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ARTICLE

Screen and Genetic Assessment of Tea Germplasms with Elevated Methylated Catechin, (−)-Epigallocatechin-3-*O*-(3-*O*-methyl)gallate

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The aim of this research was to identify tea varieties containing high levels of catechin methyl ester that could be used as important sources for genetic breeding and in the production of high quality tea. We examined 113 species that have been preserved in the Taitung Branch of the Tea Research and Extension Station of Taiwan (TTES). The average level of (−)-epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3″Me) was 0.45% for all varieties screened. Among them, 16 varieties with higher EGCG3″Me content (>0.8%) were considered good candidates for manufacturing partially fermented tea. Analysis of these tea varieties revealed that the EGCG3″Me content in leaves did not correlate with the caffeine content. Genetic assessments revealed that the lengths of their internal transcribed spacers (ITS) were in the range of 638−670 bp and that the sequence identities were in the range of 0.690−1. Two major groups were constructed by phylogenetic analysis, I and II, with a genetic distance of 0.08 based on the ITS1-5.8S-ITS2 sequences between the ribosomal genes. Our results provide genetic information about tea varieties with elevated EGCG3″Me content and indicate the need for a comprehensive genetic assessment of tea germplasms preserved in the TTES to better serve the future of tea breeding.

KEYWORDS: ***Camella sinensis*; *O*-methylated EGCG; internal transcribed spacers (ITS); phylogenesis**

INTRODUCTION

Originating in China, tea (*Camella sinensis*) is the most popular nonalcoholic drink in the world. Depending on the manufacturing process, teas are classified in three major categories: nonfermented green tea; partially fermented oolong tea; and fully fermented black or red (*Pu-Erh*) teas. Tea has been found to exhibit various biological and pharmacological properties, e.g., having antioxidative (*1*), anticarcinogenic or antimutagenic (*2*, *3*), antimetastatic (*4*), antiatherosclerotic (*5*), antihypertensive (*6*, *7*), antibacterial (*8*, *9*), and allergy preventing (*10*−*12*) properties. Catechins, a group of polyphenolic compounds, have been reported to be largely responsible for these effects.

Naturally occurring methylated catechins have been isolated from Taiwanese oolong tea (*10*, *11*). A growing body of research has shown that the *O*-methylated forms of (−)-epigallocatechin-3-*O*-gallate (EGCG) and its derivatives have potent antiallergic activity against type I and type IV allergies (*10*), preventing inflammation of the respiratory tract by suppressing FceRI expression and histamine release (*11*). A clinical study reported that the intake of Benifuuki green tea, which has a high level of *O*-methylated EGCG content in its leaves, reduces the symptoms of allergic cedar-pollinosis (*12*).

The internal transcribed spacer (ITS) of the nuclear ribosomal 18S-5.8S-26S cistron has been the most popular genomic target for systematic molecular investigations of plants at the species level (*13*). Comparison studies of homologous ITS sequences are also widely used as an improved method to authenticate medicinal herbs using DNA (*14*, *15*). Species, and even variants within species, can be distinguished by their ITS nucleotide sequences (*16*).

The germplasm of tea has gained value as a fundamental material for tea plant breeding because of its huge economic and social interest. A solid understanding of the genetic relationships within and between species of tea plants may improve the management of existing germplasm collections and maintain a broad range of genetic variability. In the present study, the content of *O*-methylated EGCGs (EGCG3″Me) was determined by high performance liquid chromatography (HPLC) analysis for 113 tea varieties preserved as experimental materials at the Tea Research and Extension Station of Taiwan (TTES). The ITS rDNA (rDNA) was used as a genetic marker to assess the genetic diversity of the tea germplasms that were determined to have high EGCG3″Me content. Tea germplasm resources from strains with a high content of methylated catechins can be used to enhance the quality of future tea breeding programs.

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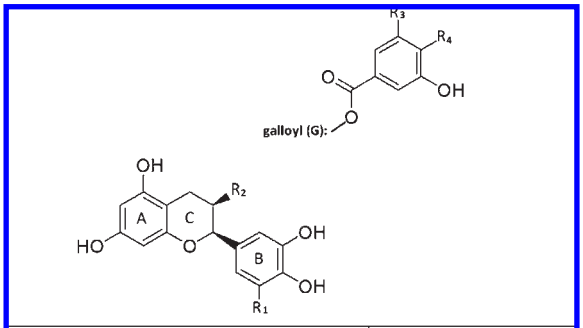
Table 1. Sixteen Varieties of Tea Germplasms with High Methylated Epicatechin Content in the Leaves and Seven *Camellia* spp. Used in the Present Study for Phylogenetic Tree Construction

code	organism	origin	clone or accession name	suitability	GenBank accession
			(A) Present Study[a]		
TD03	*Camellia sinensis*	hybrid variety	TTES No. 3	TTES	FJ004851
TD15	*C. sinensis var. sinensis*	hybrid variety	TTES No. 15	TTES	FJ004863
TD17	*C. sinensis var. sinensis*	hybrid variety	TTES No. 17	TTES	FJ004865
TD26	*C. sinensis var. sinensis*	China tea (land variety)	Bair Shin Wuu Yi	partially fermented	FJ004869
TD27	*C. sinensis var. sinensis*	China tea (land variety)	Horng Shoin Wuu Yi	partially fermented	FJ004870
TD29	*C. sinensis var. sinensis*	China tea (land variety)	Dah Terng	partially fermented	FJ004871
TD40	*C. sinensis var. sinensis*	China tea (land variety)	Guey Hua	partially fermented	FJ004872
TD64	*C. sinensis var. sinensis*	China tea (land variety)	Woan Joong	partially fermented	FJ004873
TD65	*C. sinensis var. sinensis*	China tea (land variety)	Shy Jih Chuen	partially fermented	FJ004874
TD70	*C. sinensis var. sinensis*	China tea (land variety)	Taur Ren Joong	partially fermented	FJ004875
TD79	*C. sinensis var. sinensis*	China tea (land variety)	Ruey Suey Dah Yeh Oolong	partially fermented	FJ004876
TD91	*C. sinensis var. sinensis*	Assam tea	Keemon	red tea	FJ004881
TD117	*C. sinensis var. sinensis*	China tea (land variety)	Bair Mau Hour	partially fermented	FJ004877
TD118	*C. sinensis var. sinensis*	China tea (land variety)	Dah Nan Wan Bair Mau Hour	partially fermented	FJ004878
TD120	*C. sinensis var. sinensis*	China tea (land variety)	Heh Mau Hour	partially fermented	FJ004879
TD122	*C. sinensis var. sinensis*	China tea (land variety)	Chin Shin Oolong variant	partially fermented	FJ004880
			(B) GenBank[b]		
N/A	*C. crassissima*	N/A	N/A	N/A	EF639855
N/A	*C. japonica Y-14*	N/A	N/A	N/A	AY697417
N/A	*C. liberistamina*	N/A	N/A	N/A	EF649692
N/A	*C. semiserrata*	N/A	N/A	N/A	EF649688
(A)	*C. sinensis*	N/A	N/A	N/A	AF315492
(B)	*C. sinensis*	N/A	N/A	N/A	AY096014
(C)	*C. sinensis*	N/A	N/A	N/A	EF649693

[a] Sixteen varieties with high EGCH3″Me content from the present study. [b] *Camellia* spp. ITS sequences that were available from GenBank at the time of this study.



Catechins	R_1	R_2	R_3	R_4
(-)-Epigallocatechin-3-*O*-gallate (EGCG)	OH	G	OH	OH
(-)-Epicatechin-3-*O*-gallate (ECG)	H	G	OH	OH
(-)-Epigallocatechin (EGC)	OH	OH	-	-
(-)-Epicatechin (EC)	H	OH	-	-
(+)-Catechin (C)	H	OH	-	-
(-)-Gallocatechin (GC)	OH	OH	-	-
(-)-Epigallocatechin-3-*O*-(3-*O*-methyl)gallate (EGCG3″Me; 3″-OMe)	OH	G	OCH_3	OH

Figure 1. Chemical structures of tea epicatechins and their derivatives.

MATERIALS AND METHODS

Chemicals and Reagents. (−)-Epigallocatechin-3-gallate (EGCG), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (+)-catechin (C), (−)-gallocatechin (GC) (**Figure 1**), and caffeine were purchased from Sigma Chemical Co. (St. Louis, MO). (−)-Epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3″Me) was purchased from Nacalei Tesque, Inc. (Kyoto, Japan). Sodium dihydrogen phosphate, *N*,*N*,-dimethylformamide, and acetonitrile were purchased from E. Merck Co. (Darmstadt, Germany).

Tea and Tea Leaf Samples. In this study, 113 varieties of fresh tea leaves were plucked from plants at the TTES. The young leaf samples were composed of the apical bud and the two youngest leaves. The collected tea leaves were processed using the protocol for manufacturing green tea, i.e., fresh tea leaves (600 g) are pan-fried at 280−300 °C for 5−6 min, followed by rolling at room temperature for 2−3 min, and drying at 90−100 °C for 2−4 h.

Preparation of Extracts from Tea Leaves and Tea. Each of the dry tea leaves were ground into powder and filtered through a 20-mesh net. Tea powder (0.5 g) was steeped in boiling distilled water (100 mL) for 20 min at 80 °C. The infusion was filtered through a 0.45-μm PVDF filter disk (Millipore, Bedford, MA), and the filtrate was analyzed by HPLC as described below.

Reverse-Phase HPLC Analysis of Tea Polyphenols and *O*-Methylated EGCG (EGCG3″Me). The determination of *O*-methylated EGCG was carried out by HPLC using a UV detector. The HPLC was equipped with a Mightysil RP-18 GP column (5 μm, 4.6 mm I.D. × 250 mm; Nacalei Tesque, Inc., Kyoto, Japan). The following is a brief description of the method: solution A was 100% acetonitrile; solution B was composed of 0.1 N phosphoric acid containing 0.1% acetonitrile and 5% *N*,*N*,-dimethylformamide; the product was resolved on a 1−20% gradient over 37 min; the flow rate was 1 mL/min; the injection volume was 20 μL; and the column temperature was maintained at 40 °C. The *O*-methylated EGCGs and catechins in tea leaves were detected with an L-4000 UV detector (Hitachi, Japan) at a wavelength of 280 nm and identified by comparison with standard peaks. The quantities of the compounds were obtained by integrating the area under the peaks of the spectrograms.

Genetic Diversity Assessment. DNA isolation, polymerase chain reaction (PCR) amplification, and DNA sequencing were performed according to the procedures outlined in Chiou et al. (*14*). Dried tea leaf material (100 mg) was used for genomic DNA extraction. The complete ITS1-5.8S-ITS2 fragments of 16 tea varieties with high EGCG3″Me content (**Table 1**) were amplified using the forward primer X-12 (5′-TAGAGGAAGGAGAAGTCGTAA-3′), which is complementary to the sequence located approximately 50 nucleotides from the 3′ terminus of the 18S rDNA sequence, and the reverse primer BEL-3 (5′-GACGCTTCTC-CAGACTACAAT-3′), which targets the sequence located approximately 160 nucleotides from the 5′ terminus of the 26S rDNA sequence

(**Figure 3A**). Each 50-μL PCR solution contained 2 μL of template DNA (40−80 ng), 5 μL of 10× PCR reaction buffer, 3 μL of 25 mM $MgCl_2$, 3 μL of 2.5 mM dNTP, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, 0.5 μL (5 units) of *Taq* DNA polymerase (Geneaid Biotech Ltd.; Taipei, Taiwan), 3 μL of dimethyl sulfoxide (DMSO), and 31.5 μL of sterile distilled water. The reaction mixture was heated to 94 °C for 10 min in order to denature the DNA and was then subjected to 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s. The final cycle included an extension period of 10 min at 72 °C. The PCR products were separated on a 0.8% agarose gel and subjected to sequencing analysis. Nucleotide sequences were determined using the ABI PRISM BigDye Terminator Cycle Sequencing kit and analyzed with an ABI PRISM 377 DNA sequencer (Applied Biosystems Industries; Foster City, CA, USA). At least three clones were sequenced for each specimen. The obtained sequences were compiled using BioEdit software (version 7.0.0) and confirmed after comparison to the in-house and GenBank databases. The sequence data published in this article were deposited in the GenBank nucleotide sequence database (**Table 1**).

Sequences of the complete ITS region (ITS1-5.8S-ITS2) of the 16 tea varieties with elevated EGCG3″Me content were aligned with other *Camellia* spp. by ClustalW using the BioEdit software (version 7.0.0). MEGA 3.1 (*17*) software was used to construct a sequence identity matrix and phylogenetic trees of these sequences. The phylogenetic trees were constructed without an outgroup, on the basis of the unweighted pair group method with arithmetic mean (UPGMA) methods. The default phylogeny test options used to construct the UPGMA were bootstrap (500 replicates), seed = 22607; gaps/missing data = complete deletion; substitution model = nucleotide (kimura 2-parameter); substitution to include transitions + transversions; pattern among lineages = same (homogeneous); and rate among sites = uniform rates.

Statistical Analysis. One-way analysis of variance (one-way ANOVA) was carried out on the values obtained in the experiment. Correlation and regression analyses (linear discriminate analysis, LDA) were also carried out to determine the relationship between the epicatechin content and the derivatives of the tea extracts from the 113 variants. SAS software (statistical analysis system) was used to perform the statistical analysis.

RESULTS

In the present study, 113 tea varieties from the TTES garden in Taiwan were screened for tea germplasms with high EGCG3″Me content. The 113 tested tea varieties make up five groups: 20 varieties that are new to the TTES, 15 varieties of green tea, 11 varieties of red tea, 9 varieties of wild tea, and 58 varieties of partially fermented tea.

HPLC Analysis of Authentic Standard Tea Catechins and *O*-Methylated EGCG. The catechin content of pan-fried green teas was examined. Young fresh tea leaves consisting of a bud and two leaves were plucked from 113 tea varieties during the spring. The catechin fraction was prepared from tea leaves by hot water extraction. Seven naturally occurring tea catechins were analyzed by HPLC: EGCG3″Me, EGCG, ECG, EGC, GC, EC, and C. The structures of these epicatechin derivatives are shown in **Figure 1**. A mixture of the authentic tea catechin standards was separated by isocratic HPLC combined with UV/vis detection as described above, and a baseline resolution was achieved (data not shown). The separation of these tea constituents (10 ng of each) by HPLC was accomplished in 60 min. HPLC chromatograms of Woan Joong tea, TTES No.8 tea, and TTES No.8 tea + EGCG3″Me are shown in **Figure 2A**, **B**, and **C**, respectively.

Survey of Tea Catechins and O-Methylated EGCG (EGCG3″Me) in Tea Leaves. One hundred and thirteen tea germplasms and three tea varieties were preserved in the TTES. The three tea varieties were *C. sinensis*, *C. sinensis* var. *sinensis*, and *C. sinensis* var. *assamica* (Appendix in Supporting Information). Each tea germplasm was first manufactured into three different types of tea based on the degree of fermentation, i.e., unfermented, partially fermented, and fermented tea. Then, each tea germplasm was evaluated to determine the manufacturing suitability. *C. sinensis* var. *assamica* is best used to manufacture red tea because the taste will be bitter if it is not fermented (i.e., made into unfermented or partially fermented teas) because of the higher catechin levels and caffeine content in the leaves. *C. sinensis* var. *sinensis* can be manufactured into various types of teas, but not red tea, as it is not strong enough to possess the characteristic red tea flavor. The results indicated that a total of 16 varieties contained EGCG3″Me in greater than 0.8% abundance. Most of them are suitable for the manufacture of partially fermented tea. Woan Joong contained as much as 1.63% EGCG3″Me, which was the highest of all the varieties. Most of the varieties are either wild teas or teas suitable for manufacturing red tea (**Tables 1** and **2**).

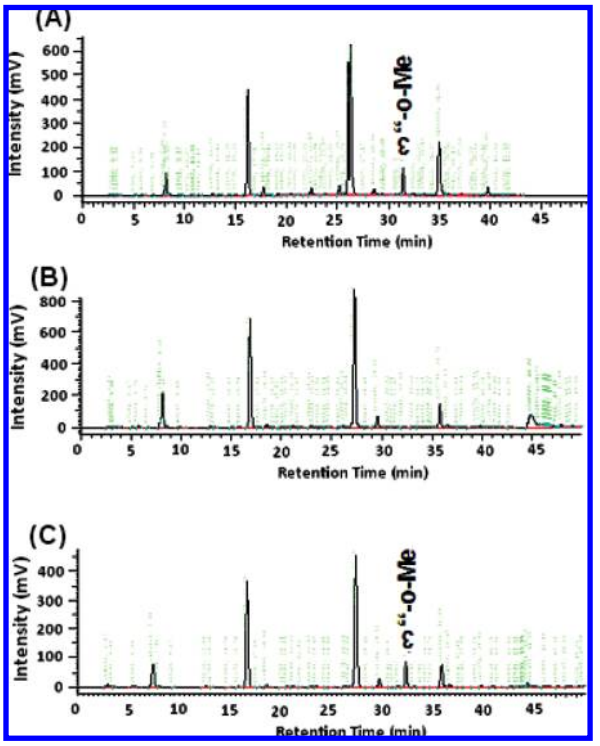


Figure 2. Isocratic HPLC separation of catechins, epicatechin, epicatechin derivatives, and *O*-methylated EGCG in tea water extracts (TWE). The following sample solutions were analyzed: (**A**) Woan Joong tea, 1% TWE; (**B**) TTES No.8 tea, 1% TWE; (**C**) TTES No.8 tea, 1% TWE + an authentic standard (EGCG3″Me; 3″-OMe).

The results also showed that the EGCG3″Me content of tea leaves varied greatly among the varieties or lines of tea plants, ranging from undetectable to 1.63%. Of the 113 varieties, 16 had an EGCG3″Me content greater than 0.8%, 46 had an EGCG3″Me content ranging from 0.8% to 0.4%, 30 had an EGCG3″Me content ranging from 0.4% to 0.1%, and 21 had an EGCG3″Me content below 0.1% (**Table 3**). The mean, maximum, and minimum EGCG3″Me contents in all 113 tea varieties were 0.45%, 1.63%, and 0.01%, respectively. In comparison to the catechin content, the variation is greatest in the coefficient of variation (cv) for EGCG3″Me (**Table 4**).

Correlation and Regression Analysis. Catechins are a group of naturally occurring compounds that are ubiquitous in tea germplasms. Seven catechins and caffeine were analyzed by HPLC. Tea catechins exist as glycosides and contain several phenolic hydroxyl groups on their ring structure (**Figure 1**). The content of EGCG3″Me in tea leaves showed a significant positive correlation with the content of total catechins (TC); interestingly, the content of EGCG3″Me in tea leaves did not correlate with the content of caffeine (**Table 5**).

Table 2. Varieties in Which the Methylated Catechin (EGCG3″Me) Content Is Greater than 0.8% (g/100g of Dry Leaf)

code	3″-OMe	EGCG	ECG	EGC	GC	EC	C	[a]TCG	[b]TCF	[c]TC	caffeine
TD064	1.63	8.34	1.05	2.38	1.14	1.35	0.22	9.39	5.10	14.50	2.27
TD117	1.52	8.59	0.78	3.15	0.75	1.16	0.20	9.37	5.27	14.65	2.43
TD122	1.32	6.81	0.52	3.66	0.65	1.11	0.28	7.33	5.72	13.06	2.43
TD118	1.26	8.88	1.08	2.10	0.94	1.19	0.23	9.97	4.48	14.45	2.46
TD026	1.17	6.94	0.56	3.94	0.83	1.24	0.21	7.51	6.24	13.76	2.05
TD029	1.11	5.79	0.48	3.64	0.87	1.15	0.18	6.28	5.86	12.15	1.93
TD120	1.10	7.40	0.95	1.79	0.59	0.98	0.23	8.36	3.61	11.98	2.41
TD027	1.01	6.78	0.50	3.89	0.89	1.12	0.21	7.28	6.12	13.41	2.23
TD079	0.98	5.81	0.51	3.36	0.78	1.11	0.20	6.32	5.47	11.80	1.97
TD015	0.89	7.92	0.62	3.74	0.73	1.26	0.22	8.54	5.96	14.51	2.86
TD070	0.89	6.38	0.56	3.20	0.80	1.29	0.24	6.95	5.55	12.50	2.08
TD040	0.86	5.86	0.46	2.83	0.79	1.25	0.21	6.32	5.09	11.42	1.77
TD003	0.84	11.13	0.95	2.57	0.86	0.98	0.25	12.09	4.67	16.77	2.84
TD065	0.88	9.66	0.90	1.70	0.77	0.73	0.22	10.57	3.43	14.01	2.46
TD017	0.84	9.41	1.02	2.46	0.78	1.25	0.24	10.44	4.74	15.19	2.68
TD091	0.82	7.55	0.78	2.35	0.67	1.22	0.30	8.33	4.56	12.90	2.50

[a] TCG: EGCG + ECG. [b] TCF: EGC + GC + EC + C. [c] TC: EGCG + ECG + EGC + GC + EC + C.

Table 3. Methylated Catechin (EGCG3″Me) Levels in the Germplasms of Tea Leaves

g/100 g of dry leaf	number	percent (%)
>0.8	16	14.2
0.8−0.4	46	40.7
0.4−0.1	30	26.6
<0.1	21	18.6

Genetic Diversity Assessment of Tea Germplasms. The complete ITS1-5.8S-ITS2 fragments of 16 tea germplasms with high methylated catechin content were amplified using the forward primer X-12 and the reverse primer BEL-3 (**Figure 3A**). Primers flanking the 18S-ITS1-5.8S-ITS2-26S rDNA spacer domain were used for PCR analysis, and the successfully amplified PCR products were separated by 0.8% agarose gel electrophoresis and visualized by ethidium bromide staining (**Figure 3B**). A phylogenetic tree of these 16 varieties and other tea germplasms (*Camella* spp.) was constructed by the UPGMA method (**Figure 3C**). A sequence identity matrix table was developed on the basis of the ITS1-5.8S-ITS2 sequence (**Table 6**).

Primers flanking the 18S-ITS1-5.8S-ITS2-26S rDNA spacer domain were used to successfully amplify the ~800-bp target from the 16 tea germplasms with elevated methylated catechin content. After removal of the 18S and 26S rDNA sequence domains, the length of ITS regions in these 16 tea germplasms ranged from 638−670 bps, and their average base compositions were calculated to be G + C (67.9%), A + T (32.1%), A (17.4%), C (35.8%), G (32.1%), and T (14.8%), with an average length of 644 bp. For comparison, the sequence compositions of three *C. sinensis* tea plant ITS1-5.8S-ITS2 domains available from GenBank were calculated as G + C (67.9%), A + T (32.1%), A (17.7%), C (34.9%), G (33.0%), and T (14.4%), with an average length of 642 bps. Four *Camella* spp. (*C. crassissima*, *C. japonica* Y-14, *C. liberistamina*, and *C. semiserrata*) ITS1-5.8S-ITS2 sequences, which are also available from GenBank, have average base compositions of G + C (67.7%), A + T (32.3%), A (17.7%), C (35.2%), G (32.4%), and T (14.6%), with an average length of 641 bps (data not shown).

A phylogenetic tree of the 16 tea varieties with elevated EGCG3″Me content and seven *Camellia* spp. plants revealed two major groups, termed I and II, at a genetic distance of 0.08 based on their ITS1-5.8S-ITS2 sequences from the ribosomal genes. Group I consists of two tea varieties, TD122 and TD079. Group II can be further divided into two subgroups, IIa and IIb, at a genetic distance of 0.04. Group IIa consists of two tea varieties, TD003 and TD065. Group IIb contains the rest

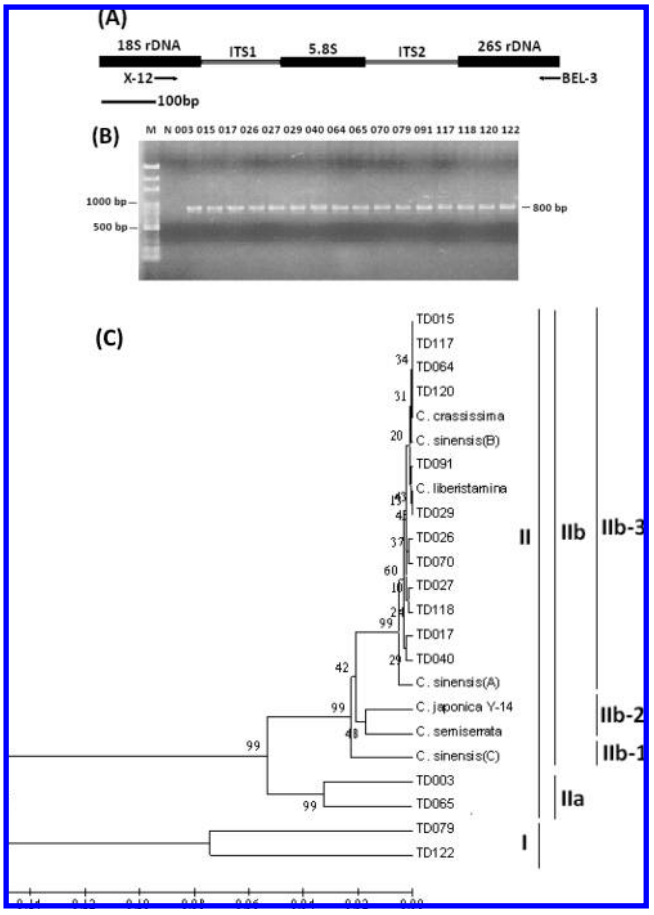


Figure 3. Genetic analysis of tea germplasms with high contents of methylated catechins: (**A**) schematic diagram of rRNA gene ITS regions and the designed primers. The coding regions of the 18S, 5.8S, and 26S rRNA genes are indicated by black boxes. (**B**) Primers (X-12 and BEL-3) flanking the 18S-ITS1-5.8S -ITS2-26S rRNA spacer domain were used for PCR analysis, and the PCR products were separated by 0.8% agarose gel electrophoresis and visualized by ethidium bromide staining. (**C**) A phylogenetic tree is shown for the 16 varieties of tea germplasms with high methylated catechin content from the present study and 7 *Camellia* spp. sequences available from GenBank, as assessed by the UPGMA method.

Table 4. Statistical Analysis of Catechin Fractions in Leaf Germplasms of the 113 Tea Varieties Used in the Present Study (g/100g of Dry Leaf; %)

	3′′-OMe	EGCG	ECG	EGC	GC	EC	C	[a]TCG	[b]TCF	[c]TC	caffeine
mean (%)	0.45	6.97	0.63	2.74	0.75	1.14	0.23	7.61	4.88	12.49	2.23
[d]SD	±0.34	±1.85	±0.23	±0.79	±0.19	±0.50	±0.06	±1.96	±1.14	±2.08	±0.52
max (%)	1.62	11.54	1.89	4.75	1.61	4.92	0.66	12.24	7.85	16.76	3.49
min (%)	0.01	0.01	0.07	0.29	0.36	0.11	0.15	0.08	1.39	1.48	0.16
[e]CV (%)	75.52	26.61	36.83	28.96	25.45	43.59	27.22	25.83	23.53	16.71	23.57
n	113	113	113	113	113	113	113	113	113	113	113

[a] TCG: EGCG + ECG. [b] TCF: EGC + GC + EC + C. [c] TC: EGCG + ECG + EGC + GC + EC + C. [d] SD: standard deviation of samples. [e] CV: coefficient of variation = SD/mean × 100%.

Table 5. Variation of Coefficients among Catechin Content in the Leaf Germplasms of the 113 Teas Used in the Present Study[a]

	3′′-OMe	EGCG	ECG	EGC	GC	EC	C	[b]TCG	[c]TCF	[d]TC	caffeine
3′′-OMe	1										
EGCG	0.107	1									
ECG	0.083	0.419**	1								
EGC	0.229**	−0.177*	−0.298**	1							
GC	0.216**	0.251**	0.060	0.331**	1						
EC	0.030	−0.299**	0.530**	0.329**	0.079	1					
C	−0.128	0.194*	0.487**	−0.152	0.162*	0.434**	1				
TCG	0.111	0.994**	0.516**	−0.203**	0.245**	−0.219**	0.241**	1			
TCF	0.201*	−0.201*	0.060	0.883**	0.440**	0.701**	0.165*	−0.182*	1		
TC	0.215**	0.825**	0.519**	0.294**	0.472**	0.179**	0.318**	0.840**	0.352**	1	
caffeine	0.043	0.703**	0.300**	−0.094	0.206**	−0.141	0.278**	0.700**	−0.077	0.616**	1

[a] * and **: $P < 0.05$ and $P < 0.01$, respectively; $n = 113$. [b] TCG: EGCG + ECG. [c] TCF: EGC + GC + EC + C. [d] TC: EGCG + ECG + EGC + GC + EC + C.

Table 6. Sequence Identity Matrix of the Sixteen Varieties with High Methylated Epicatechin Content in the Germplasms of the Tea Leaves Based on the ITS1-5.8S-ITS2 Sequence of the Ribosomal Genes

sequence	TD003	TD015	TD017	TD026	TD027	TD029	TD040	TD064	TD065	TD070	TD079	TD117	TD118	TD120	TD122	TD091
TD003	ID															
TD015	0.877	ID														
TD017	0.879	0.995	ID													
TD026	0.876	0.995	0.990	ID												
TD027	0.877	0.996	0.992	0.995	ID											
TD029	0.879	0.998	0.996	0.993	0.995	ID										
TD040	0.877	0.993	0.995	0.995	0.993	0.995	ID									
TD064	0.877	1	0.995	0.995	0.996	0.998	0.993	ID								
TD065	0.913	0.865	0.867	0.865	0.865	0.867	0.867	0.865	ID							
TD070	0.874	0.993	0.989	0.995	0.993	0.992	0.993	0.993	0.864	ID						
TD079	0.797	0.729	0.729	0.729	0.729	0.731	0.731	0.729	0.794	0.730	ID					
TD117	0.877	1	0.995	0.995	0.996	0.998	0.993	1	0.865	0.993	0.729	ID				
TD118	0.880	0.996	0.992	0.995	0.996	0.995	0.993	0.996	0.865	0.993	0.729	0.996	ID			
TD120	0.877	1	0.995	0.995	0.996	0.998	0.993	1	0.865	0.993	0.729	1	0.996	ID		
TD122	0.745	0.690	0.690	0.691	0.691	0.691	0.691	0.690	0.755	0.691	0.827	0.690	0.690	0.690	ID	
TD091	0.877	0.996	0.995	0.992	0.993	0.998	0.993	0.996	0.865	0.990	0.731	0.996	0.993	0.996	0.691	ID

of the tested tea varieties and *Camellia* spp. plants. Group IIb can be further divided into three subsubgroups (IIb-1, IIb-2, and IIb-3) at a genetic distance of 0.02 (**Figure 3**C). By comparing rDNA ITS sequences of the 16 tea varieties with elevated EGCG3′′Me content, the sequence identity values were determined to be in the range of 0.690−1. The determined sequence identity for TD015, TD064, TD117, and TD120 was 1, meaning that these four tea varieties have homologous ITS sequences (**Table 6**).

DISCUSSION

An allergy is a disorder of the immune system defined as excessive immunoreactivity. It has been estimated that 22% of people from 33 countries, representing 1.39 billion people, may suffer from some form of allergic disease based on a survey conducted by World Allergy Organization (WAO) in 2004 (*18*). The WAO's survey results highlight a pressing need for the development of allergy services and physiological/functional foods for allergy prevention worldwide.

O-methylated EGCGs, which are naturally occurring antiallergy catechins, were first isolated from oolong tea and later from various fresh tea leaves and commercial teas (*10*). The naturally occurring *O*-methyl-EGCG has a stronger antiallergy activity than EGCG (*10*, *11*). Screening tea germplasms for elevated *O*-methyl-EGCGs (3′′- and 4′′-methyl-epigallocatechin gallate, EGCG3′′Me and EGCG4′′Me) has received more attention recently (*11*, *19*−*21*). The location and degree of methylation in the galloyl moiety modifies EGCG's antiallergic activity. Compared to equal concentrations of EGCG, EGCG3′′Me has a higher inhibitory effect on nitric oxide generation and inducible nitric oxide synthase (iNOS) expression, while EGCG4′′Me was found to be less effective. Chiu and Lin reported the following order for the potency of NO synthase protein expression inhibition: EGCG3′′Me > EGCG > EGCH4′′Me > EGCG 4′,4′′-di-Me (*11*).

Studies of 200 tea germplasms containing high tea polyphenolic content in China demonstrated that the content of EGCG3′′Me is highly variable between different tea varieties.

Only six tested tea cultivars have more than 1% EGCG3″Me in their leaves (*21*). In Japan, the cultivar Benihomare and two of its offspring, Benifuuki and Benifuji, contain as much as 1% EGCG3″Me in their green tea; Benifuuki has been successfully implemented as a physiological−functional beverage for allergy prevention (*19*, *20*). Among the tea varieties in Japan, the cultivar Benifuuki contains the highest content, with approximately 2% EGCG3″Me (*19*−*21*). Our results indicate that eight tea varieties among the 113 tested tea germplasms in the Taiwan area have more than 1% of EGCG3″Me content in their tea leaves. Varieties Woan Joong (TD064) and Bair Mau Hour (TD117) contain as much as 1.63% and 1.52% EGCG3″Me, respectively, which are the first and second highest contents among the tested varieties. Our results from studying 113 tea germplasms indicate that the catechin content in tea leaves showed a significant positive correlation with the caffeine content. Unfortunately, tea varieties with a higher content of caffeine will be of limited use. It is important to note that the content of EGCG3″Me in tea leaves showed a significant positive correlation with the content of TC, and fortunately, the content of EGCG3″Me in tea leaves did not correlate with the content of caffeine. Apparently, higher EGCG3″Me content is an important characteristic for early selection in tea breeding programs. Chin Hsin Oolong tea is the most popular variety in Taiwan, while the varieties Woan Joong (TD064) and Bair Mau Hour (TD117) are relatively unpopular. Our results have pointed out the need to re-evaluate whether the old tea varieties meet the needs of today.

In official breeding programs, at least 21 years is required to breed a new tea variety. The TTES has successfully released 20 new tea cultivars. Taiwan has a long history of tea breeding; therefore, there is enough data to establish a reliable correlation between plant characteristics and tea quality, yield, fermentation ability, and some cultural behaviors. It is important to use such correlations in the early selection index of tea breeding. DNA marker approaches have been introduced in research on the germplasm and breeding of tea plants in order to study genetic diversity and variation, molecular identification, molecular phylogenetics, genetic stability and integrity of tea germplasm, and genetic linkage mapping (*22*). Among all of the target sequences used for the phylogenetic studies and polymorphism analysis, the ITS regions are valuable for their discrete phylogenetic separation of closely related species, recognition of new species, determination of conspecificity between isolates, discrimination within a species, and differentiation between piroplasm species and subspecies (*23*, *24*).

The tree topology separates the full ITS1-5.8S-ITS2 nucleotide sequences of the *Camellia* species into two well-supported branches with a common ancestor. The first branch contains a single node with two varieties, TD122 and TD079. The second branch contains two nodes, with the first node exhibiting two varieties (TD003 and TD065) and the second node grouping the rest of the tea varieties together (**Figure 3C**). By comparing the rDNA ITS sequences of the 16 tea varieties with elevated EGCG3″Me content, the sequence identity values were determined to be in the range of 0.690−1 (**Table 6**). Our results also indicate that there is sufficient sequence variation within the ITS regions to permit the identification of 12 of the 16 individual specimens of *Camella sinensis* with high EGCG3″Me content. We recommend the use of molecular authentication technology, especially in the ITS sequence of the rDNA gene, to be used in tea breeding for genetic marking and protection of a designated trait of interest.

In conclusion, the average level of (−)-epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3″Me) was 0.45% for all of the 113 varieties screened. Among them, 16 varieties with higher EGCG3″Me content (>0.8%) are good candidates for manufacturing partially fermented tea. Analysis of these tea varieties revealed that the content of EGCG3″Me in the tea leaves did not correlate with the content of caffeine. Genetic assessments revealed that the lengths of their internal transcribed spacers (ITS) were in the range of 638−670 bp and that the sequence identities were in the range of 0.690−1. A phylogenetic analysis led to the construction of two major groups, I and II, with a genetic distance of 0.08 based on the ITS1-5.8S-ITS2 sequences. The success of any breeding or genetic conservation program depends on the amount and distribution of the genetic variation present in the gene pool. Our results provide fundamental genetic information for tea varieties with elevated EGCG3″Me content and indicate a need for a comprehensive genetic assessment of the tea germplasms preserved in the TTES to better serve the future of tea breeding.

ABBREVIATIONS USED

EGCG3″Me, (−)-epigallocatechin-3-*O*-(3-*O*-methyl)gallate; ITS, internal transcribed spacers; EGCG, (−)-epigallocatechin-3-*O*-gallate; rDNA, ribosomal DNA; ECG, (−)-epicatechin-3-gallate; EGC, (−)-epigallocatechin; EC, (−)-epicatechin; C, (+)-catechin; GC, (−)-gallocatechin; TTES, Taitung Branch of the Tea Research and Extension Station; TC, total catechins; TCF, total free-catechin; WAO, World Allergy Organization; iNOS, inducible nitric oxide synthase.

Supporting Information Available: List of germplasms of the 113 teas used in the present study. This material is available free of charge via the Internet at http://pubs.acs.org.

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