ア活性を示さなかったが、MG63 に対して *Bistorta* sp.の根のエキスは接着が緩くなり形態が丸くなる現象が観察され抗腫瘍性に関連する活性と推察される。

4) 大麻の DNA 鑑定を目的としたゲノム多様性に関する研究

THCA 生合成酵素遺伝子の DNA 配列は、THC の含有率によって 2 タイプに分かれた。THC 高含有系統の THCA 生合成酵素遺伝子の DNA 配列を D 型(Drug-type)、THC 低含有系統の DNA 配列を F 型(Fiber-type)とした。D 型の系統には 2 タイプが存在し、D-1 型、D-2 型とした。D-2 型は D-1 型に対して 4 塩基の置換が認められた。F 型には F-1～F-7 の 7 タイプ存在した。F-1 型に対してそれぞれ、4 塩基(F-2 型)、3 塩基(F-3 型)、5 塩基(F-4 型)、4 塩基(F-5 型)、3 塩基(F-6 型)、4 塩基(F-7 型)の置換が認められた。形態的特徴からは THC 含有率及び THCA 生合成遺伝子型と関連する事項は確認できない。従来、文献で「植物体および葉身が細身のものは THC 含有率が高い」としたことは否定せざるを得ない。乾燥葉と種子の DNA 分析手法の開発では、ドラッグタイプ型大麻の生葉、乾燥葉、種子の 3 種類の試料から *rbcL* 遺伝子、ドラッグタイプ型 THCA 生合成酵素遺伝子の 2 領域の検出が可能であり、乾燥大麻試料や大麻種子でも DNA 鑑定が可能であることが示唆された。

D. 結論

国際的調和を踏まえた麻薬代替植物の研究の最終年度報告にあたり、これまでの 10 年にわたる研究成果を振り返り今後の対策への提言としたい。

乱用薬物のインドシナ半島最大の生産国であるミャンマーでは、不正薬物による現金収入が、少数民族の独立の財源であった。しかし、昨年新政府によって、少数民族の経済支援が打出された。それに応え、ケシの代替に薬用植物を考えてきたが、不法麻薬生産地は貧困のため医療費がなく伝統薬を使っている現状も明らかになった。そこで、現地の伝統薬栽培と我が国で要望されている漢方原料植物及び薬用果樹の栽培指導を並行して行ってきた。少数民族のカチン州とチン州で行った栽培試験と研修で、大量栽培できる候補となったのは、ショウガ、ベニバナ、ハトムギ、ミシマサイコ、コガネバナ、キバナオウギ、柑橘類、ブドウ及び薬用ランであった。新政府の方針で外国との経済交流が可能になったことを受け、日本の製薬会社や天然素材会社の協力を得て、大型栽培が始まったことは大きな前進である。伝統的な生薬からヒントを得て、経済的付加の高い新薬の開発に役立つことが期待されるのが、ミャンマーの経済植物チーク材の葉から、リュシュマニア治療薬開発を可能とする成分の発見である。この化合物の治療薬への発展研究には、更なる検討が必須であるが、有用性が確定された際には種の多様性条約の利益分配の面から重要な植物になると期待される。更に多くの素材や新しい活性試験法も開発されているので、より早い解明を期待する。アサの形態的特徴、THC 含有率、THCA 生合成酵素遺伝子型の情報を総合的にデータベース化でき、大麻関連の事犯における鑑定作業に有用な手法となるものといえる。覚せい剤については、近年、密造地がアフリカ、メキシコ、中近東とグローバルに拡散している。関係国の覚

せい剤原料規制担当者による、覚せい剤原料の横流し規制対策に役立つ化学情報の重要性が徐々に認識されつつあり、国連の担当部局が、本研究で進めてきた日本の覚せい剤プロファイリングの化学情報について公式に情報を求めてきている。1998年から継続して検討してきた本研究班での多面的な覚せい剤の安定同位体分析や有機不純物分析によるプロファイリング手法は、国内の関係機関の実務をリードし、国際空港等の水際で押収される覚せい剤についての役立つデータが蓄積されつつある。適切にこの化学情報がオープンされれば、今後の国際的な原料規制に役立つものと確信する。各国の法化学分析機関が可能なデータをとり、世界各国で流通している覚せい剤が、どのような化学情報をもっているか明らかにしていくことが必要である。同時に、関係各国の行政担当者に、覚せい剤の化学情報を収集し、有効に原料物質規制対策に役立てていく努力を期待し、最終報告とする。

E. 論文発表

1. H.Asahina, J.Shinozaki, K.Masuda, Y.Morimitsu, M.Satake, *J.Nat Med,* 64 (2010) 133-138
2. Y.Makino, *Biomed. Chromatogr.,* 26 (2012) 327-330
3. N.Kurashima, Y.Makino, Y.Urano, K. Sanuki, Y.Ikehara, T.Nagano, *Forensic Sci. Int.*, 189 (2009) 14-18
4. H.Fuchino, M.Kawano, K.Mori-Yasumoto, S.Sekita, M. Satake, T. Ishikawa, F. Kiuchi, and N. Kawahara, *Chem. Pharm. Bull.* 58 (2010) 1047-1050
5. H.Morita, Y.Nagakura, T.Hosoya, W. Ekasari, A.Widyawaruyanti, K. Mori-Yasumoto, S. Sekita, Y. Hirasawa, *Heterocycles,* 81 (2010) 441-450
6. M.Rangel, M.P.S.Cabrera, K.Kazuma, K.Ando, X.Wang, M.Kato, K.Nihei, I.Y. Hirata, T.J.Cross, A.N.Garcia, E.L.F.Mauro, M.R.Franzolin, H.Fuchino, K.M.Yasumoto, S.Sekita, M.Kadowaki, M.Satake, K.Konno, *Toxicon,* 57 (2011) 1081-1092。

F. 学会発表

1. 安元（森）等,抗リーシュマニア活性を有する有用植物の探索(その 17）-ミャンマー産植物 *Diospyros burmanica* の成分について 2-, 日本生薬学会　第 56 回年会(京都)2009.9
2. Y. K. Mori et al., Study of anticancer and antiprotozoa products from tropical plants, Pacifichem 2010 (Hawaii, USA) 2010 12
3. 黒柳正典等, パキスタン産 Withania coagulans の抗リーシュマニアステロイド, 第 54 回香料・テルペン及び精油化学に関する討論会(山梨), 2010.10
4. 渕野裕之等、Fernando Cabieses Molina、Zuno Burstein Alva: 薬用植物における抗リーシュマニア活性化合物の探索研究, 第 52 回天然有機化合物討論会(静岡) 2010.9
5. 紺野勝弘等、単独性カリバチ毒から得られた新規抗菌ペプチドの構造と生物活性, 日本生薬学会第 57 回年会(徳島) 2010.9
6. 菊池博之等、抗リーシュマニア活性を有する薬用植物の探索(その21）-ペルー産ソロモン諸島産植物の活性評価及び

Mutisia acuminataの活性成分-, 日本生薬学会　第57回年会(徳島) 2010.9

7．安元(森)加奈未等、抗リーシュマニア活性を有する有用植物の探索(その22)-ミャンマー産植物KYUN *Tectona grandis*の成分について-. 日本薬学会 128年会(静岡) 2011.3

8. 安元(森)加奈未等、抗リーシュマニア活性を有する有用植物の探索(その 23)-ミャンマー産植物KYUN *Tectonagrandis*の成分について-.日本生薬学会第58回年会(東京) 2011.9

9. 安元加奈未、熱帯植物からの抗リーシュマニア活性物質の探索,日本薬学会生薬天然物部会奨励研究受賞講演,第4回食品薬学シンポジウム, 東京.2011.10

10. 高上馬希重等、違法薬物「大麻」のDNA 鑑別手法開発に関する研究、日本生薬学会北海道支部第 34 回支部例会(札幌) 2010.5.8

11. 高上馬希重等、違法薬物「大麻(アサ)」の植物学的特徴と多様性について、日本薬学会北海道支部第 134 回支部例会(札幌) 2010.5.8

12. 高上馬希重、Cannabis 大麻の植物としての多様性、生薬若手懇談会第 4 回勉強会(筑波) 2010.8.10

13. 高上馬希重、2010.9.9、違法薬物「大麻」の植物としての多様性、日本植物学会第７4回大会シンポジウム(春日井)

II．研究成果の刊行に関する一覧表

1. Makino Yukiko “Simple HPLC method for detection of trace ephedrine and pseudoephedrine in high-purity methamphetamine”, Biomedical Chromatography, (2012); 26:327-330
2. Asahina, H., Shinozaki, J., Masuda, K., Morimitsu, Y. and Satake, M. "Identification of medicinal Dendrobium species by phylogenetic analyses using *matK* and *rbcL* sequences." *Journal of Natural Medicines,* (2010)64:133-138
3. 佐竹元吉:インドシナ半島のケシ代替植物プロジェクト、*pharmashia*, 46, 845-850 (2010)
4. N. Kurashima, Y. Makino, Y. Urano, K. Sanuki, Y. Ikehara, T. Nagano, *Forensic Sci. Int.* , 189 (2009) 14-18.
5. 佐竹 元吉, 薬用植物とその保存, *Techno Innovation* **19** (2009) 50-60.

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Simple HPLC method for detection of trace ephedrine and pseudoephedrine in high-purity methamphetamine

Yukiko Makino*

ABSTRACT: A simple and sensitive HPLC technique was developed for the qualitative determination of ephedrine and pseudoephedrine (ephedrines), used as precursors of clandestine *d*-methamphetamine hydrochloride of high purity. Good separation of ephedrines from bulk *d*-methamphetamine was achieved, without any extraction or derivatization procedure on a CAPCELLPACK C_{18} MGII (250 × 4.6 mm) column. The mobile phase consisted of 50 mM KH_2PO_4–acetonitrile (94:6 v/v %) using an isocratic pump system within 20 min for detecting two analytes. One run took about 50 min as it was necessary to wash out overloaded methamphetamine for column conditioning. The analytes were detected by UV absorbance measurement at 210 nm. A sample (20 mg) was simply dissolved in 1 mL of water, and a 50 µL aliquot of the solution was injected into the HPLC. The detection limits for ephedrine and pseudoephedrine in bulk *d*-methamphetamine were as low as 3 ppm each. This analytical separation technique made it possible to detect ephedrine and/or pseudoephedrine in seven samples of high-purity *d*-methamphetamine hydrochloride seized in Japan. The presence of trace ephedrines in illicit methamphetamine may strongly indicate a synthetic route via ephedrine in methamphetamine profiling. This method is simple and sensitive, requiring only commonly available equipment, and should be useful for high-purity methamphetamine profiling. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: HPLC; ephedrine; pseudoephedrine; high-purity methamphetamine; profiling analysis

Introduction

Clandestine manufacture of methamphetamine is an increasing problem, particularly in East and Southeast Asia, where the main precursor for illicit methamphetamine has been ephedrine or pseudoephedrine for a long time. Impurity profiling of methamphetamine hydrochloride is a very useful tool for monitoring the synthetic routes and distribution of drugs and precursors (Remberg and Stead, 1999). The chemical information on the precursor is useful for the strict control of precursor chemicals, which is a valuable countermeasure to prevent illicit synthesis. Therefore, the identification of ephedrine or pseudoephedrine as a starting material in illicit methamphetamine is essential to estimate the precursor (Makino *et al.*, 2005; Kurashima *et al.*, 2009). There have been seizures of high-purity *d*-methamphetamine hydrochloride in Japan. It is not possible to detect ephedrine and/or pseudoephedrine in these samples by GC-MS (Sasaki and Makino, 2006) or HPLC (Makino *et al.*, 2002). A number of analytical methods have been developed for the separation and determination of ephedrines in herbal materials and illicit methamphetamine hydrochloride (Makino *et al.*, 2002; Iwata *et al.*, 2006; Pellati and Benvenuti, 2008). It may be possible to determine trace ephedrines in bulk samples if using HPLC-MS or GC-TOF-MS with high sensitivity and selectivity. However, these instruments are not yet available for many forensic laboratories in East and Southeast Asia. Therefore, I have investigated a simple and practical HPLC method for the detection of trace ephedrine and pseudoephedrine in high-purity methamphetamine. Few simple methods for the determination of trace ephedrines in bulk methamphetamine hydrochloride have been reported in the literature. In our previous work, the detection limit for ephedrines was 500 ppm (Makino *et al.*, 2002). Iwata *et al.* (2006) reported the detection limit of ephedrines in bulk methamphetamine to be 40 ppm by capillary electrophoresis. In recent years, Indonesia and Malaysia have reported the increasing scale and sophistication of methamphetamine manufacture (World Drug Report by UNODC, 2010), and there is a need for forensic laboratories in Southeast Asia to obtain chemical information on seized methamphetamine quickly and conveniently, using conventional instruments in hand. Most forensic laboratories in Southeast Asia already have facilities to determine key impurities of seized methamphetamine by GC-MS and the optical activity by simple HPLC (Makino *et al.*, 1999). However, there is no decisive chemical information for estimating the precursor for high-purity methamphetamine, because there is no simple and sensitive method that would be applicable in many forensic laboratories in which only conventional HPLC is available. As a continuation of our previous work (Makino *et al.*, 2002), the present paper describes a simple and more sensitive HPLC method for the qualitative analysis of ephedrine and pseudoephedrine in high-purity methamphetamine samples from which ephedrines were not detected by GC-MS or GC as reported in our methamphetamine profiling (Sasaki and Makino, 2006).

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Experimental

Materials

l-Ephedrine hydrochloride, *d*-ephedrine hydrochloride and *d*-pseudoephedrine hydrochloride were purchased from Daiichi Fine Chemical (Takaoka, Japan). 2-Phenylethylamine hydrochloride was purchased from Tokyo Chemical Industry (Tokyo, Japan). Seven illicit methamphetamine hydrochloride samples seized in Japan were provided by the Ministry of Health, Labor and Welfare, Japan. Water was purified with a Milli-Q system (Nihon Millipore, Tokyo, Japan). All other chemicals were of analytical reagent grade.

Instruments and HPLC analysis

A Shiseido Nanospace liquid chromatograph equipped with a photodiode array detector linked to a data system (EZChromElite kit for Shiseido, Tokyo, Japan) was used for data acquisition and storage. The column effluent was monitored by measuring UV absorbance at 210 nm. The system consisted of two pumps, a column oven, an autosampler and a degassing unit. Chromatographic separation was achieved at 40°C on an octadecylsilyl (ODS)-type column (CAPCELLPAK C_{18} MGII S-5, 250 × 4.6 mm, 5 µm, Shiseido, Tokyo, Japan) using the following mobile phase: (A) acetonitrile and (B) 50 mM aqueous KH_2PO_4. Elution was started with 6% A solution (20 min hold) for detecting ephedrines, changed to 13% A solution at 20.1 min (15 min hold) for washing out bulk methamphetamine, and then turned back to 6% A solution. The flow-rate was 1 mL/min.

Sample preparation

Standard solution was prepared at a final concentration of 10 µg/mL in water (10 ng/µL) for each analyte. An internal standard solution of 2-phenylethylamine hydrochloride was prepared at a final concentration of 10 µg/mL in water (10 ng/µL). A sample without ephedrine and pseudoephedrine was prepared by recrystallization of seized high-purity *d*-methamphetamine hydrochloride in order to examine the accuracy and precision of this method. The recrystallization was repeated five times from chloroform by dropping of *n*-hexane (sample 500 mg, chloroform 30 mL and *n*-hexane 30 mL for the first step). The recrystallized sample (200 mg) was dissolved in water (10 mL), and 1 mL aliquots were added to five vials. The internal standard solution (20 µL) was added and standard aqueous solution was spiked into each vial to give final contents of 0, 3, 6, 9 and 12 ppm. Seven seized samples were selected for this study based on the preliminary impurity profiling by GC-MS carried out in our earlier work (Sasaki and Makino, 2006). The optical activities of the seven samples were all *dextro*-form, and the impurity characteristics of samples are summarized in Table 1. Each sample was dissolved at the high concentration of 40 mg per 2 mL water. Each sample solution was divided equally into two vials. The internal standard solution (20 µL) was added to each vial. Eighteen microliters of the standard solution was added into one vial to give a final content of 9 ppm. The measurement of each sample was carried out five times. The injection volume was 50 µL for all measurements.

Results and discussion

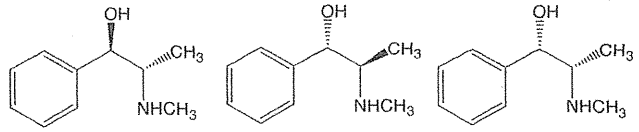
HPLC conditions

In order to develop a simple and sensitive HPLC technique for the qualitative identification of trace ephedrines (for structures see Fig. 1) in pure *d*-methamphetamine hydrochloride, I modified our previous method (Makino *et al.*, 2002), in which an ODS type UG-120 column was used with a mobile phase containing an ion-pairing reagent, because the detection limit of ephedrines in bulk methamphetamine was 500 ppm and mobile phase containing sodium dodecyl sulfate (SDS) was not good for the column's lifetime. For the present purpose, a CAPCELLPAK C_{18} MGII column was selected, which offers better retention and can be more highly loaded with basic amines than the UG-120 column. The ability to inject higher-concentration samples (20 mg/mL water) on the MGII afforded better sensitivity. The merit of this column is derived from the ultimately shielded silanol groups of packing materials and the high ligand density of octadecyl groups on the surface of polymer-coated silica (Kanda *et al.*, 2004). The optimum ratio of acetonitrile in buffer solution was investogated to obtain good separation of *l*-ephedrine hydrochloride and *d*-pseudoephedrine hydrochloride from bulk methamphetamine without the use of SDS. Too low a concentration of acetonitrile (5% and under) can degrade column performance, resulting in poor retention and slow recovery of performance (Luo *et al.*, 2008). Concentrations of 15–6% acetonitrile in potassium phosphate buffer were examined. At the acetonitrile concentration of 6%, the resolution value (R_s) was 1.9, which is sufficient for separation of *l*-ephedrine hydrochloride and *d*-pseudoephedrine hydrochloride. However, *l*-ephedrine hydrochloride and *d*-ephedrine hydrochloride were not separated with this ODS-type column. It is important to check if ephedrine and/or pseudoephedrine used as precursors are present in high-purity methamphetamine. Samples spiked in the range of 3–12 ppm ephedrines in recrystallized methamphetamine were examined. The separation was satisfactory, and no interfering peaks were seen. A chromatogram of a sample spiked at the content of 3 ppm is shown in Fig. 2(A). Next, 20 successive analyses of a bulk sample were performed by isocratic elution with 50 mM KH_2PO_4–acetonitrile (94:6 v/v %). The retention time of each analyte decreased gradually to as little as half of the initial value by impairment of the column efficiency from sample

Table 1. Characteristics of seven samples selected for this study

Sample no.	Key impurity[a]		
	Azi	Nap	Eph
1	+	–	–
2	–	+	–
3	–	+	–
4	–	+	–
5	–	–	–
6	+	–	–
7	+	–	–

+, Detected; –, not detected.
Azi, *cis*- and/or *trans*-1,2-dimetyl-3-phenylaziridine; Nap, 1,3-dimethyl-2-phenylnaphthalene and/or 1-benzyl-3-methylnaphthalene; Eph, ephedrine/pseudoephedrine.
[a]Key impuritiy was detected by GC-MS.



l-ephedrine *d*-ephedrine *d*-pseudoephedrine

Figure 1. Chemical structures of the ephedrines investigated in this study.

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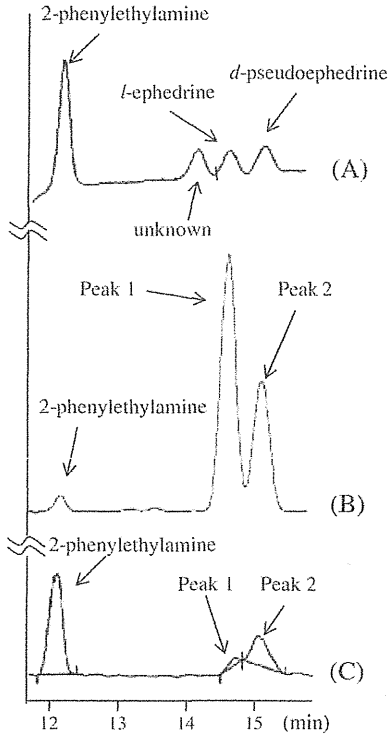

Figure 2. Chromatograms of some samples. (A) Solution spiked with the analytes at 0.06 µg each in 20 mg crystal methamphetamine (final content: 3 ppm). (B) Illicit *d*-methamphetamine seized in Japan (sample no. 1). (C) Illicit *d*-methamphetamine seized in Japan (sample no. 6).

Table 2. Reproducibility of the peaks of *l*-ephedrine and *d*-pseudoephedrine in bulk methamphetamine

	Ratio of peak area[a] ($n=5$)	
Content	*l*-Ephedrine Mean ± RSD (%)	*d*-Pseudoephedrine Mean ± RSD (%)
3 ppm	0.14676 ± 9.3	0.18609 ± 5.8
6 ppm	0.34134 ± 3.9	0.36518 ± 4.4
9 ppm	0.52041 ± 3.8	0.53887 ± 2.6
12 ppm	0.72367 ± 1.1	0.72185 ± 2.9

[a]Peak area ratio of *l*-ephedrine (or *d*-pseudoephedrine) to 2-phenylethylamine.

Table 3. Results of ephedrines in seven seized samples

Sample no.	Ephedrine	Pseudoephedrine
1	+	+
2	+	+
3	+	+
4	+	trace
5	trace	–
6	trace	+
7	+	trace

+, Detected; –, not detected.

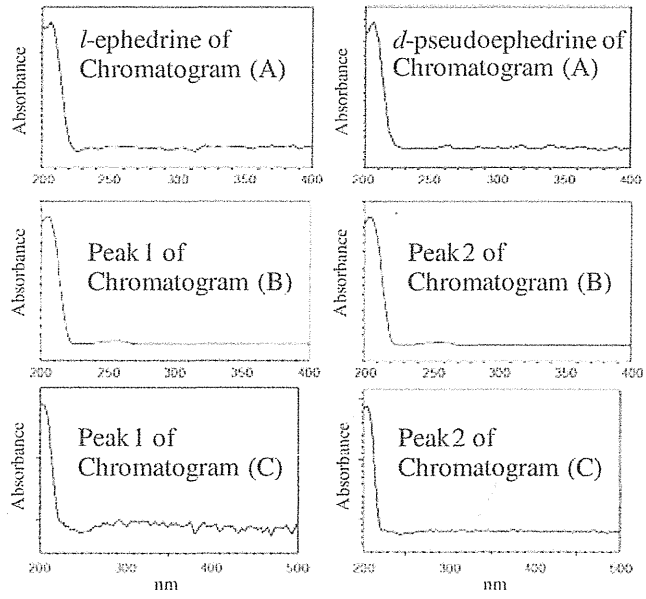

Figure 3. Spectra of ephedrines in the chromatograms of Fig. 2.

overloading. To achieve good performance in continuous analysis, bulk methamphetamine containing unknown impurities must be washed out rapidly. The cleaning of the column was performed by increasing the acetonitrile concentration in buffer solution to 13% after elution of ephedrines. The values of repeatability of retention time for 2-phenylethylamine hydrochloride (t_R, 12.1 min), *l*-ephedrine hydrochloride (t_R, 14.6 min) and *d*-pseudoephedrine hydrochloride (t_R, 15.1 min) were 0.63, 0.76 and 0.78%, respectively (RSD for retention times at samples of 3, 6, 9 and 12 ppm ephedrines spiked in bulk methamphetamine, $n=20$), which are acceptable for the qualitative determination of ephedrines in bulk methamphetamine. In this study, 2-phenylethylamine hydrochloride was used for checking the column efficiency. The reproducibility of the area for each peak in bulk methamphetamine is shown in Table 2. Peak areas of each analyte had good correlation for the content. This method is not intended for quantification of ephedrines, and so the results are sufficient for qualitative determination of trace ephedrines in bulk methamphetamine. The detection limit was examined with samples containing 3 ppm ephedrines, and the signal-to-noise ratio was 7.4 for *l*-ephedrine and 7.5 for *d*-pseudoephedrine. Based on the data in Table 2 and the chromatogram of Fig. 2(A), the limit of detection can be set at 3 ppm for qualitative analysis of trace ephedrines in bulk methamphetamine; this corresponds to 60 ng ephedrine or pseudoephedrine hydrochloride in 20 mg methamphetamine hydrochloride.

Method application

The developed method was used to determine ephedrine and pseudoephedrine in seven samples of pure *d*-methamphetamine hydrochloride seized in Japan. The qualitative detection of ephedrines was based on the match of retention time and UV spectrum with those of the standard ephedrines. The results are presented in Table 3. The trace notation in Table 3 means that the acquired area of peak was smaller than the area of peak containing 3 ppm ephedrines. Figures 2 and 3 display representative chromatograms and the UV spectra of spiked

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authentic ephedrines and samples (nos 1 and 6). The spectra of peaks 1 and 2 in the chromatogram of Fig. 2(B and C) matched the spectra of the standard solution of *l*-ephedrine and *d*-pseudoephedrine in the chromatogram of Fig. 2(A).

Conclusion

This simple and convenient HPLC method could determine ephedrine and pseudoephedrine at levels as low as 3 ppm in bulk methamphetamine. As it is very difficult to extract trace ephedrines from the structural analog methamphetamine, this method without any extraction or derivatization procedure may bring benefits for the profiling of high-purity methamphetamine. This developed method was confirmed to be effective for qualitative determination of ephedrines in illicit bulk methamphetamine hydrochloride samples, in which the contents were substantially below the detection limit of the previous method. The configuration of ephedrines is not acquired by this method, but the detection of trace ephedrines may provide valuable information in impurity profiling of high-purity samples. If the content of ephedrines is rich in seized methamphetamine sample, it's favorable to confirm the configuration of ephedrine by our previous method (Makino *et al.*, 2002). Illicit *d*-methamphetamine is prepared only from *l*-ephedrine or *d*-pseudoephedrine, not from *d*-ephedrine or *l*-pseudoephedrine. The ephedrine and pseudoephedrine detected in seized samples may be considered *l*-ephedrine and *d*-pseudoephedrine. Conventional HPLC apparatus is available at most forensic laboratories, so the present method should be widely applicable for identifying trace ephedrines in bulk methamphetamine hydrochloride, and should be helpful for monitoring trends in synthetic methods and precursors used for the illicit production of methamphetamine hydrochloride.

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References

Iwata Y, Inoue H, Kuwayama K, Kanamori T, Tsujikawa K, Miyaguchi H and Kishi T. Forensic application of chiral separation of amphetamine-type stimulants to impurity analysis of seized methamphetamine by capillary electrophoresis. *Forensic Science International* 2006; **161**: 92–96.

Kanda T, Takezawa K, Kobayashi A, Xin X and Kutsuna H. Capcell Pak C18: ultimately silanol-shielded HPLC column for improved peak shapes of basic compounds under neutral conditions. *LC-GC* 2004; **56**: 43.

Kurashima N, Makino Y, Urano Y, Sanuki K, Ikehara Y and Nagano T. Use of stable isotope ratios for profiling of industrial ephedrine samples: application of hydrogen isotope ratios in combination with carbon and nitrogen. *Forensic Science International* 2009; **189**: 14–18.

Luo H, Ma L, Paek C and Carr PW. Application of silica-based hypercrosslinked sulfonate-modified reversed stationary phases for separating highly hydrophilic basic compounds. *Journal of Chromatography A* 2008; **1202**: 8–18.

Makino Y, Suzuki A, Ogawa T and Shirota O. Direct determination of methamphetamine enantiomers in urine by liquid chromatography with a strong cation-exchange precolumn and phenyl-β-cyclodextrin-bonded semi-microcolumn. *Journal of Chromatography B* 1999; **729**: 97–101.

Makino Y, Urano Y and Nagano T. Impurity profiling of ephedrines in methamphetamine by high-performance liquid chromatography. *Journal of Chromatography A* 2002; **947**: 151–154.

Makino Y, Urano Y and Nagano T. Investigation of the origin of ephedrine and methamphetamine by stable isotope ratio mass spectrometry: a Japanese experience. *Bulletin on Narcotics* 2005; **57**: 63–78.

Pellati F and Benvenuti S. Determination of ephedrine alkaloids in Ephedra natural products using HPLC on a pentafluorophenylpropyl stationary phase. *Journal of Pharmaceutical and Biomedical Analysis* 2008; **48**: 254–263.

Remberg B and Stead A H. Drug characterization/impurity profiling, with special focus on methamphetamine: recent work of the United Nations International Drug Control Programme. *Bulletin on Narcotics* 1999; **51**: 97–117.

Sasaki T and Makino Y. Effective injection in pulsed splitless mode for impurity profiling of methamphetamine crystal by GC or GC/MS. *Forensic Science International* 2006; **160**: 1–10.

World Drug Report by UNODC, 2010; available from: http://www.unodc.org/unodc/en/data-and-analysis/WDR-2010.html

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ORIGINAL PAPER

Identification of medicinal *Dendrobium* species by phylogenetic analyses using *matK* and *rbcL* sequences

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Abstract Species identification of five *Dendrobium* plants was conducted using phylogenetic analysis and the validity of the method was verified. Some *Dendrobium* plants (Orchidaceae) have been used as herbal medicines but the difficulty in identifying their botanical origin by traditional methods prevented their full modern utilization. Based on the emerging field of molecular systematics as a powerful classification tool, a phylogenetic analysis was conducted using sequences of two plastid genes, the maturase-coding gene (*matK*) and the large subunit of ribulose 1,5-bisphosphate carboxylase-coding gene (*rbcL*), as DNA barcodes for species identification of *Dendrobium* plants. We investigated five medicinal *Dendrobium* species, *Dendrobium fimbriatum*, *D. moniliforme*, *D. nobile*, *D. pulchellum*, and *D. tosaense*. The phylogenetic trees constructed from *matK* data successfully distinguished each species from each other. On the other hand, *rbcL*, as a single-locus barcode, offered less species discriminating power than *matK*, possibly due to its being present with little variation. When results using *matK* sequences of *D. officinale* that was deposited in the DNA database were combined, *D. officinale* and *D. tosaense* showed a close genetic relationship, which brought us closer to resolving the question of their taxonomic identity. Identification of the plant source as well as the uniformity of the chemical components is critical for the quality control of herbal medicines and it is important that the processed materials be validated. The methods presented here could be applied to the analysis of processed *Dendrobium* plants and be a promising tool for the identification of botanical origins of crude drugs.

Keywords *Dendrobium* spp. · *matK* · *rbcL* · DNA barcoding · Phylogenetic analysis

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Introduction

The genus *Dendrobium*, distributed in most Asian countries and Australia, belongs to the family Orchidaceae [1, 2], and includes 900–2000 species [2]. In China, about 50 *Dendrobium* species have long been used in traditional medicines [3]. Also in Japan, Kan-ro-in, a Kampo medicine, consists of *Dendrobium* plants, referred to as Ganlou-in in the Chinese historical prescription book, Heji Jufang. Although it has been reported that *Dendrobium* plants have various pharmacological actions, including anti-cancer, anti-oxidant, immune modulation, and vasodilation effects [4], the chemical constituents that are responsible for these activities have yet to be identified. The fact that some *Dendrobium* species have been used as a tonic for hundreds of years in China suggests that these plants have potential for modern medicinal application in Japan. Jin chai shi hu, ma bien shi hu, and tie pi shi hu are listed in the latest Pharmacopoeia of the People's Republic of China [5]. According to Flora Reipublicae Popularis

Sinicae (FRPS) vol 19 [6], the scientific names of these species are *D. nobile*, *D. fimbriatum*, and *D. officinale* (*D. candidum* auct. non Lindl. [7]), respectively.

Many kinds of processed *Dendrobium* herbs, sometimes falsely sold as a more expensive variety, such as *D. officinale*, and numerous processed *Dendrobium* herbs with vernacular names are distributed in the Asian markets, making identification of their origin species more difficult. On the other hand, it is essential that processed materials of herbal medicine are validated from the point of view of quality control. Thus, the identification of the plant source as well as the uniformity of their chemical components is critical for their use as herbal medicines.

It is known that many *Dendrobium* plants are morphologically similar, making their identification based on morphology very difficult, except during flowering, when they can be easily classified. The most popular form of the processed drug of *Dendrobium* is called "Fengdou" in Chinese, and it appears in the shape of a small coil [3]. Identifying the botanical origin of such a processed drug is difficult because during the process, the natural resource is boiled, coiled, dried, and sometimes cut into pieces. As little is known about the chemical constituents of *Dendrobium* species, chemotaxonomy has not been applied for their botanical identification. Therefore, a reliable and brief method for the identification of these plant species needs to be developed for their further pharmaceutical utilization and investigation.

Recently, molecular systematics in plants as well as other organisms has been widely used for species identification and in the determination of phylogenetic relationships. In plants, the genes for maturase (*matK*), for the large subunit of the ribulose 1,5-bisphosphate carboxylase (*rbcL*), and for the *trnH-psbA* intergenic spacer on the chloroplast genome, are often used for molecular phylogenetic analysis [8–13]. For example, in a study using 1566 specimens of orchids in Costa Rica representing 1084 species, the *matK* sequence was shown to be useful for species identification and reconstructing phylogeny [14]. In addition to these three loci, several plastid DNA regions (*atpF-atpH* spacer, *rpoB* gene, *rpoC1* gene, and *psbK-psbI* spacer) [15] are also used in plant species identification.

Furthermore, the concept "DNA barcoding" has emerged and was successfully applied for many animal groups as an efficient species identification tool (e.g. [16]). In 2009, the Consortium for the Barcode of Life (CBOL) Plant Working Group has recommended the 2-loci combination of *rbcL* plus *matK* as plant DNA barcodes, which are sequences that vary extensively between species but hardly at all within them [15]. The methodology of DNA barcording could be applied to identify species, and to provide taxonomic information in clarifying the evolutionary relevance [17, 18].

In this study, five unprocessed medicinal *Dendrobium* plants and data deposited in the DNA database of DDBJ/EMBL/GenBank were used for species identification by phylogenetic analysis. Based on the proposal of CBOL Plant Working Group, we chose the two plastid genes, *matK* and *rbcL*, to conduct our phylogenetic analyses for assessing the intra- and interspecies relationship of the *Dendrobium* species.

Materials and methods

Plant materials

Leaves or stems of medicinal *Dendrobium* plants were collected from botanical gardens, individual cultivators, and a field, which are listed in Table 1. *Dendrobium* spp. noc-a to noc-c are *Dendrobium* hybrid cultivars of *D. nobile*. *Dendrobium* spp. noc-a and b are strains that were hybridized more than once. Both have different evolutionary lineages but the details are unknown. *Dendrobium* spp. noc-c is also a hybrid cultivar but its lineage is unknown. For *D. officinale*, plant material was not available in Japan. Therefore, the data deposited in the DNA database of DDBJ/EMBL/GenBank was used.

Isolation of DNA, amplification, and sequencing

Genomic DNAs were extracted from fresh plant samples by DNeasy® Plant Mini Kit (QIAGEN). Synthetic oligonucleotides for polymerase chain reaction (PCR) primers were obtained from Nihon Bioservice (Saitama, Japan). The primer sets used for amplification of *matK* and *rbcL* gene were as follows: OMAT1F (5′-CCGTTMTSACCAT ATTGC-3′) and trnK-2R (5′-AACTAGTCGGATGGAGT AG-3′) for *matK* [19]; aF (5′-ATGTCACCACAAACAGA GACTAAAGC-3′) and cR (5′-GCAGCAGCTAGTTCCG GGCTCCA-3′) for *rbcL* [11] Using Ex Taq® Hot Start Version (TaKaRa Bio.), PCR was carried out with the above primer sets and DNA (approximately 5–55 ng) as a template. The PCR conditions were 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The PCR reaction products were separated by agarose gel electrophoresis and purified by Wizard® SV Gel and PCR Clean-Up System (Promega). The purified DNA was ligated into a T-vector using pGEM®-T Easy Vector System (Promega) and *Escherichia coli* DH5α COMPETENT high (TOYOBO) was transformed with the resultant plasmid. Plasmids were isolated by illustra™ plasmid Prep Mini Spin Kit (GE Healthcare Bioscience). At least three clones were obtained for each species and used for sequencing. Sequencing was carried out by ABI 3100 Avant and 3130xl Genetic Analyzer

Table 1 Plant materials of *Dendrobium* species examined in this study

Taxon	Identifier	Source			Accession no.	
		Abbreviation	Collection site	Wild/market	*matK*	*rbcL*
D. fimbriatum Hooker	KK	fitk	Thailand, KKC	Market	AB519776	AB519784
D. moniliforme (Linn.) Swartz	SM	mot	Tochigi Pref., SMC	Wild	AB519775	AB519786
D. moniliforme (Linn.) Swartz	n/a	mom	Mikurajima, SMC	Market	AB519773	AB519788
D. monilforme (Linn.) Swartz	n/a	mok	Kagoshima Pref., SMC	Market	AB519774	AB519787
D. nobile Lindley	KK	notk	Thailand, KKC	Market	AB519772	AB519785
D. pulchellum Roxburgh ex Lindley	KK	putk	Thailand, KKC	Market	AB519778	AB519790
D. pulchellum Roxburgh ex Lindley	HB	puth	Thailand, HBG	Market	AB519777	AB519789
D. sp. noc-a	A	noca	Okayama Pref.	Market	AB519779	AB519791
D. sp. noc-b	A	nocb	Okayama Pref.	Market	AB519780	AB519792
D. sp. noc-c	A	nocc	MPG, unknown	Market	AB519781	AB519793
D. tosaense Makino	KK	totb	Kagoshima Pref., STC	Wild	AB519770	AB519782
D. tosaense Makino	KK	tosk	Shikoku, KKC	Market	AB519771	AB519783

All voucher specimens are deposited in Satake laboratory at the Ochanomizu University

KK Karasawa, Kohji; *SM* Satake, Motoyoshi; *n/a* results matched but final identification by experts only at next flowering period, *HB* The Hiroshima Botanical Garden, ID no. 5477; *A* Hybrid Cultivars of *D. nobile*; *SMC* Satake Collection; *KKC* Karasawa Collection; *HBG* Hiroshima Botanical Garden; *STC* Seki Collection; *MPG* Medicinal Plant Garden of Showa Pharmaceutical University; *unknown* unknown origins

(Applied Biosystems) using BigDye® Terminator v1.1 and v.3.1 Cycle sequencing kits (Applied Biosystems). Each procedure using the kits was carried out following the manufacturers' instructions. The nucleotide sequences of *matK* and *rbcL* reported here had been submitted to the DDBJ/EMBL/GenBank database and their accession numbers are listed in Table 1. In this study, the obtained sequence of each gene excluding the primer sequence was considered to be the full-length sequence of the genes.

DNA sequence data analysis

DNA sequences obtained from *matK* and *rbcL* were aligned with ClustalW [20]. The Neighbor-joining (NJ) method [21] was selected for the construction of phylogenetic trees. Maximum Composite Likelihood method [22] was used for computing the evolutionary distance. The output data was processed using MEGA4 [23] to draw the phylogenetic trees. A total of 1000 bootstrap replicates were calculated for the NJ tree construction [24].

Results

First, two species classified in the Chinese pharmacopoeia, *Dendrobium fimbriatum* and *D. nobile*, were chosen. The most important medicinal *Dendrobium* in China, *D. officinale*, was not available in Japan. Therefore, we used the sequence data deposited in the DNA Database of DDBJ/EMBL/GenBank for comparison. Second, *D. moniliforme* and *D. tosaense* were chosen because these were Japanese native species that have a history of medicinal use and had been exported from Japan to China in the early twentieth century [25]. Lastly, *D. pulchellum*, which is widely found in Southeast Asia as well as in India [1, 2] and considered as a medicinal *Dendrobium*, was studied.

Phylogenetic tree analysis

For the twelve samples containing the five *Dendrobium* species (Table 1), the PCR-amplified fragments of both *matK*, including parts of the *trnK* intron, and *rbcL* genes, were sequenced. The full-length *matK* gene ranged between 1870 base pairs (bp) and 1879 bp, and that of *rbcL* was 1324 bp long. The number of nucleotides in the variable site between each pair of species is shown in Table 2.

The phylogenetic tree from the *matK* sequences including parts of the *trnK* intron showed that each species formed clearly distinctive clades (Fig. 1). *D. nobile* (notk) and three hybrid cultivars of *D. nobile* formed one clade (*D. nobile* group) although a genetic distance between notk and the other species was detected. The analysis of the phylogenetic tree constructed from *matK* without the *trnK* intron revealed that species clustering was not affected by the discarded sequence (data not shown). When using the 3′-half of the *matK* sequence without the *trnK* intron, the phylogenetic tree resulted in a clustering pattern similar to that of the full-length *matK*, although the genetic variation among *D. nobile* group was absent (data not shown). To compare the sequences obtained from the present study with other sequences deposited in the DNA database, a phylogenetic tree was constructed from partial *matK*

Table 2 Number of nucleotides in the variable site between species for full-length sequences of *matK* (upper) and *rbcL* (lower)

		matK											
		fitk	notk	mot	mom	mok	putk	puth	noca	nocb	nocc	totb	tosk
rbcL	fitk	-	19	17	17	17	14	14	16	16	16	20	20
	notk	5	-	9	9	9	23	23	3	3	3	11	11
	mot	6	3	-	0	0	21	21	6	6	6	9	9
	mom	6	3	0	-	0	21	21	6	6	6	9	9
	mok	8	5	2	2	-	21	21	6	6	6	9	9
	putk	3	2	3	3	5	-	0	20	20	20	24	24
	puth	4	3	4	4	6	1	-	20	20	20	24	24
	noca	37	38	39	39	41	36	37	-	0	0	8	8
	nocb	7	2	5	5	7	4	5	40	-	0	8	8
	nocc	6	1	4	4	6	3	4	39	3	-	8	8
	totb	3	2	3	3	5	0	1	36	4	3	-	0
	tosk	3	2	3	3	5	0	1	36	4	3	0	-

Refer to Table 1 for abbreviation of samples

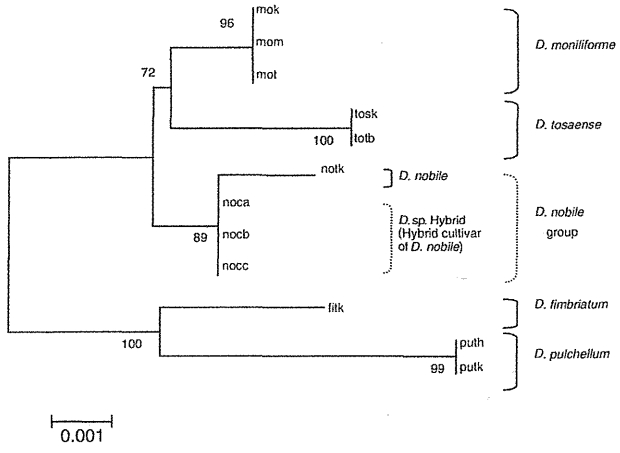

Fig. 1 Phylogenetic tree from full-length *matK* including partial *trnK* intron of 12 strains of five *Dendrobium* species. Bootstrap values (%) are shown on each branch. The indicated scale represents 0.001 nucleotide substitution per site

sequences (Nt. 537–1341, Fig. 2). In Fig. 2, most of the species formed distinct clades as shown in Fig. 1. Interestingly, sequences from *D. tosaense* and *D. officinale* excluding *D. officinale* AF445449 formed one clade.

The phylogenetic analysis using full-length *rbcL* sequences showed no species discrimination power between *D. pulchellum* and *D. tosaense* (Fig. 3). In addition, a hybrid cultivar of *D. nobile*, was not positioned within the clade of the *D. nobile* group formed from *D. nobile* and other hybrid cultivars of *D. nobile* (noc-b and noc-c). These results were also obtained in the phylogenetic tree constructed with partial *rbcL* sequences (Nt. 1–697) that were available in the database (Fig. 4). *D. officinale* (FJ216567) was located in a clade formed from *D. tosaense* but also in the clade from *D. pulchellum*.

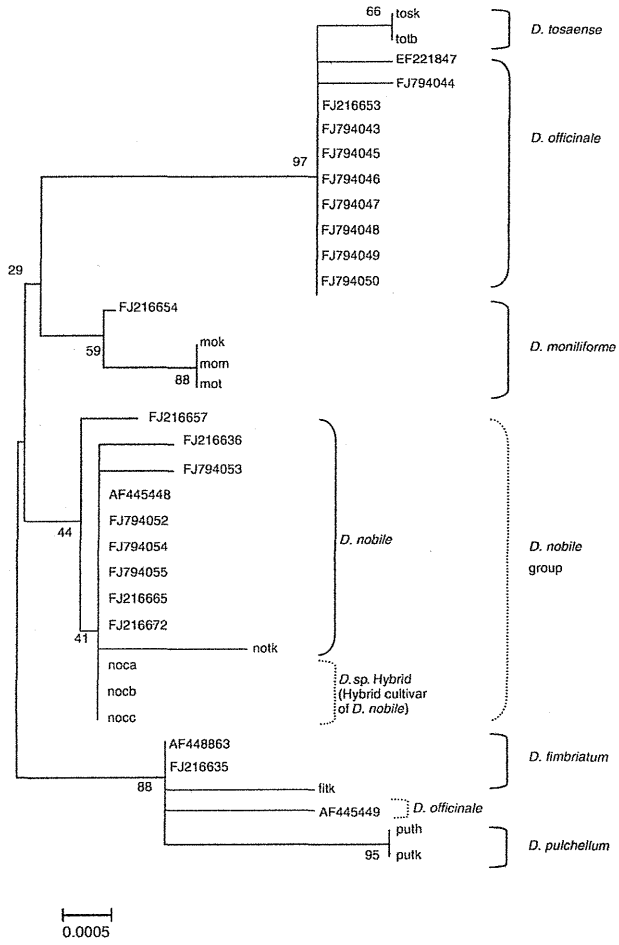

Fig. 2 Phylogenetic tree from partial *matK* (Nt. 537–1341) of 35 strains of six *Dendrobium* species. Bootstrap values (%) are shown on each branch. The indicated scale represents 0.0005 nucleotide substitution per site

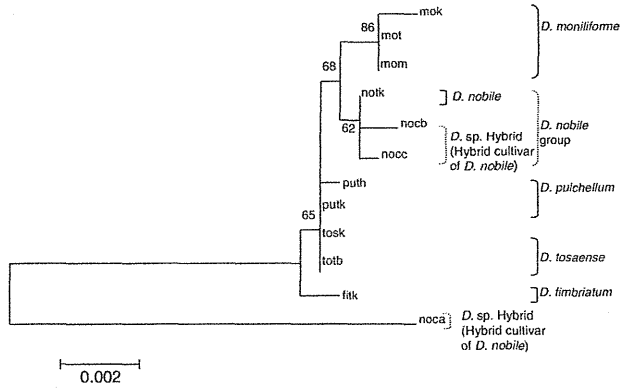

Fig. 3 Phylogenetic tree from *rbcL* of 12 strains of five *Dendrobium* species. Bootstrap values (%) are shown on each branch. The indicated scale represents 0.002 nucleotide substitution per site

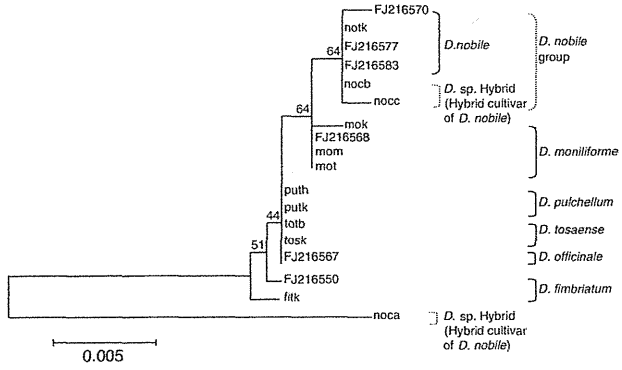

Fig. 4 Phylogenetic tree from partial *rbcL* (Nt. 1–697) of 18 strains of six *Dendrobium* species. Bootstrap values (%) are shown on each branch. The indicated scale represents 0.005 nucleotide substitution per site

Discussion

Our study showed that species identification of *Dendrobium* plants was possible using phylogenetic analyses constructed from *matK* sequences. Using the 3′-half of *matK* sequences (Nt. 944–1616), which is comparable to that of the full-length sequences, also had species discrimination power. Thus, as suggested by Lahaye et al. [14], the 3′-half of the *matK* sequence alone was useful for species identification of *Dendrobium* plants.

As shown in Fig. 1, among the species found in Honshu, Japan, *D. moniliforme* and *D. tosaense*, which seemed to have diverged later in time, were found as distinctive species in different clades. The closest species to these two species was the nobile group, although *D. nobile* formed a distinctive clade from the two species. *D. fimbriatum* and *D. pulchellum* which are morphologically categorized in the *Holocrysa* Section [1], are considered to be apart from the above three species, *D. moniliforme*, *D. tosaense*, and *D. nobile*. These three species are categorized in the *Dendrobium* Section [1]. *D. fimbriatum* and *D. pulchellum* also formed distinct clades from each other. These results demonstrate that a phylogenetic analysis using the *matK* sequence is a useful tool for the species identification of five *Dendrobium* plants.

In contrast to using the *matK* sequence, the phylogenetic tree from *rbcL* sequences had less species discrimination power. It was not possible to distinguish between *D. tosaense* and *D. pulchellum* (Fig. 3). These two species are classified as species in different sections, those of *Dendrobium* and *Holochrysa*, respectively [1]. The failure of species discrimination with the *rbcL* sequence could be due to its slow nucleotide substitution rate in comparison with other nuclear genes leading to a functional constraint that reduces the evolutionary rate of nonsynonymous substitutions [26]. From Fig. 1 and 3, we concluded that the *matK* sequence alone is probably sufficient to distinguish among these five species.

We also conducted a phylogenetic analysis of *D. officinale* and *D. tosaense* and the results pointed to the high likelihood of their being in the same clade (Fig. 2). *D. officinale* is one of the most popular and rare medicinal *Dendrobium* species in China [27], and *D. tosaense* was thought to be distributed mainly in Japan and Taiwan [28]. Based on morphological and biogeographic information, some researchers have regarded them as two different species [6, 28, 29], while Wood saw them as a common species [1]. Our phylogenetic analysis using *matK* data showed that the two species were positioned within a common clade (Fig. 2). The latest morphological and biogeographic information (refer to [2, 6, 30]) show that these two species seem to be identical and our results did not contradict this. Further sequencing of numerous data samples collected from various regions and data from traditional approaches, could finally confirm their common identity. As a whole, species identification by molecular phylogenetic analysis will help resolve the ambiguity of plant origins of crude drugs.

Regarding the *rbcL* sequence, the phylogenetic tree from the *rbcL* data showed that the hybrid cultivar, noc-a, was distantly positioned from the clade of *D. nobile* group (Fig. 3). This strain is a hybrid derived from *D. nobile* and is considered to have undergone many hybridization events, which may have caused this positioning on the phylogenetic tree.

In this investigation, the relationship between five medicinal *Dendrobium* species based on phylogenetic tree analyses constructed from *matK* and *rbcL* genes was clarified. With regard to species discrimination, *matK* rather than *rbcL* offered a higher resolution and was better suited in identifying medicinal *Dendrobium* species. Although phylogenetic analysis is a brief and rapid method, and a

powerful tool for species identification, there were still some ambiguities. Nevertheless, using a large sample of sequence data accumulated from a wide range of regions can improve the significance of the results. Overall, the use of the *matK* sequences as barcodes for the first identification process was confirmed to be very efficient. Combining data from phytochemical analysis could help standardize the species identification process of medicinal *Dendrobium* plants.

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References

1. Wood HP (2006) The *Dendrobiums*. Gantner Verlag, Ruggel
2. Baker MLB, Charles O (1996) Orchid species culture: *Dendrobium*. Timber Press, Portland
3. Bao XS, Shun QS, Chen LZ (2001) The medicinal plants of *Dendrobium* (Shi-hu) in China. Shanghai Medicinal University Press and Fudan University Press, Shanghai (in Chinese)
4. Lin P, Bi Z, Xu H, Wang Z, Xu L (2003) Advances in studies on pharmacology of plants from *Dendrobium* Sw. Chin Tradit Herb Drugs 34(11)Suppl.:19–22 (in Chinese)
5. Chinese Pharmacopoeia Committee of People's Republic of China (ed) (2005) The Pharmacopoeia of the People's Republic of China: I. Chemical Industry Press, Beijing (in Chinese)
6. Tsi Z, Chen S, Luo Y, Zhu G (eds) (1999) Flora, Reipublicae Popularis Sinicae. Angiospermae, Monocotyledoneae, Orchidaceae (3), Tomus 19. Science Press, Beijing (in Chinese)
7. Institute of Botany, Academy of Science (ed) (1976) Iconographia Cormophytorum Sinicorum Tomus V. Science Press, Beijing (in Chinese)
8. Hilu K, Liang H (1997) The *matK* gene: sequence variation and application in plant systematics. Am J Bot 84:830–839
9. Wilson CA (2004) Phylogeny of Iris based on chloroplast *matK* gene and *trnK* intron sequence data. Mol Phylogenet Evol 33:402–412
10. Kress WJ, Erickson DL (2007) A two-locus global DNA barcode for land plants: the coding *rbcL* gene complements the non-coding *trnH-psbA* spacer region. PLoS ONE 2:e508
11. Hasebe M, Omori T, Nakazawa M, Sano T, Kato M, Iwatsuki K (1994) *rbcL* gene sequences provide evidence for the evolutionary lineages of leptosporangiate ferns. Proc Natl Acad Sci USA 91:5730–5734
12. Fazekas AJ, Burgess KS, Kesanakurti PR, Graham SW, Newmaster SG, Husband BC, Percy DM, Hajibabaei M, Barrett SCH (2008) Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. PLoS ONE 3:e2802
13. Steven GN, Subramanyam R (2009) Testing plant barcoding in a sister species complex of pantropical Acacia (*Mimosoideae*, *Fabaceae*). Mol Ecol Res 9(Suppl. 1):172–180
14. Lahaye R, van der Bank M, Bogarin D, Warner J, Pupulin F, Gigot G, Maurin O, Duthoit S, Barraclough TG, Savolainen V (2008) DNA barcoding the floras of biodiversity hotspots. Proc Natl Acad Sci USA 105:2923–2928
15. CBOL Plant Working Group (2009) A DNA barcode for land plants. Proc Natl Acad Sci USA 106:12794–12797
16. Hebert PD, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proc Biol Sci 270:313–321
17. Vogler AP, Monaghan MT (2007) Recent advances in DNA taxonomy. J Zool Syst Evol Res 45:1–10
18. Chase MW, Salamin N, Wilkinson M, Dunwell JM, Kesanakurthi RP, Haidar N, Savolainen V (2005) Land plants and DNA barcodes: short-term and long-term goals. Philos Trans R Soc Lond B Biol Sci 360:1889–1895
19. Topik H, Yukawa T, Ito M (2005) Molecular phylogenetics of subtribe Aeridinae (Orchidaceae): insights from plastid *matK* and nuclear ribosomal ITS sequences. J Plant Res 118:271–284
20. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
21. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
22. Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 101:11030–11035
23. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599
24. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
25. Suzuki H, Keimatsu S, Ito K (1934) Nachtrag zum "Chin-Shih-Hu". Yakugakuzasshi 54(9):820–823 (in Japanese)
26. Wolfe KH, Li WH, Sharp PM (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. Proc Natl Acad Sci USA 84:9054–9058
27. Ding G, Zhang D, Feng Z, Fan W, Ding X, Li X (2008) *SNP*, *ARMS* and *SSH* authentication of medicinal *Dendrobium officinale* KIMURA et MIGO and application for identification of Fengdou drugs. Biol Pharm Bull 31(4):553–557
28. Kimura K (1936) Pharmacological study of Dendrobiinae plants as the Chinese drug shih-hu. J Shanghai Sci Inst Sect III 3(Suppl. 1):1–60 (in Japanese)
29. Kimura K, Migo H (1936) New species of *Dendrobium* from the Chinese drug shih-hu. J Shanghai Sci Inst Sect III 3:121–124
30. Karasawa K (2003) Species Orchidacearum I text. Japan Broadcasting Publishing, Tokyo (in Japanese)

インドシナ半島のケシ代替植物プロジェクト

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1 はじめに

タイ北部の標高2,000 mほどの山岳地帯にある閑静なメオソルト村を初めて訪れたのは，1968年12月であった．村の高台に外観は綺麗な丸木小屋が何軒かあり，その中は真っ暗で，床には5，6名の人が静かに横たわっていた．部屋全体に生臭いアヘン特有の匂いが立ち込めており，よく見ると竹でできた水タバコのパイプのようなものを持ってぷかぷか煙を出していた．ここは阿片窟だった．この地域一帯の住民たちはケシの栽培で生計を立てており，その多くが阿片中毒者であった．それから20年後，再度この村を訪れた．村は大変な変わりようで，畑ではケシの替わりに切り花が作られ，観光客が花園を鑑賞していた．これはタイ国王プミポン殿下の肝いりのケシ撲滅計画，キングズ・プロジェクトの成果であることを知った．この経験から，私はミャンマーでも「ケシ栽培をなくすことができる」と確信した．

1999年，日本の麻薬対策の1つにミャンマーでのケシ撲滅計画があり，国連麻薬統制委員会からの要望に沿ってそのプロジェクトの具体的な計画を提出することになった．私は筑波薬用植物栽培試験場で得たケシの栽培研究に関する経験から，ケシの代替植物に薬用植物を提案した．まず，国内の薬用植物需要動向を調査し，30品目をミャンマーでの栽培候補に選んだ．この内容を国立衛研の麻薬課長がウイーンの国連麻薬統制委員会で報告したところ，予想外の好反響があり，2000年，定年を控えた私は薬務局長からミャンマー行きを要請された．

2001年，私のミャンマー行きは，後に最良の友となる我妻 豊 氏(当時，読売新聞ミャンマー緑化計画担当者)との出会いにより決定的になった．彼は，約10年間のミャンマーにおける活動から豊富な人脈を持ち，語学が堪能である点で，私の計画における最適のパートナーとなった．早速，具体的な行動計画を作り上げた．そして2001年3月，厚生労働省からの資金援助のないまま，最初のミャンマー訪問を決行した．ミャンマーでは，林業大臣が私の計画を全面的に支持してくれた．ここに，ミャンマープロジェクトが本格的に開始された．

2 背景

1. なぜ，ミャンマーはケシの不法栽培をするのか

ミャンマー最大の問題点は，少数民族と中央政府との平和協定に秘められた貧困問題にあり，我妻氏はその解消策を模索していた．代替植物による試みは国連のトウモロコシ栽培と日本政府のソバ栽培であったが，少数民族の多くがケシ栽培で生計を維持している現実から，十分な成果は得られなかったのである(図1)．

2. ケシの代替植物はあるのか

ケシ栽培で得られる収益は，少数民族の独立のための兵器購入と経済的自立に使われる．地下資源豊富な一部の地域では，ルビーやヒスイの鉱山収益を当てたところもあるが，代替植物プロジェクトはほ

図1 カチン州での焼畑でのケシ栽培(現在は薬用植物栽培が行われている)

とんど不成功だった．その理由の1つは，皮肉なことに代替植物を提案し持ち込む国際組織のプロジェクト運営に対する多額の資金提供にあった．この大きな資金はもっぱら貰い得の人たちに渡り，少数民族の人たちの利益には決して繋がらなかったのである．航空写真でケシ栽培がなくなったことが明示された地域に隣接する畑では，ケシ栽培がなくなった土地と同程度の面積でケシが栽培されていた．ケシ撲滅を条件に橋が架けられ，学校ができ，道路が舗装される．しかしケシ栽培が完全になくなった後は何も援助されないことを，地元の指導者達は熟知していたのである．

そこで，まずは高価な薬用植物として薬用ニンジンの栽培を選んだ．また，それ以外の様々な薬用植物も導入して，適応性についていろいろな地域で検討した．生薬の生産だけではなく，地元で消費されそうな薬用果樹も試みた．しかし，ケシのように簡単に換金できる植物はない．ケシの撲滅とともに，少数民族の人たちが自立して生活するためには，劣悪な生活環境と教育環境の改善を視野に入れながら，自ら有用植物の栽培技術の習得に取り組むプロジェクトが必要であると考えて，以下の活動を10年にわたり展開してきた．

3 プロジェクト第一期(2001～2005年)

1. ミャンマーで活動開始

我妻氏はミャンマーの軍事内閣に人脈があり，政府の林業大臣を2001年3月に訪問し，研究事業計画を作成した．私もミャンマー林業省の幹部会議に出席し，意見交換を行った．経済担当局長からは，ODAなどの大型資金がないと実施困難ではないかという意見が出たが，少ない予算であっても両国の努力次第で成果は上げられると話したところ，林業大臣から理解が得られ，担当は森林局長とするとの決定が下された．ここで事業計画を作成，調印後，いよいよ実行段階に入った．2002年4月2日にミャンマー政府は活動許可書を閣議了解し，私たちはミャンマー国内で自由に活動ができるようになった．

2. 活動拠点の決定

林業省は，私たちの活動拠点を北部カチン州の2か所(ガイテイ地区とセイロン地区)と決定し(図2；濃い色の部分がカチン州)，30 haの土地を提供するとともに，同省の職員同行を認めてくれた．早速，ガイテイ地区のケシ栽培していた焼畑跡地に研修薬草園を開設し，日本から持ち込んだ約30種の薬草を植えた(表1，図3)．翌年，セイロン地

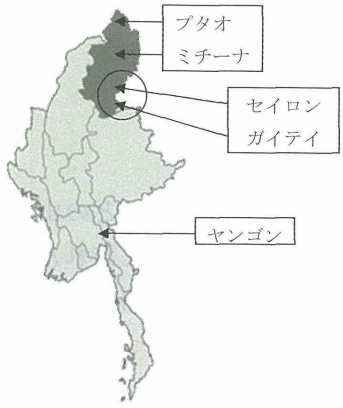

図2　ミャンマーの活動地区

表1　日本国内で使用されている主な生薬のミャンマーにおける生育状況(2001～2005年)

	生薬名		植物名	成育状況
1	ウイキョウ	茴香	ウイキョウ	良い
2	オウゴン	黄芩	コガネバナ	良い
3	オウバク	黄柏	キハダ	良い
4	オウレン	黄連	セリバオウレン	良い
5	ガジュツ	我朮	ガジュツ	良い
6	カミツレ	カムツレ	カミツレ	良い
7	カンゾウ	甘草	カンゾウ	やや良
8	キキョウ	桔梗	キキョウ	良い
9	キクカ	菊花	キク	良い
10	ケイヒ	桂皮	シナニッケイ	良い
11	コウカ	紅花	ベニバナ	良い
12	サンキライ	山帰来	サンキライ	良い
13	サンシシ	山梔子	クチナシ	良い
14	サンショウ	山椒	アサクラザンショウ	良い
15	シコン	紫根	ムラサキ	やや困難
16	シャクヤク	芍薬	シャクヤク	良い
17	シャゼンソウ	車前草	オオバコ	良い
18	シュクシャ	縮砂	シュクシャ	良い
19	ソヨウ	蘇葉	シソ	良い
20	ダイオウ	大黄	ダイオウ	困難
21	タイソウ	大棗	ナツメ	良い
22	チクセツニンジン	竹節人参	トチバニンジン	良い
23	トウキ	当帰	トウキ	良い
24	ナンテンジツ	南天実	ナンテン	良い
25	ハンゲ	半夏	カラスビシャク	良い
26	ビャクジュツ	白朮	オケラ	良い
27	ボタンピ	牡丹皮	ボタン	栽培中
28	ヤクチ	益智	ヤクチ	良い
29	ヨクイニン	薏苡仁	ハトムギ	良い
30	リョウキョウ	良姜	リョウキョウ	良い

図3　ガイテイ農園全景

図4　セイロンの栽培研修用の薬草園

図5　オウレンの植え付けを指導する京都薬大の後藤氏

区にも研修薬草園を作り，100種の薬草と果樹を導入した(図4)．薬草園作りは日本人専門家の指導の下に，現地の林業省の職員と地元の人たち(ケシ栽培者も含めた)とともに栽培を開始した．

3．植栽植物の生育状況と研修開始

両地区でほとんどの植物が良く育ったが，一部は暖かすぎたせいか枯れてしまった．生育結果からミャンマーに適する植物が分かってきたので，第一候補としてベニバナ，ハトムギ，ウイキョウを選び，本格的生産を開始した．一方，地元の野生植物にもニンジン類(人参)，ツクバネソウ類(重楼)，ナルコユリ類(黄精)，薬用ラン(石斛)など重要なものが多く，これらの栽培も大きな目標となった．農園周辺の人たちは，薬草の花が咲きミカンやブドウの実が実ると，これらの栽培にますます興味を持つようになった．

地元でも原住民を研修指導員として育成し，その中から林業省の職員に採用して栽培研修指導に当たらせた．さらに，各地のケシ栽培者のグループから研修の希望が寄せられた．また，少数民族の族長から研修者を派遣したいとの要望とともに，指導員候補者が派遣された．これらの人たちが，今ではカチン州全体への代替植物導入のリーダーとして活躍している．

4．日本からの栽培指導者派遣

日本からは我妻氏が派遣研究員として半年間，8回にわたり継続的な指導を行った．また私たち以外の栽培指導者は，日漢協及び京都薬科大学薬草園に所属するメンバーであった(図5)．日本では薬用果樹の専門家育成のために，筑波，天童及び甲府でミャンマーの研修生を受け入れた．特に，天童市の果樹農家で接木，剪定，日常管理など，接木の技術習得を目的とした研修を実施した．

5．第一期の成果

ミャンマー林業省との5年間の契約の成果を，以下にまとめた．

① 代替植物として薬用植物・果樹が生産可能であることが分かった(表1)．

② ニンジン類(人参)，ツクバネソウ類(重楼)，ナルコユリ類(黄精)，カギカズラ類(釣藤鈎)など，国際マーケットに適する現地薬用植物を見いだした．なお薬用ラン(石斛)も，無菌培養による増殖方法が確立できれば有望である．

③ カチン州のセイロン地区では，種苗の供給，栽培指導，流通面での積極的な支援が必要であることが分かった．

④ 南部のガイテイ地区は，熱帯植物の栽培に適することを明らかにした．

⑤ 育成された植物や苗をメイミョウ地区へ移植後，そこから各地へ種子や果樹の苗木を供給した．また，剪定技術の指導等を行った．

上記の他に学術的な成果として，カチン州における野生薬用植物の分布調査と化学成分の同定を行った．キノコ類のニセショウロ属 *Scleroderma* sp. を採集し，栽培と成分の検討を行った．ユリ科ツクバネソウ属 *Paris* sp. は中国の重楼と呼ばれる生薬に類し，その成分としてステロイドサポニンを単離した．ナルコユリ属 *Polygonatum* sp. は葉の先を鉤状に巻き込む特徴があり，黄精に類似する(図 6)．ウコギ科 Araliaceae のチクセツニンジン属 *Panax* sp. は，地上部はトチバニンジンに類似し根茎も竹の節状に横走しているが，主根茎部分がやや大きい特徴があった(図 7)．成分として，ニンジンサポニン G-Rb_1，G-Rc，G-Rd，G-Rg_1，チクセツサポニンⅤとⅣを確認したが，G-Re 及びマジョノサイド R_2 は検出しなかった．ブータン産のものと類似傾向が見られ，周辺のベトナムニンジン，デンシチニンジン，ニンジンとは異なっていた．その他，ナルコユリ属 *Polygonatum* sp.，ウリ科 Cucurbitaceae の *Hodgsonia macrocarpa*(油瓜と思われる)，及びラン科 Orchidaceae のセッコク属 *Dendrobium laciniosum*, *D. aphyllum* 及び *D. crystallium* を同定した．これらミャンマー産のランは，日本での繁殖を試みている．なおその実施にあたり，現地栽培品であることを示すミャンマー政府の証明書(CITES)が発行されている．


図 6　野生の重楼 *Paris* sp.


図 7　ミャンマーの野生の *Panax* sp.

これら第一期の成果を受け，第二期では多くの植物を北部の中央研修薬草園に移植し，実用的な栽培計画を企てた．栽培可能な薬用植物として，ベニバナ，ハトムギ，ウイキョウ，ミシマサイコ，トウキ，センキュウなどを選定した．これらの販売ルートとして，中国市場及び中国を経由する日本市場を考えたからである．

4　プロジェクト第二期(2006～2010 年)

1. 実用栽培試験

カチン州中部と北部を，実用栽培拠点として選定した(図 2)．同地域は資源環境が保存され，少数民族の人たちの貧困追放には最適の場所である．栽培可能な植物の実用栽培として，ミチーナ付近の中洲でベニバナ栽培を開始した(図 8)．また，ソバの栽培と乾麺の生産が可能になった．最北部のプタオでは，ブドウ，モモ，チャノキの栽培を開始し，また薬用植物として，漢方薬の原料となる植物の試験的栽培を開始した．なお，カチン州は第二次世界大戦で日本軍が侵入し，多数の兵士が死亡した地域である．ここには，日本軍と戦った長老達がいまだ健在である(図 9)．カチン族の部族長の協力体制も確立した(図 10)．

2. 植物調査研究・新しい資源植物の研究

インドジー湖(ミャンマー最大の湖)の植物調査を実施した．湖畔には，イネの原種とハトムギの原種


図 8　ベニバナの摘み取り

図9　日本の軍歌を歌ってくれたピンチョウさん(92歳)

図10　カチン族長との交流

が生育していた．イネは水面下数メートルに達し，浮稲といえる生態型である．ハトムギはイネと同様に，茎は太く多孔質であった．雌しべの柱頭は赤色であり，成熟した果実は黒色の外面にストライプは見られず，硬いものであった．一方，東ヒマラヤの雪山を望む川沿いに，バナナが野生していた．背の高い種と背の低い種で，花序の形と種子，果実に大きな違いが見られた．

ミャンマーのケシ栽培地の代替植物として栽培を検討している薬用植物 *Diospyros burmanica* の有効利用について検討した．まず，熱帯リーシュマニア原虫に対する殺虫活性を検討し，有用な成分を見いだした．チークの葉にも活性が見られた．[1]

医薬資源としてミャンマーからセッコク(石斛)の供給を期待して，ミャンマー国内，中国市場，日本の種の生薬学的調査を行った．ミャンマーで入手した薬用種 *Dendrobium finbriatum*，*D. pulchellum* 及び日本産のセッコク *D. moniliforme*，キバナセッコク *D. tosaense*，園芸品種の *D. nobile* の特性をそれぞれ明らかにした．[2]

3．生活環境の調査研究・伝統医療の推進

生活環境や教育環境の改善も本プロジェクトの重要なテーマであり，ミャンマーにおける米の調理方法や食生活の調査，生活周辺の水環境の調査，生糸やハス繊維と伝統的な天然染料の調査，有用植物資源調査及び特異な化粧文化の調査を行った．一方，山奥にあるコンニャクの原種を採取し，加工製品作りを指導した．

日本財団とともに東南アジア諸国連合(ASEAN)加盟国の伝統医薬振興に努め，カンボジアの薬用植物園創設に向けた協力，伝統医薬の配置薬方式による配布，カンボジアの伝統医師の資格制度の確立，ミャンマー薬局方の制定などへの協力を行っている．また最北のプタオでは，伝承薬医マチャンボ氏から現地の薬草に関する聞き取り調査を行った．

4．違法薬物の流通経路の解明

国際的に押収された違法薬物の流通経路を解明するために，押収物の化学分析を行い，押収物の成分構成を年次ごとに報告している．[3] また，MDMAの合成原料であるサフロールを多く含む植物 *Cinnamomum pathylcathum* の生態調査も行っている(図11)．

5　おわりに

本事業の広報活動として，ミャンマーフォーラムを日本で開催している．第1回は虎ノ門発明会館で，2〜9回はお茶の水女子大学で行った．毎回，プロジェクトに興味を持つ100〜200名が参加し，様々な情報交換が行われている．本フォーラムは，日本のミャンマー関係者の連絡拠点としての役割を果たしている．第9回フォーラムには，インパール作戦に参加された軍医の塩川優一先生(元 順天堂大学付

図11　カンボジアの *Cinnamomum pathylcathum*

属病院長)と桑木崇秀先生(千代田漢方クリニック,元 北里大学東洋医学研究所臨床部長)をお招きした.

本プロジェクトは2014年まで継続予定であり,これまでの活動を更に発展させていきたい.例えば,ミャンマー国民に自国の薬用植物について正確に把握してもらうために薬用植物図鑑(Medicinal Plant in Myanmar, 2002年, 2009年)[4]を作成し,広く配布した.

ミャンマーは政治的に不安定であり,予定した計画の変更を余儀なくされる場合もある.しかし本プロジェクトは,相手国政府の活動許可書により,現地では比較的自由に活動できる.また政府の理解の下に行動できるため,女子学生でも安心して調査活動が展開できる.最近ミャンマー北部の平和協定軍政府から,ケシ栽培地の非政府組織地域内での指導を要請された.ミャンマー政府もこれを認めたので,異例なことではあるが,私たちの活動が非政府軍地域でもできるようになった(図12).

当初,ミャンマーに限定した活動も,タイ,ラオス,カンボジアと周辺諸国や地域に広がった.またケシ代替植物をキーワードとするプロジェクトが,いつのまにか覚せい剤やMDMAの流通ルートの一掃にも寄与した.10年間の継続により,国内における違法薬物追放にまで貢献できた.これからも多くの人たちの協力のもと,更によい成果を挙げていきたいと願っている.[5]

図12 山岳地域の薬草園への協力者一行

引用文献

1) Mori K. *et al*., *J. Nat. Prod*., 71, 18-21 (2008).
2) Asahina H. *et al*., *J. Nat. Med*., 64, 133-138 (2010).
3) Kurashima N. *et al*., *Forensic Sci. Int*., 189, 14-18 (2009).
4) Htun N., Satake M., "Myanmar Medicinal Plants," 2009.
5) 藤井基之, "亡国のドラッグ," 医薬経済社, 東京, 2010.

新刊紹介

「健康とくすりシリーズ」 食べものとがん がんを遠ざける食生活

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日本における死亡原因の1位はがんであり,この10年間変わっていない.近年では,3人に1人ががんで亡くなっている.このためか,各種メディアにおいて「がん」「生活習慣」「食事」,これらの文字の躍らない日はないといっても過言ではない.とりわけ,日々口にする食品とがんとの関係については関心が高く,がんの予防効果を謳う記事や番組をしばしば見かける.しかしながら,信頼性の高い情報とそうでない情報が,入り混じって氾濫しているのが現状であるといえる.

本書は「食べものとがん」に関し,現状において明らかとなっている正しい知識を提供することを目的として,疫学研究に基づいた情報と,そこから導き出された判断について分かりやすく解説されている.科学的根拠とは何か,国際的評価により発がんリスクとの関係が確立されている食品や食品成分,まだ評価は確立していないものの近年の研究により解明されつつあるもの,抗酸化栄養素のサプリメントによるがん予防,さらには日本人のためのがん予防法について,章ごとに分かりやすくまとめられており,エビデンスに基づいたがん予防の食生活について,一読で理解することができる.

誰しもがんは避けたいと願っている.無論,筆者もそうである.本書の副題のとおり,少しでもがんを遠ざけるため,今日から最終章のがん予防法を実践したいと思う.

池田幸弘 Yukihiro IKEDA

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Book Review