	OPO-09 730
	OPA-07 420	OPA-08 1930	OPA-08 1790	OPO-15 600	OPO-16 860	OPO-16 360
	OPA-09 1010	OPA-09 440	OPA-10 610	OPO-18 1470	OPO-18 1030	OPO-19 1030
	OPA-11 1040	OPA-13 900	OPA-13 710	OPO-19 700	OPO-19 630	OPO-20 1140
	OPA-13 470	OPA-14 2150	OPA-14 1610	OPO-20 460	OPO-20 240	
	OPA-14 1370	OPA-14 960	OPA-14 550			
	OPA-15 1120	OPA-15 1010	OPA-16 1050			
	OPA-16 300	OPA-17 1280	OPA-18 990			
	OPA-18 900	OPA-18 700	OPA-18 350			
	OPA-19 1520	OPA-19 880	OPA-19 450			
	OPA-20 2090	OPA-20 950	OPO-01 3450			
	OPO-01 1770	OPO-01 1660	OPO-02 2250			
	OPO-03 1840	OPO-03 1280	OPO-04 1410			
	OPO-04 570	OPO-05 1140	OPO-05 820			
	OPO-06 540	OPO-06 440	OPO-07 1650			
	OPO-07 1450	OPO-07 690	OPO-08 920			
	OPO-14 470	OPO-15 410	OPO-16 1540			
	OPO-16 1370	OPO-16 1140	OPO-16 530			
	OPO-18 1830	OPO-18 1530	OPO-18 1400			
	OPO-18 1200	OPO-18 800	OPO-19 1830			
	OPO-19 960	OPO-19 750	OPO-20 640			
	OPO-20 500					

Note ; "Present" and "Absent" indicate presence or absence of the specific bands in the 16 species or ecotypes. For example, 『OPA-05$_{700}$』 in "Present" column means that the band at 700 bp produced by primer OPA-05 is specifically present in Hokkaikisso. 『OPO-14$_{570}$』 in "Absent" column means that the band at 570 bp produced by primer OPO-14 is present in the other 15 species or ecotypes, but specifically absent in Hokkaikisso.

and Tengudaira, Himehara and Ezokanokoso were each clustered at high similarity value.

Surprisingly, *V. officinalis* was more closely related to the 13 ecotypes of *V. fauriei* than to Akkado. Although Akkado is an ecotype of *V. fauriei* according to the traditional classification, the DNA level assay and the morphological characters suggest that it is a unique ecotype. Akkado is morphologically more similar to *V. officinalis* than to *V. fauriei*. For example, Akkado has many leaflets and leaf serrations, and as *V. officinalis* and *V. wallichii* it contained only small amounts of KAC, KG8A, KG2A and KGD. On the other hand, *V. fauriei*, except Akkado, contained one to three of these 4 constituents, though their contents varied greatly among the ecotypes of *V. fauriei*. It is reasonable to conclude that Akkado is not to be used for the same medicinal purpose as other *V. fauriei*.

These results indicate that RAPD marker can be used for the estimation of the genetic relationship among the *Valeriana* plants. This phylogenetic systematics using the RAPD markers is a simple and convenient procedure which requires only a small amount of the materials. Furthermore, the result obtained in this study suggest that detection of hybrids may be possible in the breeding program.

REFERENCES

1) The committee of Japanese Pharmacopoeia Guide Book, ed., "The Guide Book of Japanese Pharmacopoeia 13th Ed.", p. D198-200, Hirokawa shoten, Tokyo (1996).

2) Suzuki, H., Zhang, B.-C., Harada, M., Iida, O., Satake, M., The 35th Annual Meeting of the Japanese Society of Pharmacognosy, Niigata, Sept., Abstract Papers A-4, p. 4 (1988).

3) Suzuki, H., Zhang, B.-C., Harada, M., Iida, O., Satake, M., *Shoyakugaku Zasshi*, **47**. 305-310 (1993).

4) Eickstedt, K. W., Rahman, S., *Arzneimittel Forschung*, **19**. 316-319 (1969).

5) Eickstedt, K. W., *Arzneimittel Forschung*, **19**. 995-997 (1969).

6) Stahl, E., Schild, W., *Arzneimittel Forschung*, **19**. 314-316 (1969).

7) Fujino, H., Suzuki, S., Tatsuo, Y., Yamazaki, N., Yoshizaki, M., The 37th Annual Meeting of the Japanese Society of Pharmacognosy, Chiba, Nov., Abstract Papers 1C-1, p. 65 (1990).

8) Hikino, H., Hikino, Y., Kato, H., Takeshita, Y., Takemoto, T., *Yakugaku Zasshi*, **89**. 118-121 (1969).

9) Hikino, H., Hikino, Y., Takeshita, Y., Isurugi, Y., Takemoto, T., *Yakugaku Zasshi*, **91**. 650-656 (1971).

10) Hikino, H., Hikino, Y., Kato, H., Takeshita, Y., Takemoto, T., *Yakugaku Zasshi*, **91**. 766-769 (1971).

11) Hikino, H., Ono, M., Takemoto, T., *Yakugaku Zasshi*, **92**. 479-481 (1972).

12) Hikino, H., Hikino, Y., Nakamura, R., Ono, M., Takemoto, T., *Yakugaku Zasshi*, **92**. 498-502 (1972).

13) Hikino, H., Kato, T., Takemoto, T., *Yakugaku Zasshi*, **95**. 243-245 (1975).

14) Takamura, K., Kakimoto, M., Kawaguchi, M., Iwasaki, T., *Yakugaku Zasshi*, **93**. 599-606 (1973).

15) Takamura, K., Nabata, H., Kawaguchi, M., *Yakugaku Zasshi*, **95**. 1205-1209 (1975).

16) Hikino, H., Hikino, Y., Kobinata, H., Aizawa, A., Konno, C., Oh'izumi, Y., *Shoyakugaku Zasshi*, **34**. 19-24 (1980).

17) Rogers, S. O., Bendich, A. J., *Plant Mol. Biol.*, **5**. 69-76 (1985).

18) Williams, J. G. K., *Nucleic Acids Res.*, **18**. 6531-6535 (1990).

19) Nei, M., Li., W. H., *Proc. Natl. Acad. Soc. USA*, **76**. 5269-5273 (1979).

20) Nei, M., "Molecular Evolutionary Genetics", p. 247-287, Baifukan, Tokyo (1996).