



Development of 23 novel microsatellite markers of *Amomum tsao-ko* (Zingiberaceae) based on restriction-site-associated DNA sequencing

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Abstract

Amomum tsao-ko (Zingiberaceae) is a traditional Chinese medicine and condiment, and an important economic crop in the tropical forest of southwest China. However, few simple sequence repeat (SSR) markers are available in *A. tsao-ko*, which is hindering genetic research in this species. The aim of this study was to develop and characterize microsatellite markers for *A. tsao-ko* using restriction-site-associated DNA sequencing. A total of 115,482 microsatellites were identified using MISA software, and 13,411 SSR primer pairs were designed. 100 pairs of SSR primers were selected at random and used to evaluate polymorphisms among 4 *A. tsao-ko* samples. Finally, 23 pairs of SSR primers with clear bands and obvious polymorphism were selected for genetic diversity analysis of 72 *A. tsao-ko* accessions. The number of alleles and effective number of alleles per locus ranged from 2 to 6 and from 1.315 to 3.776, respectively. The observed heterozygosity ranged from 0.208 to 0.779, and the expected heterozygosity was from 0.239 to 0.735. The average values of the polymorphic information content were 0.454. Hardy–Weinberg equilibrium (HWE) analysis showed that 10 loci significantly deviated from HWE ($P < 0.05$). The pairwise F_{ST} and genetic distance values revealed low levels of genetic differentiation and high genetic similarity among six *A. tsao-ko* populations. These microsatellite markers developed will provide a valuable tool for further germplasm characterization, genetic diversity, and breeding studies in *A. tsao-ko*.

Keywords *Amomum tsao-ko* · Microsatellite · Genetic diversity · Zingiberaceae

Introduction

Amomum tsao-ko, commonly known as black cardamom, is a perennial evergreen tufted herb belonging to the family Zingiberaceae. It is mainly distributed in Southwest China and Northern Vietnam, and found at altitudes ranging from 1300 to 1800 m. It is a type of economical crop that can grow in humid forests in tropical and subtropical areas [1, 2]. Yunnan province is the main *A. tsao-ko*-producing area in China, contributing to 95% of the nation's output [3]. The whole plant of *A. tsao-ko* has a spicy smell, is edible and

used medicinally. Its dried fruit is often used to treat internal resistance to cold and dampness, epigastric distension, fullness and vomiting, and malaria among other ailments [4, 5]. Recently, *A. tsao-ko* was one of the clinical prescriptions for patients with light, ordinary and severe cases of COVID-19 in China, demonstrating its important role in antiviral therapy [6, 7]. In food, *A. tsao-ko* has become a top-grade seasoning, and widely used in food processing [8].

Crop breeding effectiveness not only depends on the number of germplasm resources but also largely depends on the mastery of genetic characteristics of the diversity of these germplasm resources. Previous studies on *A. tsao-ko* have primarily concentrated on chemical composition analysis and pharmacological action, and there are few reports on genetic diversity [9–11]. Microsatellite markers, also called simple sequence repeat (SSR) markers, are effective in estimating genetic diversity and relationships within and among populations, and are widely used in plant genetic research. Yang et al. [12] developed 24 pairs of SSR primers by FIASCO technique for *A. tsao-ko*, but only nine of them were polymorphic, such limited SSR markers are not enough

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to conduct comprehensive genetic studies. The objectives of this study were to develop a set of novel microsatellite markers using restriction-site-associated DNA sequencing (RAD-seq) technique. These markers will provide a useful tool for *A. tsao-ko* genetic research.

Materials and methods

A total of 72 accessions from six cultivated populations of *A. tsao-ko* were collected from Yunnan Province, China (Fig. 1, Table S1). The collections covered the main producing area, with the longitudes ranging from 99°49'12" (LC, Lancang population) to 103°31'48" (PB, Pingbian population), latitudes ranging from 22°53'24" (LVC, Lvchun population) to 24°17'24" (YX, Yunxian population), and elevations spanning from 1665 to 2108 m. Each population consisted of 12 individuals, and the linear distance between individuals in the same population is not less than 50 m to decrease the possibility of sampling from the same clone.

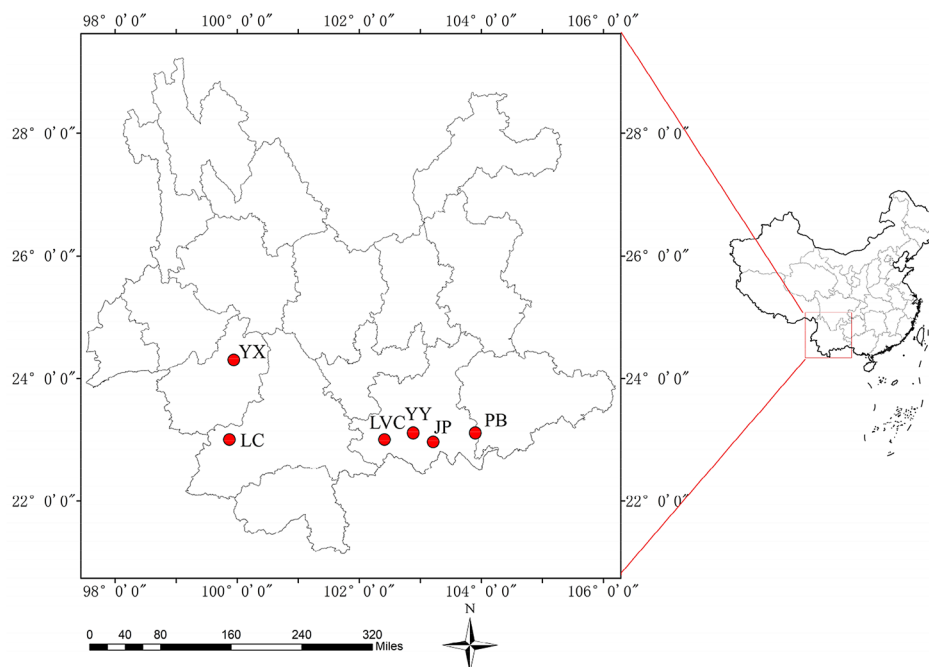
The genomic DNA of *A. tsao-ko* was extracted using a cetyltrimethylamine bromide method [13], and the concentration and quality of DNA were detected by NanoDrop 2000 ultra-micro spectrophotometer. The working solutions of genomic DNA (20 ng/ μ L) of different samples were stored at 4 °C. Six genomic DNA samples (one sample from each population) of *A. tsao-ko* were mixed with the same amount,

and the RAD-seq technique was used to simplify sequencing. The bidirectional sequencing of 350–650 bp recovery sequence was carried out by HiSeq 4000 sequencing platform with PE150 mode. To obtain clean reads, the adapters and sequences <50 bp and low-quality reads were removed. The clean reads were assembled using SOAPdenovo2.04 [14]. The sequencing data were deposited in the Sequence Read Archive (SRA) of NCBI database (SRA Accession: PRJNA529582).

The software MISA was used to search the SSR sites of the DNA sequences obtained by sequencing [15]. The configuration parameters are as follows: the minimum repetition times of single nucleotide, dinucleotide, trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide were 10, 5, 4, 4, 4 and 4, respectively, and the sequence length between two adjacent SSR is ≥ 12 bp. SSR primers were designed based on the flanking sequence of SSR locus using Primer 3.0 software [16]. The parameters were set as follows: GC content 40–60%, primer length 18–27 bp, annealing temperature 50–65 °C, and amplification product length 100–300 bp.

100 pairs of SSR primers were randomly selected for synthesis and were screened by four *A. tsao-ko* samples. Finally, 23 pairs of SSR primers with clear bands and obvious polymorphism were selected for genetic diversity analysis of 72 *A. tsao-ko* accessions. The 10 μ L polymerase chain reaction (PCR) system consists of 1.0 μ L template DNA (20 ng/ μ L),

Fig. 1 Map of *A. tsao-ko* samples collection sites. PB, Pingbian population; JP, Jinping population; YY, Yuanyang population; LVC, Lvchun population; YX, Yunxian population; LC, Lancang population (see Table S1 for full description of the populations)



0.8 μL SSR primers (0.2 $\mu\text{mol/L}$), 0.8 μL deoxyribonucleoside triphosphates (dNTPs) (2.5 mol/L), 1.2 μL 10 \times PCR buffer (including Mg^{2+}), 0.2 μL Taq DNA polymerase (2.5 $\text{u}/\mu\text{L}$), and 6 μL ddH₂O. The PCR procedure included pre-denaturation (95 $^{\circ}\text{C}/5$ min), followed by 95 $^{\circ}\text{C}/30$ s, 51–55 $^{\circ}\text{C}/30$ s, 72 $^{\circ}\text{C}/30$ s, a total of 30 cycles, and finally extended at 72 $^{\circ}\text{C}$ for 10 min to preserve at 4 $^{\circ}\text{C}$. All of the PCR amplifications were conducted using a thermal cycler (ETC-811, Eastwin). The amplified products were separated on 8% non-denaturing polyacrylamide gel (acrylamide:bisacrylamide = 19:1), stained with 1% silver nitrate at the end of electrophoresis and photographed after the bands were clear.

For each locus, the evaluation parameters including the observed number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (F), genetic distance, and pairwise F_{ST} values were calculated by GenAlEx 6.501 program [17], which are also used for the Hardy–Weinberg equilibrium (HWE) test. Frequencies of null alleles were estimated using Micro-checker 2.2.3 [18]. The polymorphism information content (PIC) of polymorphic loci was calculated using PowerMarker V3.25 [19]. 72 accessions were assessed by multivariate principal coordinate analysis (PCoA) performed with GenAlEx 6.501. A Mantel test was performed to determine the correlation between genetic distance and geographic distance matrices using NTSYS-pc 2.11 software [20].

Results and discussion

By RAD-seq, 5,269,456 (1,317,364,000 bp) raw reads were obtained, and 5,117,655 (1,185,485,454 bp) clean reads were obtained after filtering. A total of 115,482 microsatellites were identified using MISA software, and 13,411 SSR primer pairs were designed using Primer 3.0. 100 pairs of randomly selected primers were validated in four *A. tsao-ko* accessions, of which 95 pairs could amplify bands in the target area, 64 pairs were polymorphic (Table S2). 23 primer pairs with clear bands and obvious polymorphism were selected for further analysis (Table 1).

A total of 90 bands were amplified by 23 SSR markers in 72 *A. tsao-ko*, with an average of 3.913 bands per primer pairs. The total number of alleles (N_a) per loci varied from 2 (AM65) to 6 (AM49 and AM89), and the effective number of alleles (N_e) ranged from 1.315 (AM43) to 3.776 (AM89). The average N_e (2.305) was lower than the N_a (3.913) thus reflecting imbalanced allelic frequencies in populations, which could imply the presence of rare or unique alleles.

The observed heterozygosity (H_o) and expected heterozygosity (H_e) in each locus vary from 0.208 (AM43) to 0.779 (AM42) and from 0.239 (AM43) to 0.735 (AM89), respectively. All the genetic parameters were higher than those of SSR markers developed by Yang et al [12]. The PIC ranged from 0.228 (AM43) to 0.709 (AM89). The mean fixation index was 0.072, and values ranged from -0.181 (AM42) to 0.272 (AM13), indicating that there was no inbreeding depression in *A. tsao-ko* population. Among all the loci, 5 loci (AM4, AM12, AM13, AM40, and AM44) were exhibited heterozygote deficiency, as indicated by the positive and significant fixation index. Heterozygote deficiency could be the result of rare alleles, correlations among loci, or the sampling of closely related individuals. HWE analysis showed that 10 loci (AM4, AM6, AM8, AM12, AM13, AM42, AM43, AM44, AM45, and AM49) significantly deviated from HWE ($P < 0.05$), and the remaining 13 loci (AM1, AM7, AM9, AM17, AM27, AM29, AM36, AM40, AM41, AM65, AM89, AM94, and AM99) were in accordance with HWE (Table 2). Micro-checker analysis showed that null alleles frequencies were lower than 0.2 for each locus, indicating these HWE deviations may be caused by anthropic selection and short-cloning history in cultivated rather than null alleles [12, 21].

In this study, PCoA analysis did not clearly separate the populations, and the germplasm from different populations were mixed with each other (Fig. 2). Consistent to this result, the Mantel test revealed that there was no significant correlation between geographic and genetic distance ($r = 0.076$, $p = 0.579$), which suggested no significant geographic restriction to gene flow among the populations. The pairwise F_{ST} values ranged from 0.029 (LVC and LC) to 0.074 (YY and LC), with an average of 0.047. The genetic distance varied from 0.056 (JP and YX) to 0.146 (YY and LC) with an average of 0.072 (Table 3). These results showed a relatively low degree of genetic differentiation and high genetic similarity among these populations. *A. tsao-ko* is a small group of species that is geographically restricted to southern China (Yunnan), northern Laos and Vietnam at altitudes ranging from 1300 to 1800 m, where it thrives in well-drained, moist, organic-matter-rich soil in shade or partial shade [4, 22]. Narrow distribution areas and special habitats lead to the high genetic similarity between *A. tsao-ko* populations, similar results were also observed in *Dendrocalamus membranaceus* [23], *Thuja sutchuenensis* [24] and *Stemona parviflora* [25]. Furthermore, a high level of gene flow ($N_m = 5.425$) was detected among *A. tsao-ko* populations based on random amplified polymorphic DNA

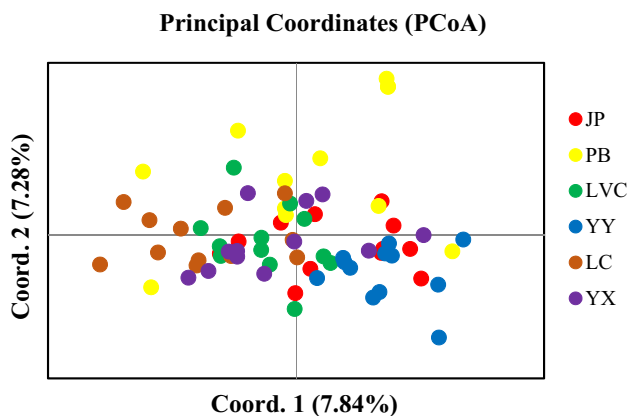
Table 1 Characterization of 23 microsatellite loci developed for *A. tsao-ko*

Locus	Primer sequences (5'–3')	Repeat motif	Ta (°C)	Size (bp)	GenBank accession number
AM1	F: CGCAGAATTAACCACCAGCG R: TGCCTCTCAATTGTTTCAGGACA	(A)16	55	135	MT495611
AM4	F: AGGGATAGAAGACAGCCAGGA R: TGAGGTTTCTCCGCACTGT	(AAAC)5	55	150	MT525020
AM6	F: GCCAAGAGCATATTCAGTGAGT R: ATAACCGACCCACCTAGTG	(AAC)7	53	137	MT525021
AM7	F: CGCGCTTGTGTTGGTTGGTTA R: TCCCTTGCTTGCCTGCTATT	(AAT)10	55	146	MT525022
AM8	F: CGAACAAACATGCCCGTGAA R: CGACAAGGTAACGGGTTC	(AAT)8	55	141	MT525023
AM9	F: TGGGAGATCGAGGAAGGAAGA R: GCCTGCACCGCCATTTTAT	(AATT)5	55	135	MT525024
AM12	F: AGCAAGTGACTCTCATCT R: ACTTGAGAGATACACAATGTGTT	(AG)13	51	133	MT525025
AM13	F: GACGTAGCTGCACTGCAATG R: CCGAACAAACCAACCGAACC	(AG)8	55	144	MT525026
AM17	F: TGTGGTAATCAAACGCGACG R: CGTCGAAGTCTGTCATATCTGC	(AGAGGG)5	54	137	MT525027
AM27	F: GAGGGAAGCGAATCTCCGAG R: GTCCTCTCCTTCTCGGCATC	(ATCGAA)5	55	151	MT525028
AM29	F: ACGTACCAGTACAAACCTCAGA R: ACGTGTTAGCGACGAAATTAGT	(ATT)8	54	143	MT525029
AM36	F: ACAAGAATAAATGCCCCGAA R: TGTTTGTAGTGTTCGCGTGC	(CTATTC)5	53	176	MT525030
AM40	F: TGGTACCACAAAACCTGCCA R: TGCCTGGCCAGTAAATTAAGGA	(CCT)6	55	154	MT525031
AM41	F: GGCCTCAAGGTGTTCTGTGTA R: AGGAGCTCCTCAACATCATCG	(CCT)8	55	143	MT525032
AM42	F: GTAATTAGCAGGCACGACGC R: ATCGTTGAAGTGTGGACGCT	(CCTCTG)5	55	149	MT525033
AM43	F: ACGACTAACCGATCGCCTTC R: CGTTCTCATCTCCGGAGTCG	(CGA)8	55	137	MT525034
AM44	F: CTTCCGACAAAGGGCTGAGT R: CCACGATGGAGTTAAGGCTGA	(CGC)11	55	133	MT525035
AM45	F: GAGCAAGGCAGCAGATTTTCG R: CGCTTCTCCGGAGTCGTA	(CGC)6	55	143	MT525036
AM49	F: TCGGCTTGGAGGTAGCTAGA R: AGGTAGGTAGGTGGTGCTGT	(CT)11	55	151	MT525037
AM65	F: TTCCCCGACAAAACCTAGGGC R: AGCAACGAGATCAAGGGCAA	(GCA)6	55	149	MT525038
AM89	F: GTCGTCGATTGACTCCGTCA R: TCCATGTTCTGCTGCGAAGT	(TCT)7	55	157	MT525039
AM94	F: GATGTGCGGCGAGACAGATA R: GGCTGAGCCTGAGAGTACAG	(TTAA)5	55	146	MT525040
AM99	F: AACCCTCGAACGACCCTTC R: CAACGTCCAAAAGCAGAAGCA	(TTGCCG)5	55	138	MT525041

Table 2 Genetic variation of the 23 polymorphic microsatellite loci in 72 accessions of *A. tsao-ko*

Locus	Na	Ne	Ho	He	PIC	F	FNA	HWE
AM1	4	2.444	0.514	0.591	0.516	0.130	0.073	0.233 ^{ns}
AM4	3	2.461	0.479	0.594	0.533	0.193*	0.129	0.046*
AM6	3	2.056	0.514	0.514	0.395	-0.001	0.013	0.000***
AM7	4	1.877	0.472	0.467	0.413	-0.011	0.039	0.961 ^{ns}
AM8	3	1.650	0.366	0.394	0.359	0.070	-0.022	0.006**
AM9	5	2.555	0.571	0.609	0.580	0.061	0.038	0.972 ^{ns}
AM12	4	1.403	0.211	0.287	0.302	0.265*	0.139	0.000***
AM13	3	2.062	0.375	0.515	0.454	0.272*	-0.033	0.000***
AM17	4	1.864	0.493	0.463	0.399	-0.064	0.154	0.202 ^{ns}
AM27	3	2.223	0.606	0.550	0.479	-0.101	-0.051	0.401 ^{ns}
AM29	4	1.704	0.394	0.413	0.387	0.045	0.041	0.860 ^{ns}
AM36	4	1.706	0.431	0.414	0.387	-0.040	0.124	0.220 ^{ns}
AM40	4	2.741	0.486	0.635	0.571	0.235*	0.060	0.153 ^{ns}
AM41	5	1.810	0.403	0.447	0.415	0.100	-0.096	0.916 ^{ns}
AM42	4	2.939	0.779	0.660	0.655	-0.181	0.080	0.000***
AM43	4	1.315	0.208	0.239	0.228	0.129	0.107	0.010*
AM44	4	2.598	0.486	0.615	0.543	0.210*	0.008	0.001**
AM45	5	2.377	0.556	0.579	0.515	0.041	0.079	0.001**
AM49	6	1.942	0.414	0.485	0.460	0.146	0.068	0.000***
AM65	2	1.591	0.324	0.371	0.323	0.128	-0.022	0.281 ^{ns}
AM89	6	3.776	0.771	0.735	0.709	-0.049	0.058	0.673 ^{ns}
AM94	3	1.584	0.333	0.369	0.328	0.096	-0.009	0.342 ^{ns}
AM99	3	2.267	0.569	0.559	0.492	-0.019	-0.025	0.979 ^{ns}
Mean	3.913	2.305	0.468	0.500	0.454	0.072		

Na, number of alleles; Ne, effective number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; PIC, polymorphism information content; F, fixation index, *significance value from 95% confidence interval; FNA, frequency of null alleles; HWE deviation from Hardy–Weinberg equilibrium (ns, no significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

**Fig. 2** Principal coordinate analysis based on the genotyping of 72 accessions of *A. tsao-ko* with 23 microsatellite loci

(RAPD) markers in our previous study [8]. Such gene flow was enough to resist the genetic differentiation caused by genetic drift. In conclusion, we developed and characterized 23 novel microsatellite markers in *A. tsao-ko*. The set of markers significantly increases the availability of polymorphic molecular markers for the species, and will provide a valuable resource for genetic diversity, core collection construction, genetic mapping and marker-assisted selection breeding in *A. tsao-ko*.

Table 3 Pairwise of F_{ST} (above diagonal) and genetic distance (below diagonal) for six *A. tsao-ko* populations

Population	JP	PB	LVC	YY	LC	YX
JP	–	0.052	0.034	0.034	0.052	0.032
PB	0.095	–	0.047	0.064	0.057	0.059
LVC	0.058	0.096	–	0.043	0.029	0.033
YY	0.059	0.141	0.081	–	0.074	0.056
LC	0.093	0.122	0.060	0.146	–	0.046
YX	0.056	0.112	0.057	0.111	0.084	–

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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