



The DNA fingerprint in food forensics part II: the Jasmine rice case

KEYWORDS: Thai Agricultural Standard, Cambodian Rice Standard, French Rice Code, Microsatellites, SSRs (simple sequence repeats); food fraud; fragrance gene *fg*; NJPlot dendrogram.

ABSTRACT

Jasmine rice is the customary name for premium fragrant cultivars originating from the lowlands of the Central East of Thailand and the North-western part of Cambodia. In contrast to the term "Basmati rice", which is well defined in the pioneering UK Code of Practice (CoP) by a joint effort of all stakeholders in the UK, India and Pakistan, there is no common understanding about Jasmine rice authenticity. The French Rice Code includes the 3 varieties KDML 105, RD15 and Pathum Thani 1 as Thai Jasmine rice and cultivars from Cambodia with similar characteristics as Jasmine rice. In contrast the Thai standard defines 8 varieties and the Cambodian standard five cultivars as Jasmine rice. The success of the UK CoP is due to its clarity and restriction to certain cultivars, geographical regions and specific characteristics affecting the cooking and appearance of the rice. DNA fingerprinting is defined as the standard method for authenticity testing. This report reveals that the genetic fingerprinting method based on 15 microsatellite or SSR (Simple Sequence Repeat) markers, which was recently developed for the differentiation of the 41 Basmati varieties in the revised UK CoP, can be also applied to Jasmine rice. Based on authentic reference materials obtained from the Cambodian Agricultural Research and Development Institute (CARDI) and from the trade the test not only allows the application of the French Rice Code, but also of the Cambodian Milled Rice Standard. This might lead to more transparency for the trade and consumers, increase the quality of this premium rice and contribute to its success on the world markets.

INTRODUCTION

DNA fingerprinting was invented by Sir Alec Jeffreys and first applied as a forensic tool in an immigration dispute (1). Since then it became the gold standard in paternity testing and human forensics to identify individuals during criminal investigations. Also in food forensics it is a standard tool for the differentiation of plant varieties and animal breeds (2, 3, 4). Authenticity testing of Basmati rice is the first example of its routine application for food control (4, 5, 6). Basmati is a premium fragrant rice with defined geographic origins and typical cooking characteristics. Due its higher price adulterations with cheaper common long grain rice have been frequently observed. As a consequence the UK Rice Association, British Rice Millers Association and British Retail Consortium developed the UK Code of Practice (CoP, 7), which defines Basmati authenticity and determines DNA fingerprinting as the standard method for the application of the Code.

Jasmine rice is an aromatic rice speciality from Southeast Asia with superb cooking characteristics. Its fragrance is similar to Basmati rice or pandan grass with 2-acetyl-1-pyrroline as the major aroma compound. Recently it has

been observed that Jasmine rice is increasingly adulterated with cheaper rice of lower quality. Accordingly laboratory testing methods are needed to protect the consumer against misleading product claims. Furthermore the EU Vietnam Free Trade Agreement (EVFTA) of 2019 provides for the import of about 30,000 metric tons of 9 specific fragrant Vietnamese rice varieties at zero duty and the authenticity of these cultivars has to be verified by the customs authorities.

Rice exports are of great commercial importance for the countries of Southeast Asia with a total value of 8.5 billion USD in 2018 (8). A large fraction of this is fragrant rice, which accounts for 17.4 % of all rice exports from Thailand in 2018 (9) and 22 % from Vietnam in 2016 (10). The largest products of fragrant rice are Hom Mali (registered mark, wording means fragrant Jasmine in the Thai language) from Thailand, Phka Malis (also sold under the collective mark "Malys Angkor™", registered with WIPO, the World Intellectual Property Organization) from Cambodia and Jasmine 85 from Vietnam (10).

For testing the authenticity of Basmati rice a DNA fingerprinting method had been developed by Bligh (11), which became the standard test in the UK Code of Practice on Basmati rice (CoP, 7). This test is based on a second generation fingerprinting procedure with SSR markers and allows the identification and quantification of all rice varieties also in mixtures, as long as the genetic fingerprints have been determined from reference materials (2). Recently a method based on 15 SSR markers and the fragrance gene *fg* was introduced for the differentiation of the 41 Basmati varieties, which are listed in the revised CoP of 2017 as genuine Basmati (12). This report reveals that this method is also suitable for the authenticity testing of Jasmine rice and might lead to more consumer transparency and improve the quality of this Southeast Asian speciality.

RESULTS AND DISCUSSION

Authenticity definition of Jasmine rice by Southeast Asian and European standards

Similar to Basmati rice the authenticity and quality of Jasmine rice is defined by varieties and their physical, cooking and biochemical characteristics. According the Thai specifications for Jasmine rice (13, 14) the milled kernel has to be long, slim (average length of whole kernels > 7 mm, length/width ratio > 3.2:1), pearly and white as the Jasmine flower. When cooked the rice is moist, soft in texture and somewhat sticky. The amylose content is low with a maximum of 20 % and the alkali spreading value of 6 to 7 indicates a low gelatinization temperature

between 55 and 69 °C. The following fragrant Thai varieties fall under these specifications: the premium Hom Mali cultivars KDML105 and RMD15 (table 1 A, 14), and furthermore Pathum Thani 1, Khao Jow Hom Khlong Luang 1, Khao Jow Hom Suphan Buri, Khao Jow Hom Pitsanuloke1, RD33 (Hom Ubon80) and Kor Khor 45 (table 1 B, 15) . The characteristics of the other aromatic rice varieties in the Thai Agricultural Standard TAS 4001-2017 (15) differ from Jasmine rice (table 1 C, D and E).

Variety	Rice standard including the variety	Growing seasons	Origin of variety	Reference material	Literature reference
A. Hom Mali (fragrant jasmine), superior Thai Jasmine rice quality					
KDML105	TAS 4000-2017; French Rice Code	wet season	landrace selection	Trade	14, 17, 18
RD15 (Kor Khor 15)		wet season	KDML105 mutant; gamma irradiated	Trade	14, 17, 18
B. Thai Jasmine rice, soft cooking non-glutinous aromatic rice					
Pathum Thani 1	TAS 4001-2017; French Rice Code	all seasons		Trade	13,15,17,19
Khao Jow Hom Khlong Luang 1	TAS 4001-2017	all seasons		-	13, 15
Khao Jow Hom Suphan Buri		all seasons		-	13, 15
RD33 (Hom Ubon 80)		all seasons		-	13, 15
Khao Jow Hom Pitsanuloke 1		wet season		-	13, 15
Kor Khor 45		wet season		-	13, 15
C. Thai loose cooking non-glutinous aromatic rice					
Nhang Mon S-4	TAS 4001-2017	wet season		-	15
Dok Pa-yom		wet season		-	15
D. Thai hard cooking non-glutinous aromatic rice					
Pathum Thani 80	TAS 4001-2017	wet season		-	15
Chai Nat 2		all seasons		-	15
E. Thai white glutinous aromatic rice					
RD 6	TAS 4001-2017	wet season	KDML105 mutant		15, 18
Khao Pong Krai		wet season		-	15
Sakon Nakorn		all seasons		-	15
R258		all seasons		-	15
F. Cambodian Jasmine rice, premium aromatic					
Phka Rumduol	Cambodian Milled Rice Standard	wet season	landrace selection	CARDI	16, 30
Phka Rumdeng		wet season	landrace selection	CARDI	16, 30
Phka Rumduol		wet season	landrace selection	CARDI	16, 30
Somali		wet season	landrace mixture	CARDI	16
Neang Malis™		wet season	landrace mixture	CARDI	16
G. Cambodian soft cooking non-glutinous aromatic rice with characteristics similar to Jasmine					
Sen Kra-ob	Cambodian Milled Rice Standard	all seasons		CARDI	16
Sen Pidao		all seasons		CARDI	16
H. Cambodian premium non-glutinous white rice					
Ginger rice, Phka Knhey	Cambodian Milled Rice Standard	wet season		CARDI	16
Phka Chan Sen Sar		wet season		CARDI	16, 30
CAR4		wet season		CARDI	16
CAR6		wet season		CARDI	16
Riang Chey		wet season		CARDI	16
I. Cambodian non-glutinous white rice					
Pearl Rice	Cambodian Milled Rice Standard	all seasons		CARDI	16
Neang Khon		all seasons		CARDI	16
Ponla Pdao		all seasons		CARDI	16
Neang Minh		all seasons		CARDI	16
IR66		all seasons		CARDI	16
Chulsa		all seasons		CARDI	16
J. Vietnamese aromatic rice with characteristics similar to Jasmine					
KDM (Khao Dok Mali)		wet season	same as KDML105	Trade	18, 21
Jasmine 85		all seasons	IR262/KDML105	Trade	22
Nang Hoa			KDML105/ Jasmine 85	-	21
VD20				-	21
K. Myanmar hard cooking non-glutinous aromatic rice					
Pearl Rice (Paw San)				Trade	27

Table 1. Aromatic rice varieties in Thailand, Vietnam and Myanmar and Cambodian indica type cultivars. DNA fingerprinting was performed, where reference materials became available.

In Cambodia the varieties Phka Rumduol, Phka Romeat and Phka Rumdeng, the landrace Somali and rice under the trade name Neang Malis™ are defined as premium fragrant Jasmine rice (table 1 F, 16) and the cultivars Sen Kra-ob and Sen Pidao as fragrant rice (table 1 G, 16).

In the French Rice Code (17) only Hom Mali (with KDML105 and RD15) and Pathum Thani 1 qualify as Thai Jasmine and Cambodian rice with a similar quality as Jasmine. The specifications for amylose and the alkali spreading value differ from the Thai standard as well (13, 14). Fragrant rice from other origins including Vietnam can be labelled as perfumed or fragrant rice (Riz Parfumés, Riz Fragrant).

Purity levels are defined with minimum 92 % for Hom Mali and 80 % for Pathum Thani 1 in the French Rice Code and the Thai agricultural standards. The Cambodian Milled Rice Standard sets a purity limit of ≥ 90 % for extra well milled and ≥ 85 % for well milled premium aromatic rice.

History of Jasmine rice varieties in Southeast Asia

Traditional Jasmine rice originates from the lowlands of the Central East of Thailand and the North-western part of Cambodia. Due to a photoperiodic sensitivity its growth cycle is adapted to the climate conditions in this region with heavy monsoon rainfalls in September and October and dry weather conditions from November to April. Inflorescence is initiated, once the days reach a critical length at the end of September. In the second half of October the plants are in anthesis and harvest can start in the second week of November with the beginning of the dry season.

In Thailand the cultivar KDML105 was selected from several landrace varieties, which had been collected in the Chachoengsao province and was officially named "Khao Dawk Mali 4-2-105" (18). The variety RD15 was evolved from KDML105 by gamma radiation mutagenesis to achieve an earlier maturation (18). In Cambodia CARDI selected the pure lines Phka Rumduol, Phka Romeat and Phka Rumdeng from land races cultivated in the country traditionally.

From these landraces new Jasmine rice varieties have been evolved by breeding with non-fragrant long grain rice, which are photoperiod insensitive and can be harvested during the whole year. In Thailand the most common variety for export is Pathum Thani 1 with cooking qualities and kernel appearance not quite matching the premium priced Hom Mali (19). The variety RD33 (Hom Ubon80) was selected at IRRI (International Rice Research Institute) by crossing KDML105 with IR70177-76-3-1, a blast resistant non-fragrant variety (18). In Cambodia Sen Kra-ob and Sen Pidao were developed as photoperiod insensitive varieties.

Many fragrant cultivars are available in Vietnam (table 1 J, 20). Commercially important for the export are Jasmine 85, KDM (supposedly identical with KDML105), VD20 and Nang Hoa rice (21), a cross bred between Jasmine 85 and KDM. Jasmine 85 (IR841-85) is a midseason fragrant long-grain variety, which was derived from the cross IR262/KDML105 at IRRI. Jasmine 85 is also grown in the rice belt of the USA (22). The EU Vietnam Free Trade Agreement exempts the varieties Jasmine 85, VD 20, Nang Hoa 9 (NangHoa 9), ST 5, ST 20, RVT, OM 4900, OM 5451 and Tai nguyen Cho Dao (Tàinguyên Chợ Đào) from import duty. Except for the first 3 varieties it is unknown, which of these cultivars reveal the typical Jasmine characteristics, as defined above.

DNA fingerprinting of Jasmine rice

DNA fingerprinting in food forensics depends on the availability reference materials. In a project funded by the International Finance Corporation of the World Bank, CARDI provided these materials for most of the fragrant and non-fragrant indica-type varieties in the Cambodian Milled Rice Standard (16, table 1 F, G, H and I). Further reference materials of fragrant rice varieties from Thailand, Vietnam and Myanmar were provided by the commercial sector and routine analyses of several thousands of samples from lots exported to the EU revealed their authenticity. Availability and sources of reference materials are summarized in table 1 and their DNA fingerprints in table 2.

Based on the data from SSR fingerprinting a dendrogram was calculated by the UPGMA (unweighted pair group method with arithmetic mean) cluster analysis with the Populations 1.2.32 software (Figure 1). UPGMA analysis is a simple bottom-up hierarchical clustering method used in bioinformatics to establish phylogenetic trees (23). Results reveal that the analysed varieties can be categorized

As was expected the fragrant variety Paw San from Myanmar differs significantly from all other cultivars.

The wet season photoperiodic sensitive Jasmine varieties from Thailand and Cambodia in group IV are closely related to each other. Thai Hom Mali and Cambodian Phka Romeat reveal identical fingerprints (Table 2). They are probably sister lines selected from the same pool of landraces. The other two Cambodian wet season varieties Phka Rumdeng and Phka Rumduol differ

Variety	SSR Markers																	lgr
	RM1	RM223	M16	RM202	RM44	RM201	RM229	RM241	RM171	RM55	RM263	RM72	RM440	RM525	RM152	RM212	RM252	
Cambodian fragrant rice																		
Phka Romeat	Z+5	V	Y	Y	W+2	W	X	Y-2	Z	Z+2	190	146	202	99	143	114	233	+
Phka Rumduol	Z+5	V	Y	Y	W+2	W	X	Y-2	Z	Z+2	180	146	202	99	143	114	233	+
Phka Rumdeng	Z+5	V	Y	Y	W+2	W	X	Y-2	Z	Z+2	190	146	202	99	148	114	233	+
Sen Kra-ob	W+6	V-8	Y	X	W+2	W	Y+2	Y	Z	Y	202	146	202	99	154	134	193	+
Sen Pidao	Z	V	Y	Y	W	Z	Y+2	Y	Z+4	Y	185	155	200	102	154	114	193	+
Somali	Z+5	V	Y	Y	W+2	W	X	Y-2	Z	Z+2	190	146	202		143	114	231/233	+
Neang Malis	Z+5	V	Y	Y	W+2	W	X	Y-2	Z	Z+2	190	146	202	99	143	114	233	+
PRD-Line1	Z+5	V	Y	Y	W+2	W	X	Y-2	Z	Z+2	180	146	202	99	143	114	233	+
Thai fragrant rice																		
Hom Mali	Z+5	V	Y	Y	W+2	W	X	Y-2	Z	Z+2	190	146	202	99	143	114	233	+
Pathum Thani 1	Z+5	V	Y	Y+4	W	W	X	Y	Z+4	Y	185	155	-	-	143	112	247	+
Vietnamese fragrant rice																		
Jasmine 85	Y	V	Y	Y	W+2	W	X	Y	Z	Y	190	146	-	-	155	112	215	+
Myanmar fragrant rice																		
Paw San	W+10	W-2	Y	Y+2	Y-2	W	Y+2	Y	X-3	Z	161	170	143	139	144	116	240	-
Cambodian non-fragrant white rice (wet season)																		
Phka Knhey (Ginger)	Z	Y	Y	Y+4	X+2	W	X	Y	Z+4	Y	183	146/164	-	-	140	112	215	+
CAR4	Z+2	V-8	Y	X	W	W-2	Y+2	Y-2	W	Y	188	146	211	99	148	114	250	-
CAR6	Z+2	V-8	Y	X	W	W-2	Y+2	Y-2	W	Y	188	152	202	99	148	114	254	-
Riang Chey	Z+2	V-8	Y	X	W	W-2	Y+2	Y-2	W	Y	188	152	202	99	148	114	256	-
Damnoeb Sbai Mongkul	Z	V-8	Y	X+4	W	W	X	Y-2	Z	Y	190	152	205	99	154	132	225	-
Phka Chan Sen Sar	Z	V-8	Y	X+4	Y+4	Z	Y+2	Y-6	Z	Y	188	152	206	99	132	114	229	-
Cambodian non-fragrant white rice (all year)																		
IR66	Z	Y	Y	Y+4	W	W	Y+2	Y-4	W	Z	190	161	207	99	154	134	248	-
Chul'ea	Z	V	V	V	W	Z	Y+2	Y	Z+4	Y	202	155	196	102	154	134	215	-

Table 2. Allele patterns of rice varieties from Southeast Asia, determined by DNA fingerprinting based on 17 SSR marker genes (28). For the first 10 SSR markers the letter code from V to Z was adapted from the DNA fingerprinting method published by the FSA in 2004 (29). Each character defines a DNA fragment of a distinct length for each SSR marker. Reference materials of Cambodian rice varieties were provided by the CARDI and all other cultivars were obtained from the trade.

in 4 distinct groups. Group I consists of the non-fragrant international variety IR66 and the Cambodian varieties Damnoeb Sbai Mongkul and Phka Chan Sen Sar. Group II includes the Cambodian non-fragrant varieties Rieng Chey, CAR4 and CAR6 and group III comprises the fragrant varieties Pathum Thani 1 and Sen Pidao and the non-fragrant cultivars Chul'sa and Phka Knhey. Group IV finally includes the fragrant varieties Hom Mali, Phka Romeat, Phka Rumdeng, Phka Rumduol, Sen Kra-ob and Jasmine 85.

from each other and from Hom Mali and Phka Romeat in only one out of 15 SSR markers. The all year photoperiodic insensitive Jasmine 85 is a progeny of KDML105 (see above) and differs from these varieties in 4 out of 15 SSR markers. The photoperiodic insensitive Sen Kra-ob differs in 10 out of 15 markers from the traditional cultivars, but still belongs to the same group IV cluster. This is in contrast to Pathum Thani 1 and Sen Pidao, which are also fragrant photoperiod insensitive varieties, but are included in the distinct group III.

Somali is a land race cultivar of Cambodian Jasmine rice. Analysis of a reference material obtained from CARDI revealed a mixture of different, but closely related cultivars, which is expected for such a collection from the traditional agriculture.

Neang Malis™ is a trade name for Phka Malis and our study revealed that a reference material of this rice mainly contained the cultivar Phka Romeat, but was not 100 % pure.

Recently a new variety from Thailand is showing up during routine testing of samples sent in by the trade, which differs from Hom Mali in marker RM252 only with an allele length of 231 instead of 233 bp. As described by Vanavichit et al. (18) new varieties like RD33 and Hom Mali 84 with higher yields and tolerances against plant diseases and pests have been developed in Thailand from the original landrace line KDML105 by gene pyramiding using marker-assisted selection.

These superior new varieties are displacing the older lines KDML105 and RD15, but cannot be identified due to the lack of reference materials. As many of them are near-isogenic lines they do not differ significantly from their ancestors. Similarly CARDI developed the new variety PRD-Line1 from Phka Rumduol with an extended growth period beyond the photosensitive season, which cannot be distinguished from the original line by our DNA fingerprinting method (table 2).

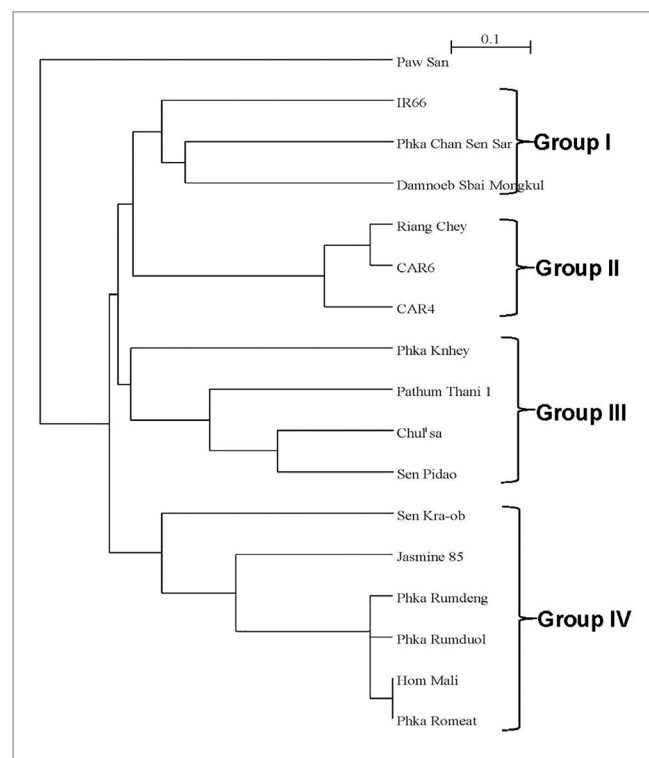
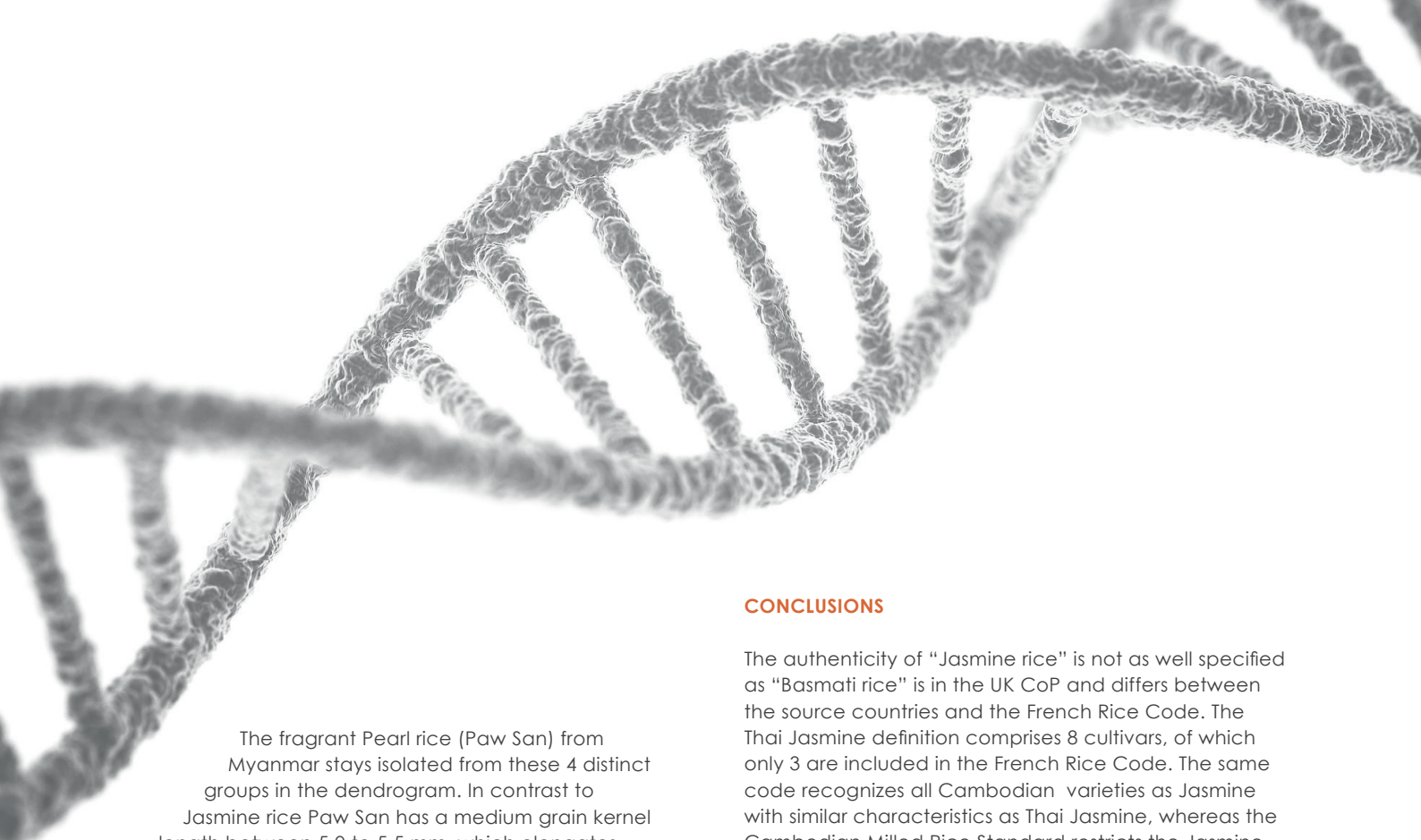


Figure 1. Dendrogram of the analysed rice varieties from Southeast Asia calculated by the UPGMA cluster analysis (23) with the Populations 1.2.32 software based on the SSR markers in Table 2. Results were visualized with the NJPlot programme.



The fragrant Pearl rice (Paw San) from Myanmar stays isolated from these 4 distinct groups in the dendrogram. In contrast to Jasmine rice Paw San has a medium grain kernel length between 5.0 to 5.5 mm, which elongates during cooking two to three times. The amylose content is intermediate (20 to 25 %) in contrast to Jasmine rice (maximum 20 %), which contributes to its hard cooking characteristics (24).

Analysis of the fragrance gene *fgr*

The genotype *fgr* is an 8 base pair deletion in exon 7 of the *bad2* gene coding for the enzyme betaine-aldehyde dehydrogenase 2 (25, BAD2) or amino-aldehyde dehydrogenase (26, AMADH), which oxidizes 4-aminobutanol to 4-aminobutyric acid (GABA). Due to the inactivation of this enzyme 4-aminobutanol accumulates and interconverts spontaneously to 1-pyrroline. It is assumed that this leads to the formation of 2-acetyl-1-pyrroline, the major aroma compound of fragrant rice (26). This genotype occurs in most Basmati and Iranian Sadri varieties tested so far. Also all Jasmine rice varieties from Thailand, Cambodia and Vietnam exhibit the same 8 bp deletion in exon 7 of the *bad2* gene. The Cambodian ginger rice Phka Knhey is considered a non-fragrant premium white rice, but interestingly also contains the *fgr* genotype.

Paw San does not exhibit the *fgr* genotype (table 2), but is still a fragrant rice due to a 3 bp insertion in exon 13 of the *bad2* gene (27).

Experimental section

The DNA fingerprinting method was described in detail in various anterior publications (2, 5, 12). Its qualitative and quantitative efficiency was proven in several studies including an extensive validation for ISO 17025 accreditation at Eurofins and several laboratory proficiency tests and ring trials (2, 5). The dendrogram was calculated with a hierarchical clustering algorithm by the software Populations 1.2.32 (23). This algorithm was developed for the analysis of genetic linkages based on differences in alleles of SSR markers. Results were visualized with the NJPlot programme.

CONCLUSIONS

The authenticity of “Jasmine rice” is not as well specified as “Basmati rice” is in the UK CoP and differs between the source countries and the French Rice Code. The Thai Jasmine definition comprises 8 cultivars, of which only 3 are included in the French Rice Code. The same code recognizes all Cambodian varieties as Jasmine with similar characteristics as Thai Jasmine, whereas the Cambodian Milled Rice Standard restricts the Jasmine definition to 5 cultivars only. Furthermore rice cultivated as Jasmine in Vietnam is not recognized as such by the French Code. Unclear definitions open doors for adulterations, which might damage the reputation of this premium quality rice. The DNA fingerprinting method was a starting point of the UK CoP in 2005 and is an important tool for its application. This study reveals that this method, which had been recently further developed for the identification of the 41 Basmati varieties in the revised UK CoP of 2017, is also suitable for Jasmine rice. Cultivars can be identified and quantified in mixtures with other varieties, which allows the control of the purity levels of Jasmine rice defined in the rice standards. A major prerequisite for the application of the method is the access to reference materials from official sources. Consequently a condition for the inclusion of Basmati varieties in the UK CoP is the availability of reference materials. In Cambodia this was achieved for Jasmine and other rice varieties by making reference materials available, which allows the enforcement of the Cambodian Milled Rice Standard.

Similarly reference materials of all rice cultivars defined as Jasmine in Southeast Asia should be made available to the laboratories to allow the application and enforcement of rice standards defining Jasmine authenticity. As the recent EU Vietnam Free Trade Agreement allows the duty free import of 30,000 metric tons of 9 specific Vietnamese fragrant rice varieties, reference materials should be provided to verify the authenticity of these cultivars by EU customs authorities.

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ABOUT THE AUTHORS

Dr. **Werner Nader** is a biologist, 68 years old and was until his retirement the managing director of Eurofins Global Control GmbH in Hamburg. He worked as a postdoctoral fellow at the Massachusetts Institute of Technology, Texas A&M University and Max-Planck-Institute, in the biotech industry until his retirement in food control. Currently he is engaged in various senior consulting activities.



Dr **Ouk Makara** is the Director of the Cambodian Agricultural Research and Development Institute (CARDI) of the Ministry of Agriculture, Forestry and Fisheries. He is a PhD in Agricultural Sciences (University of Queensland, Australia). As a plant breeder his team released 45 rice varieties and 14 varieties of other crops. He received several awards as an Outstanding Scientist and was nominated ASEAN Rice Science and Technology Ambassador during the 50th Anniversary of this intergovernmental organization.

