European Journal of Integrative Medicine 32 (2019) 100987

Contents lists available at ScienceDirect

European Journal of Integrative Medicine

journal homepage: www.elsevier.com/locate/eujim

Research paper

DNA-based phylogenetic analysis of mugwort for moxibustion from Japan, China, and South Korea

Takeshi Matsumoto[a,b,*,1], Shuichi Katai[c,d,1], Masaya Ando[e,1], Hitoshi Watanabe[e,1]

[a] Chiba University Hospital, 1-8-1 Inohana, Chuo-ku, Chiba-shi, Chiba, 260-8677, Japan
[b] Kashiwanoha Acupuncture and Moxibustion Clinic of Chiba University, 6-2-1Kashiwa-no-ha, Kashiwa City, Chiba, 277-0822, Japan
[c] Tsukuba University of Technology, 4-12-7 Kasuga, Tsukuba City, Ibaraki, 305-8521, Japan
[d] DO-HO Park Acupuncture and Moxibustion Clinic, 2-17-10 Ninomiya, Tsukuba City, Ibaraki, 305-0051, Japan
[e] Center for Environment, Health and Field Sciences, Chiba University, 6-2-1Kashiwa-no-ha, Kashiwa City, Chiba Prefecture, 277-0822, Japan

ARTICLE INFO

Keywords:
Moxafloss mugwort
Japan
China
South Korea
DNA

ABSTRACT

Introduction: Moxibustion, along with acupuncture, is an important therapeutic approach in traditional East Asian medicine. As such, the burning of moxa floss on the skin to thermally stimulate body surfaces.

Presently, moxa floss is manufactured from mugwort leaves mainly in Japan, China, and South Korea. The genus *Artemisia*, to which the mugwort belongs, reportedly includes more than 250 wild species in the world and more than 30 in Japan.

This study was conducted to characterize Japanese moxa floss by identifying various varieties of moxa floss mugwort (MFM) material collected from leading places of moxa floss production in the world by DNA-based phylogenetic analysis.

Methods: DNA was extracted from Japanese mugwort (9 families), South Korean dry mugwort (1 family), and Chinese dry moxa floss (4 types), and the nuclear DNA ITS and chloroplast DNA*rpl32-trnL*, *trnQ-5′rps16*, and *trnH-psbA* regions were sequenced.

Results: All Japanese mugwort samples were found to have the same DNA sequence in the ITS region; there were no location-specific sequences. Chinese and South Korean mugwort samples were found to have species-specific mutations, and their mutated sequence was found to be identical to the sequence of*A. argyi*, a species remote from Japanese *Artemisia*.

Discussion/conclusions: By identifying the various MFM varieties in Japan as the same species, this study showed that Japanese moxa floss can be characterized not only by manufacturing process, but also by raw material. The identification is also believed to provide basic information to understand the historical changes of places of MFM production.

1. Introduction

Being East Asian traditional medicine, oriental medicine includes acupuncture and moxibustion, the latter of which is a type of thermotherapy. The heat generated by burning moxa floss* has long been used as the heat source for moxibustion, with mugwort leaves serving as the raw material for moxa floss. Since they are also used as a material for Kampo medicines, mugwort leaves are indispensable for Oriental medicine. The members of the genus *Artemisia* are perennials of the family *Asteraceae*. More than 250 species [1] are distributed throughout the world, and there are more than 30 species in Japan [2]. The Japanese mugwort (*Artemisia princeps* Pamp, hereinafter referred to as "*A. princeps*") has long been popular in Japanese culture. The mugwort used as the raw material for moxa floss for moxibustion treatment is collected at only a few places in Japan. In addition, the method of moxa floss production [3] and the purity ranking system differ among China, South Korea, and Japan, depending on natural features and therapeutic approaches.

Descriptions of moxibustion are available in the Chinese classical literature. Mention that the mugwort was used as a heat source of moxibustion is made in 'Prescriptions for Fifty-two Diseases,' unearthed from the ancient Mawangdui tombs in current Changsha City, Hunan

*Abbreviations:*MFM, moxa floss mugwort

* Corresponding author at: Chiba University Hospital, 1-8-1 Inohana, Chuo-ku, Chiba-shi, Chiba, 260-8677, Japan.
E-mail address: t.matsumoto@faculty.chiba-u.jp (T. Matsumoto).

[1] All research was conducted by the authors.

https://doi.org/10.1016/j.eujim.2019.100987
Received 26 May 2019; Received in revised form 13 October 2019; Accepted 14 October 2019

Province, China, which were built in the early period of the Western Han dynasty in the 2nd Century BC [4]. Also, in 'Miscellaneous Records of Famous Physicians,' published in the 5th Century [5], "gaiyo (Artemisia Leaf)" is described as a material of moxibustion. Published later, in 1596, 'Compendium of Materia Medica' by Li Shizhen [6] describes the mugwort from current Qichun County, Hubei Province, as a suitable material. As such, the mugwort produced in that location is even now highly valued, itself a brand product in China. In addition, Nanyang City, Henan Province, China, a place noted in connection with the Chinese physician Zhang Zhong-jing, is known for large production of moxa floss [7]. These two locations are representative of moxa floss production in China.

In South Korea in recent years, large amounts of moxa floss have been imported from China; Ganghwa Island is the only place in South Korea where mugwort is cultivated and produced in large scale. Two mugwort cultivars "Sajabal and Sajual" are grown there. Several studies reported that their components differ from those of mugwort grown in other locations [8–13]. These cultivars are cultivated under the guidance of the national government.

A historical view of moxa floss production in Japan shows that the collection and the production were prevalent in and around Mt. Ibuki in Shiga Prefecture roughly by the Late-Edo period (1830) [14], with the moxa floss produced there being popular, with the brand name "Ibuki Moxa." The brand name is still used commercially, even after the production site moved near to current Itoigawa City in Niigata Prefecture.

Japanese moxa floss mugwort** (MFM) is characterized by high operating efficiency, because it has a mild odor, is soft to the touch, and is easy milled, and is advantageous compared with other varieties as larger yields are expected with its large leaves (Fig. 1). Another reason for their high evaluation is their richness in trichomes (white hairs on the backs of leaves).

Artemisia montana Pamp, a large-leaved mugwort species, also occurs in Japan, and is commonly distributed in Hokkaido. Having hard, deeply lobed leaves, it is said to be unsuitable for moxa floss production because of its hardness.

Oriental medicine, which originated in ancient China and was transmitted to Japan via the Korean Peninsula, has been established as a part of Japanese traditional culture while influencing and being influenced by the Japanese culture and history in various eras. In addition, moxa floss manufacturing processes and modes of use have changed, apparently depending on the climate and other natural features of the places of collection of the mugwort as the raw material for the moxibustion tool, moxa floss.

Varieties of MFM have so far been distinguished merely based on morphological differences. The objective of this study was to characterize Japanese moxa floss in terms of not only manufacturing process, but also raw material, by identifying species of mugwort for moxa floss from three countries under different circumstances, i.e., China, South Korea, and Japan, by DNA analysis. With this aim, we attempted to characterize families of mugwort for moxa floss in and outside Japan by phylogenetic analysis.

Fig. 1. Japanese large-leaved mugwort as the raw material for moxa.

*The term "moxa floss" as used in this paper is defined as "a cotton-like material for moxibustion made from mugwort leaves" in the WHO International Standard Terminologies on Traditional Medicine in the Western Pacific Region (WHO IST) [15].

In addition, "mugwort" refers to the Artemisia leaf. It is referred to as "moxa" in the WHO IST [15].

**The term "Moxa floss mugwort" refers to the mugwort growing naturally and gathered for moxa production in Japan.

2. Materials and methods

2.1. Mugwort plants studied

Moxa floss and raw material mugwort samples were collected from various countries and processed to extract DNA. The Japanese samples consisted of young leaves of 9 families, designated as "JA001—009," morphologically identified as mugwort naturally occurring in Niigata Prefecture (Fig. 2). The South Korean sample consisted of 1 family, "KO001," of dry mugwort of the cultivar Sajabal cultivated on Ganghwa Island. The Chinese samples consisted of 1 type, "CH001," of moxa floss purified from dry mugwort collected in Nanyang City, Henan Province, 1 type, "CH002," purified from dry mugwort collected at Mt. Yimeng in Shandong Province, and 2 types, that of manufacturer A, "CH003," and that of manufacturer B, "CH004," purified from dry cultivated mugwort samples in Qichun County, Hubei Province.

2.2. Species identification

Species were identified by comparing the DNA sequences of the test materials with GenBank-registered DNA sequences and the DNA sequence of the *Artemisia argyi* purity test reagent from FUJIFILM Wako Pure Chemical Corporation, Ltd. (Osaka, Japan) (hereinafter referred to as "*A. argyi*").

DNA sequences were determined by direct sequencing. About 20 mg of moxa floss or mugwort was frozen with liquid nitrogen, after which DNA was extracted using a NucleoSpin Plant II Kit (MACHEREY-NAGEL). Various regions of the extracted samples (ITS, *rpl32-trnL, trnQ-5′rps16, trnH-psbA*) were then amplified by PCR using a universal primer. Amplifications were performed using a KAPA Taq EXtra PCR Kit (KAPA Biosystems) and confirmed by agarose gel electrophoresis. Amplification products were purified using ExoSAP-IT (Affymetrix). The purified products were each subjected to a cycle-sequencing reaction using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and their sequences were then analyzed using an ABI 3500 Genetic Analyzer (Applied Biosystems).

3. Results

1 In the ITS region, the leaves of the 9 families of morphologically identified MFM samples collected in Niigata Prefecture in Japan were found to share the same DNA sequence. The sequence was found to be identical to the GenBank-registered sequence of *Artemisia princeps* Pamp. [JX051726] (Table 1). No region-specific sequences were found.

The Chinese and South Korean mugwort samples, unlike the Japanese mugwort, were shown to have a common sequence at seven sites, which was found to be identical to the GenBank-registered sequence of *A. argyi* [JX051681] and the reagent from FUJIFILM Wako Pure Chemical Corporation.

This finding, along with morphological aspects, demonstrates that the Chinese and South Korean MFM samples and the Japanese samples were from far-related species.

2 With regard to chloroplast DNA (*trnQ-5′rps16, trnH-psbA*), the

Fig. 2. Mugwort produced in Niigata Prefecture, Japan subjected to DNA-based phylogenetic analysis.

Chinese and South Korean mugwort samples and the Japanese mugwort samples had different DNA sequences in the *trnH-psbA* region at site No. 309. With regard to the *trnQ-5′rps16* region, distinct differences were found at site Nos. 672–675 among the Chinese, South Korean, and Japanese mugwort samples (Table 2).

In this region in GenBank-registered sequences, the sequence was found to be identical to that of *A. argyi* [NC_030785], with the exception of sample CH004.

3 The Chinese and South Korean moxa floss and mugwort samples were found to have different sequences in the chloroplast DNA: the *rpl32-trnL* region differed among different localities and manufacturers (Table 3). The GenBank-registered sequence and the reagent sequence also showed differences in the same *A. argyi*.

A comparison with the GenBank-registered sequence revealed a common mutation between the Japanese mugwort and *A. princeps* [MF034021].

4. Discussion

In Japan, a wide variety of mugwort plants grow naturally, and have long been classified according to their morphological characteristics. Mugwort leaves delivered by collectors to moxa floss factories comprise a wide variety of morphologically different mugwort types in mixture; the mugwort species used in the products were unknown.

In “Tenkyu treatment” (also known as direct moxibustion, in which a rice-grain-size portion of d moxa floss is combusted on the skin), a moxibustion technique unique to Japan, highly purified moxa floss with a yield of 3% (30 g of moxa floss produced from 1 kg of mugwort) is

Table 1
Nucleotide sequence variation and the classification of ITS.[a]

specimen vouchers[c]	Collection site	*ITS*																			
		34	46	50	61	76	86	102	109	201	209	238	240	445	507	521	524	591	602	619
JA001～009	Japan Niigata	C	T	T	T	G	C	G	C	T	T	C	T	C	C	T	C	A	A	C
CH001	China Henan	C	C	C	C	G	C	G	C	Y	C	C	C	C	C	C	C	G	G	T
CH002	China Shandong	C	C	C	C	G	C	G	C	T	C	C	C	C	C	C	C	G	G	T
CH003	China Hubei-A	T	C	C	C	A	C	G	T	Y[b]	C	C	C	C	C	C	C	G	G	T
CH004	China Hubei-B	C	C	C	C	G	C	G	T	Y	C	C	C	C	C	C	C	G	G	T
KO001-SAJA	Korea Ganghwado	T	C	C	C	A	C	G	T	T	T	T	C	T	T	C	T	G	G	T
A. princeps (JX051726)	Gen Bank	C	T	T	T	G	C	G	C	T	T	C	T	C	C	T	C	A	A	C
A. argyi (JX051681)	Gen Bank	T	C	C	C	A	C	A	T	T	T	T	C	T	T	T	T	G	A	T
A. argyi (AR)	FUJIFILM Wako Pure Chemical Co., Ltd.	C	C	C	C	G	C	G	C	T	C	C	C	C	C	C	Y	G	G	T

[a] Number indicates position of the variable site in intergenic region based on the sequence of JA001.
[b] Y = T or C.
[c] “JA”fresh leaves, “CH”,“KO”dry specimen belong to Chiba University.

Table 2
Nucleotide sequence variation and the classification of cpDNA (trnQ-5′rps16, trnH-psbA).[a]

specimen vouchers[c]	Collection site	*trnQ-5′rps16*			*trnH-psbA*		
		245	268	672 -675	272	274	309
JA001 ~ 009	Japan Niigata	A	A	–[b]	G	A	C
CH001	China Henan	A	A	ATTT	T	A	A
CH002	China Shandong	A	A	ATTT	T	A	A
CH003	China Hubei-A	A	A	AATT	T	A	A
CH004	China Hubei-B	A	A	–	G	A	A
KO001-SAJA	Korea Ganghwado	A	A	ATTT	T	A	A
A. princeps (MF034021)	Gen Bank	A	C	–	G	A	A
A. argyi (NC_030785)	Gen Bank	A	A	ATTT	T	A	A
A. argyi (AR)	FUJIFILM Wako Pure Chemical Co., Ltd.	A	A	–	T	A	A

[a] Number indicates position of the variable site in intergenic region based on the sequence of JA001.
[b] Dash indicates deletion.
[c] "JA"fresh leaves, "CH","KO"dry specimen belong to Chiba University.

needed. Japanese domestic mugwort is indispensable to the manufacture of moxa floss of such high purity [16]. This is because the Japanese moxa manufacturers consider that Chinese mugwort is not suitable for highly purified moxa floss, due to its hard leaves, relatively small amount of trichomes on the back of leaves, and other factors [17].

The questionnaire-based survey was conducted with Japanese acupuncture practitioners, who were asked to perform "Tenkyu" with both Japanese MFM and foreign MFM and to answer questions about their use in a blind fashion [18]. As a result more than 70% recognized a greater ease of use for the Japanese MFM. Hence, the MFM manufactured from the Japanese mugwort as the raw material using the traditional Japanese process was highly evaluated by both the manufacturers and the practitioners. With this situation in mind, we considered that it will be increasingly important to characterize Japanese moxibustion treatment from the viewpoint of the raw material MFM, and attempted to identify various mugwort varieties by DNA analysis to clarify their differences.

Recently, classification by DNA analysis has identified in the field of crude drugs. There have been increasing imports of mugwort produced outside Japan, and a crude drug test is performed as required by the Japanese Pharmacopoeia to completely identify *A. princeps* and Chinese *A. argy*i to prevent the mixing of *A. argyi* into the mugwort used in Kampo medicine in Japan [19].

In addition, the International Organization for Standardization (ISO) is working to standardize medical devices used in Oriental medicine. Likewise, the moxibustion heat source moxa floss may be subject to consideration of its standardization. In that case, the quality and features of mugwort as the raw material for moxa floss manufactured in various countries must be clarified as key factors. It will also be important to identify chloroplast DNA regions according to mugwort differences.

For this reason, and in reference to published reports, we used the *trnH-psbA* region, which is widely variable within the genus *Artemisia* [20], and also used the *rpl32-trnL* and *trnQ-5′rps16* regions, as they were reported to be widely variable among different plant species [21], to clarify the interspecific variations of mugwort depending on the place of sample collection. As a result, distinct species were identified. These findings in the present study showed that the mugwort species used as the raw material for Japanese moxa floss of high purity belonged to a group different from that of the Chinese and South Korean MFM species in terms of gene sequence coding, suggesting that they are different species not only morphologically, but also genetically.

In South Korea, moxa floss processed in China is commercially available in large amounts, with *A. princeps* cv. Sajabal, a mugwort cultivar grown on Ganghwa Island, being widely used as the raw material for moxa floss. Many studies have reported Sajabal as a "cultivar" or "variety" of *A. princeps* [22–25]. However, Jeong Hoon Lee et al. [26] reported that *A. argyi* and Sajabal were identical in the trnL-F region. In our present study, Sajabal was found to be identical to *A. argyi* in the ITS region and the chloroplast DNA *trnH-psbA*, *rpl32-trnL*, and *trnQ-5′rps16* regions. In addition, out of the Chinese MFM samples, the mugwort used as the raw material for the moxa floss manufactured in Nanyang City, Henan Province, was found to have the same chromosome sequence as the South Korean mugwort sample.

The amount of moxa floss produced in the South Central region of China, where Nanyang is located, accounts for approximately 70% of the nation's domestic production [7], and moxa sticks are manufactured prevalently. In addition, the "QiAi" mugwort from Qichun County, Hubei Province, is famous for its high quality, as appreciated by Li Shizhen in 'Compendium of Materia Medica' [6]. This mugwort reportedly contains higher levels of aromatic components than those in mugwort from other places [27] and is deemed highly valuable. At present, efforts are made to maintain the purity of the species through cultivation. The manufacturers of "CH003" have been cultivating the species to maintain its brand value while ensuring the fulfillment of its own standards through component analysis and other approaches.

In the *rpl32-trnL*, *trnH-psbA*, and *trnQ-5′rps16* regions, sequences differed depending on manufacturer even in samples from the same mugwort production site in Qichun County. In addition, sequences differed even within the same *A. argyi* sample that we used this study, depending on GenBank-registered DNA sequence and crude drug test

Table 3
Nucleotide sequence variation and the classification of cpDNA (rpl32-trnL).[a]

specimen vouchers[c]	Collection site	*rpl32-trnL*							
		173	242	473	497	532	570	652	795
JA001 ~ 007	Japan Niigata	-[b]	A	A	G	T	A	G	C
JA008,009	Japan Niigata	A	A	A	G	T	A	G	C
CH001	China Henan	–	–	A	T	C	G	A	C
CH002	China Shandong	–	A	A	G	T	A	G	C
CH003	China Hubei-A	–	–	A	T	C	G	A	C
CH004	China Hubei-B	–	A	A	G	T	A	G	C
KO001-SAJA	Korea Ganghwado	–	–	A	T	C	G	A	C
A. princeps (MF034021)	Gen Bank	–	A	A	G	T	A	G	C
A. argyi (NC_030785)	Gen Bank	–	–	A	T	C	G	A	C
A. argyi (AR)	FUJIFILM Wako Pure Chemical Co., Ltd.	–	A	A	G	T	A	G	C

[a] Number indicates position of the variable site in intergenic region based on the sequence of JA001.
[b] Dash indicates deletion.
[c] "JA"fresh leaves, "CH","KO"dry specimen belong to Chiba University.

reagent. These differences are considered to be associated with the locations of mugwort collection sites. Further examination will be necessary to identify the collection sites, because “Gen Bank” registration documents of “*A.argyi,*” which we used in this study, do not include the records of collection site details. Also, in this study, we focused on the statistical analysis of chloroplast DNA in three regions. However, there may be a region or regions more suitable for mugwort identification besides these three, also requiring further study going forward.

This study was conducted to identify and characterize Japanese MFM in comparison with samples from other countries. There are reportedly 30 species of mugwort in Japan, which are morphologically divided into even more subtypes due to intraspecific mutations, including leaf shapes and colors, as well as branching. Therefore, characterizing distinct Japanese MFM is expected to allow selected varieties to be cultivated as in China, thus contributing to advances in the field of Japanese moxibustion in the future.

We consider that Japanese moxa floss can be characterized not only by manufacturing process, but also by raw material. This study is also expected to provide information that will help us to understand the historical changes of places of mugwort for moxa floss production in Japan. We would like to expand the geographical coverage, and to conduct further phylogenetic analysis using larger sample sizes.

5. Conclusions

DNA analysis was performed to characterize mugwort samples collected from various places in Japan, mainly the mugwort produced in Niigata Prefecture, the most widely distributed moxa floss material in Japan, in comparison with South Korean and Chinese MFM samples. All the Chinese and South Korean mugwort for moxa floss samples were shown to have the same sequence as *A. argyi*. All the Japanese mugwort for moxa floss samples were shown to have the same sequence as *A. princeps*. These findings revealed genetic differences between the Japanese varieties of mugwort for moxa floss and the Chinese and South Korean varieties. These differences were found to be effectively revealed by using the *trnH-psbA*, *rpl32-trnL*, and *trnQ-5′rps16* regions.

Financial support

The researchers did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

None.

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