

Article

Developing Novel Microsatellite Markers for *Kaempferia parviflora* by Microsatellite Capture Sequencing (MiCAPs)

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Abstract: *Kaempferia parviflora*, a medicinal plant widely used in Southeast Asia, has been validated clinically for its diverse pharmaceutical applications. Despite extensive research in pharmacology, there is a notable lack of cytogenetic and genomic research, primarily due to limited genetic information. Simple Sequence Repeat (SSR) is considered a robust class of molecular markers frequently used in biodiversity studies. In this study, we adopted Microsatellite Capture Sequencing (MiCAPs) to obtain SSR sequences for marker development. We identified 13,644 SSRs and developed and validated ten sets of SSR markers through capillary electrophoresis. The ten primer sets generated 27 alleles, with an average Polymorphism Information Content (PIC) of 0.36. Principle Coordinate Analysis (PCoA) distinguished two types of *K. parviflora*, consistent with classification by leaf margin color (red and green). A neighbor-joining dendrogram of seven Zingiberaceae species was constructed with the SSR-containing sequences. The 2-c value of *K. parviflora* is first reported here as 3.16 ± 0.03 ; the genome size is estimated at 3090.48 Mbp. The newly developed molecular markers are crucial for variety identification and the conservation of wild resources. Additionally, the cytogenetic and phylogenetic information provides valuable insights into the genetic diversity and evolutionary relationships.

Keywords: SSR markers; MiCAPs; *Kaempferia parviflora*; biodiversity



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1. Introduction

Substantial traditional, complementary and alternative medicine (TCAM) use is evident across most socioeconomic groups in most cultures and amongst ethnic minority populations—groups of individuals with racial, national, cultural and/or religious origins dissimilar from the dominant population of the country where they reside—and constitutes a significant healthcare resource [1–3]. Globally, there has been an exponential increase in the use of TCAM, wherein traditional herbal medicines are important components [1,4].

Kaempferia parviflora Wall. ex Baker, also known as “Thai ginseng” or black ginger, is a medicinal plant in *Kaempferia* Linn, a genus which comprises approximately 60 species. *K. parviflora* is herbaceous perennial and conventionally propagated via rhizome. It has a dark purple rhizome with several succulent roots in a fascicle, which has been used as folk medicine in Thailand, Myanmar, Bangladesh, and India for many centuries and is now attracting increasing economic and scientific interest [5–7].

In Thailand and Laos, *K. parviflora* is traditionally known as a health-promoting herb, and its rhizomes are frequently used for the treatment of swelling, colic disorder, wounds, improve blood flow, increase vitality, and are employed against cough, stomachache, asthma, peptic and duodenal ulcers [8–10]. Clinical evidence has proved that *K. parviflora* has a wide variety of uses including in physical or exercise performance, erectile response, pain indicators, and energy expenditure [5]. Several pharmacological studies have reported its benefits for various ailments such as anti-plasmodial, anti-obesity, anti-inflammatory, anti-allergenic, anti-cancer, anti-cholinesterase, and gastroprotective benefits [9,11–16]. Moreover, *K. parviflora* is also reported to be a potential cure for weakness, lower blood glucose levels, and male impotence [9,12,13]. The rhizomes are also used as food ingredients and aphrodisiacs or made into tea and wine [10].

However, multiple medicinal and traditional uses of *K. parviflora* have led to its increased mass collection as a raw material, causing serious issues threatening the quality and consistency of wild populations and pharmaceutical products [7]. Compared to the extensive research in pharmacology, there is a disparity in the amount of cytogenetic and genomic research. Considering that the production ratio of bioactive metabolites, which the pharmaceutical industry heavily depends on, is significantly affected by genetic backgrounds, an evaluation of genetic diversity and the identification of cultivars become paramount [17].

Kaempferia species exhibit remarkable morphological similarities, presenting significant challenges in their identification and classification [18]. For *K. parviflora* species, the varieties can be divided into two main groups by leaf margin color: green and red types. In previous studies, HPLC analysis suggested that chemical composition differs in these two types [19,20]. Yet, the high degree of resemblance in their rhizomes complicates the distinction relying solely on physical examination of the rhizomes or derived products. In this context, molecular markers are considered as valuable tools for accurate identification. Genotyping by sequencing (GBS) has been applied to differentiate between the two types of *K. parviflora* [19]. However, due to limitations in cost and experimental conditions, single-nucleotide polymorphism (SNP) markers might not be the most ideal choice in scenarios requiring the rapid testing of a large number of samples.

SSRs or microsatellites are stretches of DNA consisting of only one or a few tandemly repeated nucleotides, which have been widely applied as molecular markers since their discovery in the 1980s [21]. The microsatellites mutate rapidly to provide differentiation of even closely related samples [22]. SSR markers are highly preferred among numerous molecular markers for diversity study due to their high polymorphism, stability, cost-effectiveness, ease of use, and co-dominance features [23]. SSR markers are multiallelic and co-dominant at a single locus, rendering them more informative per locus than SNP markers. Additionally, microsatellite PCR amplification protocols are standard and require only a small amount of DNA. This makes them highly practical in scenarios with modest throughput requirements or in settings where experimental conditions are limited [24].

With the advent of new next-generation sequencing (NGS) platforms, large volumes of sequencing data are being generated, which enables the easier, cheaper, and rapid identification of microsatellite markers [25]. However, for *K. parviflora*, there are currently no available SSR markers, nor is there genome or transcriptome information available for SSR marker development. Therefore, we adopted a sequencing method called Microsatellite Capture Sequencing (MiCAPs) that enriches microsatellites using probes to develop novel SSR markers. Through enrichment, the library size can be significantly reduced, further saving costs. This approach is considered to be friendly for underutilized species since it does not require a whole reference genome and can provide relatively high polymorphism information [26].

High-throughput sequencing data can easily detect a vast array of SSRs, presenting a new challenge of selecting those with high polymorphism for marker development. SSR length is positively correlated with polymorphism, the highest level being demonstrated in the SSR length range of 51–70 bp [27]. Before proceeding to PCR testing, several in silico

tests, such as electronic PCR (ePCR) [28,29] and Polymorphic SSR Retrieval (PSR) [30], can assist in the screening process, helping to identify the most promising SSRs for further analysis.

In this study, we sequenced eight *K. parviflora* accessions, developed SSR molecular markers, and identified ten highly polymorphic markers through a series of screening methods. These markers were validated for their polymorphism in PCR. The newly developed molecular markers are crucial for variety identification and the conservation of wild resources. Additionally, cytogenetic and phylogenetic information provides valuable insights into genetic diversity and evolutionary relationships. With these molecular tools and information, we hope to contribute to the conservation and sustainable utilization of this species.

2. Materials and Methods

2.1. Plant Materials

Eight *K. parviflora* accessions and thirteen other Zingiberaceae accessions from six different species were used in the current study (Table S1, passport data are also provided). The rhizomes were collected from Myanmar and Thailand and grown in controlled greenhouse conditions at the Tsukuba Plant Innovation Research Center—the University of Tsukuba (T-PIRC-UT), Tsukuba, Japan. Young leaf materials were sampled in September 2021 and kept at -80°C until DNA extraction in December.

These eight *K. parviflora* accessions consist of two morphotypes: the green type (Gene Research Center—University of Tsukuba, accession no. Z1064) and the red type (the other seven accessions) [31]. The red type was characterized by red-colored leaf margins absent in the green type [19].

The plant genetic resources used in this study were obtained through transfer in accordance with the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (ABS).

2.2. DNA Isolation, Library Establishment and Sequencing

For each sample, DNA was isolated from 100 mg frozen young leaf material using a modified CTAB method [32] with an additional 0.02 g PVP (polyvinylpyrrolidone) powder. DNA integrity was confirmed using 2% agarose gel electrophoresis at 100 V, 15 mA, for 25 min. DNA quality and purity were assessed using a NanoDrop 2000c spectrophotometer (Thermo Fisher, Waltham, MA, USA).

Subsequently, 100 ng of the DNA was fragmented by digestion with EcoRI-HF (New England Biolabs, Ipswich, MA, USA) and HindIII-HF (New England Biolabs, Ipswich, MA, USA). Fragmented DNA was ligated with custom fork adapters and size-selected using AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA). This series of steps is based on the Flexible ddRAD-seq approach [33]. The size-selected DNA was amplified using 25 high-fidelity PCR cycles with dual-index primers. PCR product quality and concentration were assessed using an Agilent 4200 TapeStation (Agilent Technologies, Waldbronn, Germany) and D5000 ScreenTape (Agilent Technologies, Santa Clara, CA, USA). Each product was diluted to a concentration of 100 nM and mixed in equal amounts.

After purification using AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA), the purified product was mixed with 1 μL of a customized biotinylated SSR probe (GA)₁₀ from a 100 μM stock in TE buffer (one of the probes is typically used for SSR enrichment), incubated at 95°C for 10 min, and then placed on ice. The mixture was hybridized by incubating at 60°C for 60 min. After washing 20 μL of the Dynabeads MyOne streptavidin C1 beads (Life Technologies, Carlsbad, CA, USA), they were resuspended in 29 μL of $6\times$ SSC buffer, added to each hybridized mixture, and incubated at 25°C for 30 min. The mixture was washed with $2\times$ SSC buffer (once) and $1\times$ SSC buffer (twice). Next, 20 μL of the SSR-enriched library was amplified using 15 high-fidelity PCR cycles with Illumina P5/P7 primers. Subsequently, the amplified product was purified to a 20 μL volume via a cleanup step using AMPure XP magnetic beads (Beckman Coulter). The

library quality and concentration were assessed using an Agilent 4200 TapeStation (Agilent Technologies) and D1000 ScreenTape (Agilent Technologies). The specific concentrations were determined using quantitative real-time PCR using a KAPA library quantification kit (Kapa Biosystems, Wilmington, MA, USA). The library was then sequenced using 2×300 bp paired-end sequencing using MiSeq (Illumina, San Diego, CA, USA).

The data were deposited with links to BioProject number PRJDB18485 in the DDBJ BioProject database: BioSample accession(s) SAMD00801926-SAMD00801947.

2.3. Pre-Processing, In Silico Polymorphic Detection, and Phylogenetic Analysis

Initially, the raw sequencing data were processed by fastp [34] version 0.23.2 with default settings to produce clean reads. The paired reads were integrated using the FLASH [35] version 1.2.11 with a maximum overlapping length of 500 and a minimum overlapping length of 60. Furthermore, the integrated reads with similar sequences were clustered using the CD-HIT-EST [36], version 4.6.

SSR polymorphism can be detected from the sequence redundancy when common sequences with SSR regions among samples are prepared as a reference sequence. To achieve this, we attempted two approaches, both based on mapping results, to detect polymorphisms.

Firstly, we adopted the in silico polymorphic detection and phylogenetic analysis method from MiCAPs protocol [26]. The sequences of all samples were combined into one and then re-clustered by CD-HIT-EST [36] version 4.6 as reference sequences. Following this, the sequence data of each sample were mapped to the reference using the CLC Genomics Workbench 9.5 (CLC Bio-Qiagen, Aarhus, Denmark). Consensus sequences for each sample were created from the mapped data. The SSR data were detected using SSRIT [37], from which polymorphism information was extracted to construct a polymorphic table. The genetic distance among samples was calculated using a distance matrix method in Populations [38] version 1.2.30, and a dendrogram established in MEGA7 [39].

The second approach was based on Polymorphic SSR Retrieval [30], a Perl package developed to identify polymorphic SSRs from NGS data and provide quantitative information for each call. PSR has two modules named PSR_read_retrieval and PSR_poly_finder, which are aimed at the identification of all the reads that cover full-length perfect SSR and the detection of length polymorphism, respectively. PSR_read_retrieval takes two files as input. The SSR information was detected by MicroSATellite identification tool [40,41] (MISA) v2.1, and the required BAM file was generated using bowtie2 [42]. However, when proceeding to PSR_poly_finder, we were unable to successfully invoke the module due to issues with the versions of support libraries. Therefore, a custom Python script was employed to extract SSR polymorphism from the output files of PSR_read_retrieval. A principle coordinate analysis (PCoA) based on genetic distance was processed with Microsoft 365[®] Excel[®] plug-in GeneA1Ex 6.503 [43,44].

2.4. SSR Marker Development and In Silico Evaluation

The clustered reads which included SSR regions were searched using MISA [40,41] v2.1. Definitions of unit size and min repeats were 2-6, 3-5, 4-5, 5-5, and 6-5 (unit size-min repeats); interruption was set as 100 bp.

Detected SSRs were filtered using a custom python script based on motifs, SSR type and size. Since all libraries prior to sequencing were enriched using an AG-motif probe, only those SSRs containing AG, GA, TC, and CT were included. Considering polymorphic possibility, only perfect SSRs with a size between 30 and 70 nt were used to develop markers.

Primer3 [45,46] v2.6.1 was utilized in novel SSR marker development. Five primer sets were designed for each SSR locus. All markers were listed together and deduplicated based on SSR and its flanking region using a custom script, and the similarity threshold was 90%.

An in silico simulation was then conducted with ePCR [28]. The sts size range was set as 100–1000, word size 12, margin 3000, max mismatches and indels 0. Thereafter, we used the custom script to select ten marker sets with the most alleles for further PCR evaluation.

2.5. PCR Validation of Novel SSR Markers

Ten novel SSR markers were first tested with PCR and agarose gel electrophoresis to check for amplicons of the expected size. The PCR reaction mixture was 10 µL in total, made of 0.1 µL Takara ExTaq (5 U/µL), 1 µL 10× ExTaq buffer, 0.8 µL dNTP mixture, 1 µL each of forward and reverse primer, 2 µL DNA sample (20 ng/µL) and 4.1 µL double distilled water. PCR reactions were carried out in GeneAmp PCR System 9700 (Thermo Fisher, Waltham, MA, USA). The PCR program consisted of an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 65 °C for 30 s and an extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. The PCR product was then mixed with 6× Midori Green Advance DNA stain (Nippon Genetics, Tokyo, Japan) and Sample Treatment for Tris Acid (Nacalai Tesque, Kyoto, Japan), following the manufacturer's instruction, and a total 6 µL of mixture was added in one well of the 2% agarose gel. Electrophoresis was carried out at 100 V, 15 mA for 25 min. The gel was scanned, and the bands were identified using the Gel Doc XR Imaging System (Bio-Rad, Hercules, CA, USA).

Then, fluorescently labeled universal primer was added through a three-primer PCR approach [47], which aimed to provide accurate size measurements. We chose Strategy II—Singleplex using multiple fluorophores—to design this PCR experiment. PCR was performed using extended forward primers and four labeled universal primers: Tail A~D labeled with FAM, VIC, NED and PET, respectively. The forward primer and tail combinations are shown in Table S2. This PCR setup was similar to the previous parameters, except in the reaction mixture, primers were replaced with 0.4 µL of the fluorescently labeled universal primer, 0.3 µL of the forward tailed primer, and 1 µL of the reverse primer. The reaction process was adjusted to an initial denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C (30 s)/65 °C (30 s)/72 °C (60 s), then 8 cycles of 95 °C (30 s)/56 °C (30 s)/72 °C (60 s), and a final extension at 72 °C for 10 min. The PCR products were subsequently screened in capillary electrophoresis (the service provided by Fasmac, Kanagawa, Japan).

The obtained allele information was processed using Microsoft Excel® plug-in GeneAlix 6.503 [43,44].

2.6. Flow Cytometry Analysis

To determine the relative nuclear DNA content (2C value) and estimated genome size (EGS) of *K. parviflora* (GRC-UT accession no. Z1064), young leaf samples were collected and analyzed by flow cytometry (Quantum PA, GmbH, Munster, Germany) following the instructions of CyStain® UV Precise P kit (Sysmex, Partec GmbH, Münster, Germany). At least three independent measurements were performed. Only measurements with CVs of less than 5% were included in the analysis. *Zingiber officinale* Roscoe (GRC-UT accession no. Z012-1), with a 2C DNA value of 3.23 pg [48], was used as an external standard.

The relative 2C value was computed based on the following formula [49]:

$$\text{Sample 2C value} = \text{Reference 2C value} \times \frac{\text{sample 2C mean peak position}}{\text{reference 2C mean peak position}}$$

Thereafter, the EGS was calculated using 1 pg = 978 Mbp [50].

3. Results

3.1. Estimating 2C Value and Genome Size Using Flow Cytometry

The mean relative nuclear DNA content of *K. parviflora* was 3.16 ± 0.03 pg, with a mean EGS of 3090.48 Mbp. Species ploidy was also confirmed as diploid. Further details

are provided in Table 1. The relative fluorescence intensity histogram results are presented in Figure S1.

Table 1. The computed 2C value and estimated genome size of *K. parviflora* compared to the standard, *Z. officinale*.

Species	Accession Number	Mean 2C Value (pg)	Genome Size (Mbp)	Number of Cells	CV (%)	Ploidy
<i>K. parviflora</i>	Z1064	3.16 ± 0.03	3090.48	565	2.1	2×
<i>Z. officinale</i>	Z012-1	3.23 ± 0.00	3158.94	1372	3.1	2×

3.2. Sequencing Results Statistics

For all 21 accessions, Illumina sequencing generated a total of 2.80 million raw reads, with 1.03 million originating from *K. parviflora*. The average Q30 score was 92.42%, indicating a high sequencing quality. After removing adapters and filtering out low-quality reads, each *K. parviflora* accession had an average of 126.00 thousand reads with a total length of 28.79 million bases, which constituted only a very small fraction of the estimated genome size of 3090.48 Mbp for the species (Table 2).

Table 2. Sequencing metrics of raw and processed data.

		Average All Accessions	Average <i>K. parviflora</i>	Total
Raw data	Total Reads (K)	133.34	128.37	2800.22
	Total Bases (M)	32.00	30.02	671.98
	Q30 Bases (%)	92.42	93.35	N/A
After fastp	Total Reads (K)	130.21	126.01	2734.46
	Total Bases (M)	30.57	28.79	641.91
	Q30 Bases (%)	94.09	94.79	N/A

3.3. Phylogenetic Study Based on Sequence Mapping

The sequence sets of all 21 accessions from seven species were merged into one file and re-clustered using CD-HIT-EST as the reference sequence. Then, the sequence data of each sample were mapped to this reference to create consensus sequences. The SSR data of consensus sequences were identified using SSRIT and its polymorphism was utilized to build a neighbor-joining tree (Figure 1).

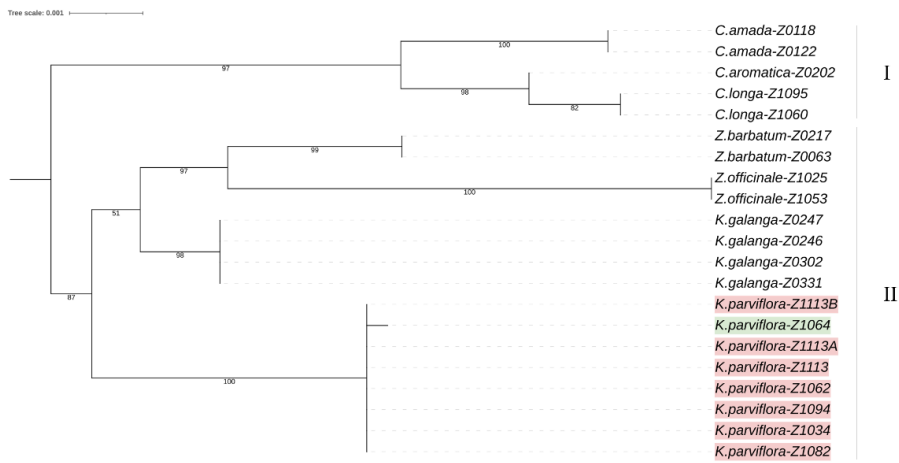


Figure 1. Neighbor-joining dendrogram constructed from SSR polymorphism. Bootstrap values are shown below the line. Two types of *K. parviflora* are labeled with the color of their margin.

Two clades are recognized in the dendrogram, wherein Clade I is exclusively composed of *Curcuma* species and has very strong bootstrap support, with values exceeding 95%; Clade II is composed of *Kaempferia* and *Zingiber* species, with significant bootstrap support ranging from 87 to 100% at major nodes. Within *K. parviflora*, the accession Z1064, morphologically classified as the green type, is of particular interest. The branch length associated with Z1064 is substantially longer than those of other accessions, hinting at potential genetic divergence.

3.4. Novel SSR Marker Development and Evaluation

SSRs were detected from integrated sequences of *K. parviflora* accessions using MISA. In total, 496.92 sequences with a size of 120.74 million bases were scanned, and 13,644 SSRs were found. Among all sequences, 92.63% of them contain at least one SSR (Table 3).

Table 3. Summary of SSRs detected in each *K. parviflora* accessions.

Accession	Z 1034	Z 1062	Z 1064	Z 1082	Z 1094	Z 1113	Z 1113A	Z 1113B
Examined sequences (Mbp)	14.62	12.13	14.42	19.19	14.09	13.49	17.26	15.55
Identified SSRs	1607	1362	1568	2233	1654	1479	1936	1805
SSR density (per Mbp)	109.91	112.33	108.77	116.34	117.41	109.67	112.20	116.05

Since all libraries prior to sequencing were enriched using the AG-motif probe, motifs without AG, GA, TC or CT were excluded. Subsequently, based on the length of the repeat sequences, perfect SSRs with repeat lengths between 30 and 70 bp were selected for marker development. A total of 1819 SSRs met the above conditions, accounting for 13.33% of all SSRs.

Primer3 was used to develop five markers for each locus. Since the SSRs are derived from eight accessions of the same species, it is necessary to remove duplicate markers. We used Python ‘Bio.Align’ library for sequence clustering, and considered those with a similarity greater than 0.9 as the same locus. Then, we compared the forward primer and the reverse primer to remove identical markers.

After removing identical ones, 3920 markers (in 796 clusters) were evaluated in the ePCR simulation. As a result, 2111 markers produced two or more than two alleles, 708 produced only one allele, and 1101 did not produce alleles. All SSR and designed markers are provided in Table S3. The ten most polymorphic markers were synthesized and tested with PCR. The PCR products were separated in agarose gel electrophoresis, all of them produced amplicons of the expected size.

To further test the performance of these novel markers in genetic diversity analysis, we added a fluorescently labeled tail through nested PCR and separated the PCR products in capillary electrophoresis. A representative electropherogram (Z1082-Kp10) is shown in Figure S2. The ten markers produced 27 alleles across eight accessions, averaging 2.7 alleles per locus. The Polymorphism Information Content (PIC) values range from 0.19 to 0.61, with an average of 0.36 (Table 4).

Table 4. Characteristics of 10 selected SSR marker sets for *K. parviflora*.

ID	Motif	Forward Primer	Reverse Primer	Expected Size (bp)	PIC
Kp01	GA	TGGCGAAGAAATCCAAGGATG	AGGAATCAAAACTTGAGCTTTCTTCT	101	0.19
Kp02	GA	TGGGCAACAATTATAGGAGAGGA	TGTCTATGCTCCGTTGACACA	103	0.29
Kp03	TCCTC	CCTCCATCTCTGCTAGCTCTC	AAAGCAGAGGAAATGGCCGA	112	0.36
Kp04	TC	GGAGGGGTTTCCACCGAAAT	TCGAAGAAGCAGCCGAAGAG	141	0.44
Kp05	CCTCT	CTCAATTCCTCACCAGACC	TAGAGCTCCCTTTGCTTGGC	164	0.21
Kp06	GGAGAG	CCGATCCCAAGGGTGTAAG	TCCCTCATTCACACATTCTCT	176	0.30
Kp07	AG	GGACTGATGTGCGCTAGTGA	TCGTACTCCTAGAACATCCACCT	186	0.61
Kp08	GA	ACTCGGTGAAGTTAGGCGTG	CGGAAAGGTGGAAAATCGGC	206	0.30
Kp09	CT	GCTCGAAATCCACCACCTCA	TTCATAGGTCAGCCGTTGCA	214	0.51
Kp10	AG	AGGTGTCCACTAACATACTAGCA	CGGGAGCCTAGTGACAAAGT	217	0.38

3.5. Diversity Analysis Using Novel SSR Markers

PSR was conducted using merged sequences as references and SSR information from MISA. An allele was considered heterozygous if over 30% of the reads supported the minor allele or homozygous if over 70% supported the major allele. A polymorphic table was established from counting the valid alleles.

PCoA was performed based on both PCR genotyping and PSR. The first two coordinates can explain 58.74% of the variations in PCR genotyping and 49.26% variations in PSR. From both sources, accession Z1064, which is recognized as the green type, is separated from the red type accessions (Figure 2).

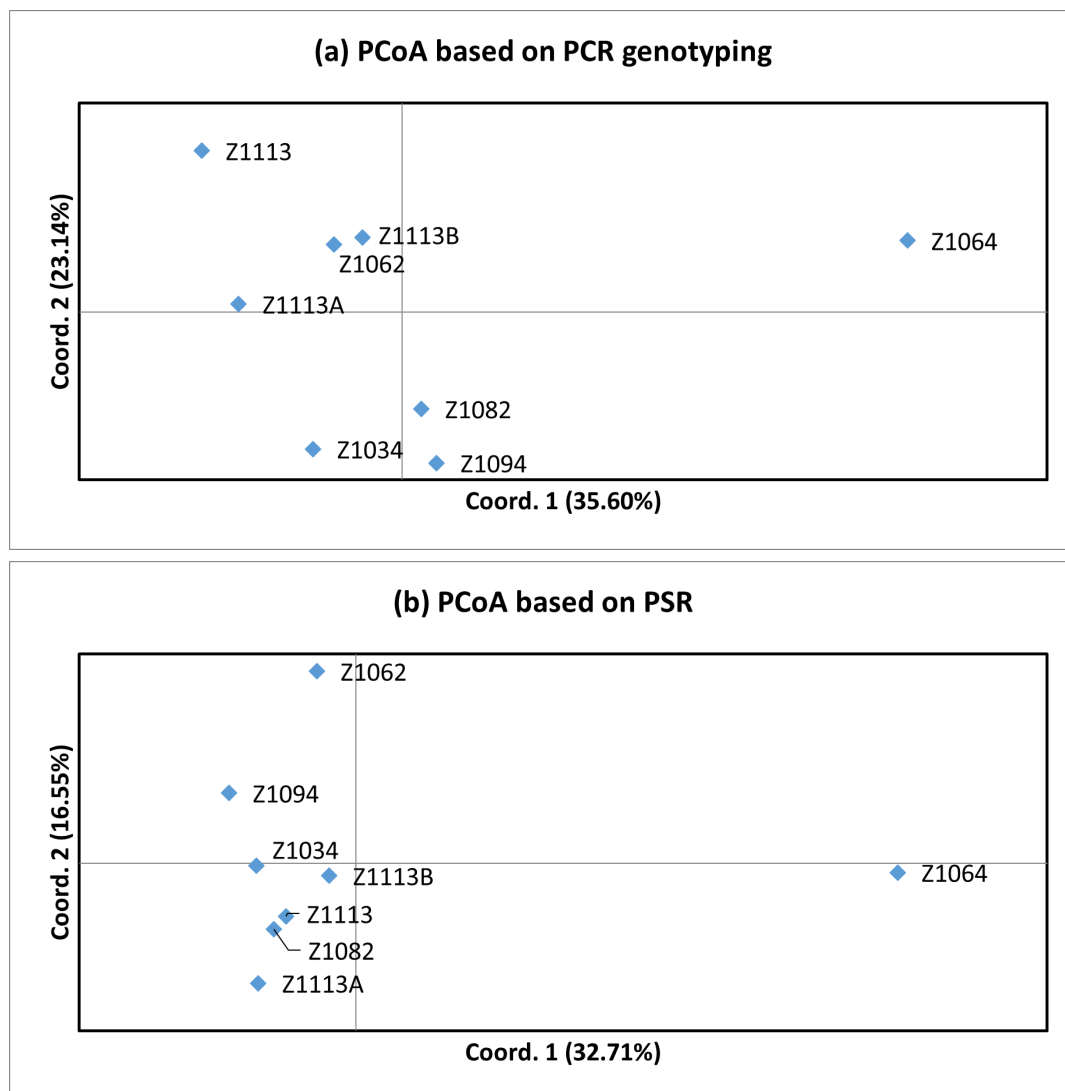


Figure 2. PCoA of eight *K. parviflora* accessions based on (a) novel SSR marker and (b) Polymorphic SSR Retrieval.

4. Discussion

Currently, there are no reports on the 2C value of *K. parviflora*. The only report on the genus *Kaempferia* was for *K. scaposa*, with a 2C value of 2.333 ± 0.007 [51]. This study measured the 2C value *K. parviflora* using FCM, which was found to be 3.16 ± 0.03 . Based on this, its genome size was estimated as 3090.48 Mbp. *K. parviflora* and ginger (*Zingiber officinale*) both belong to the subfamily Zingiberoideae, tribe Zingibereae [52]. Several high-quality complete genome assemblies for ginger have been reported, with genome

sizes of 3.1 Gb [53], 3.04 Gb [54], and 2.99 Gb [55]. This suggests that the estimated genome size of *K. parviflora* is similar to that of these closely related species.

The conventional approach of SSR development was based on Sanger sequencing, which was expensive and time consuming, usually yielding a small number of useable markers [24]. With the advent of NGS, sequencing costs have drastically reduced. NGS is therefore considered a superior approach to develop molecular markers including SSR [56]. However, for under-utilized species, it is still challenging to attract sufficient funding for whole genome sequencing using NGS. Yet, the survey and conservation of the genetic diversity of these species are urgent matters. In this study, SSR-containing fragments were enriched using probes before sequencing, which allowed us to obtain the necessary sequence information for SSR molecular marker development and phylogeny study at a very low cost [26]. MiCAPs yielded 28.79 Mbp of clean data for each accession, which was a very small fraction compared with the estimated entire genome of 3090.48 Mbp.

While NGS provides a vast amount of data for the development of numerous SSR markers, sifting through these to find truly polymorphic markers remains a challenge. Conventionally, the screening is conducted via expensive and low-throughput electrophoretic methods [24]. Yet, in this study, due to significantly reduced sequencing costs, we managed to sequence multiple accessions of the same species. This redundancy in sequence data enabled us to conduct *in silico* checks before it went to PCR genotyping. As a result, ten SSR markers were developed from the loci that produced the most alleles in the ePCR simulation.

These novel SSR markers were then evaluated via PCR amplification. All the validated SSR markers showed low-to-moderate levels of informativeness (PIC 0.19 to 0.61, average 0.36). The PIC values are lower than those of EST-SSR markers in *Zingiber officinale* [57] and similar to those in *Curcuma longa* [58,59], indicating moderate utility for genetic diversity studies in these species. The relatively low PIC values observed in *K. parviflora* may be partially attributed to its asexual reproduction via rhizomes, which often results in reduced genetic diversity.

In principle, PSR retrieves all reads located at the same locus and estimates the repeat number of motifs at that locus based on their quantity, whereas ePCR is based on sequence alignment and does not count the number of reads; thus, it is more of a qualitative analysis. Therefore, in this study, we used ePCR for preliminary screening to help in marker selection and PSR along with PCR to test the effectiveness of the newly developed markers. PSR retrieves all possible SSR loci and provides polymorphism information, whereas PCR is based solely on the ten selected loci. Z1064 was found to be distinctly differentiated from the other seven accessions in the PCoA plots obtained from both approaches. This indicates that the ten SSR markers we developed can provide sufficient polymorphism information for genetic diversity studies or variety identification. Since studies have shown significant differences in the levels of several key pharmaceutical compounds in rhizomes between the two varieties, distinguishing and identifying these varieties has become critically important [14,19,20,60].

Polymorphism information obtained from sequence mapping can be used for phylogenetic studies. In this study, in addition to the eight *K. parviflora* accessions, MiCAPs was also performed on thirteen other Zingiberaceae accessions from six different species. The results obtained show that the phylogenetic relationships of *Curcuma*, *Zingiber*, and *Kaempferia* are consistent with previous studies based on other molecular sequences, including nuclear internal transcribed spacer (ITS) and plastid *matK* regions [52].

In a previous population structure study based on over 3000 SNP markers, *K. parviflora* samples could be divided into two groups, which were consistent with the classification by morphological features (mainly by red and green leaf margins). The HPLC results also indicate significant differences in the contents of compounds such as PMF, TMF, DMF, 3,5,7,4'-tetramethoxyflavone, and 3,5,7-trimethoxyflavone between the two types [19]. In the current study, SSR-based polymorphic information also suggests genetic divergence between these two types, which is consistent with previous research.

5. Conclusions

We developed SSR molecular markers for *K. parviflora* through MiCAPs and verified the utility of ten sets. In addition, we constructed a phylogenetic tree of seven Zingiberaceae species and reported the 2C value of *K. parviflora*. These novel molecular markers will contribute to future studies on the genetic diversity, population structure, and phylogenetics of this species. Moreover, our method establishes a feasible, low-cost protocol for diversity evaluation and developing SSR markers for other underutilized species.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy14091984/s1>, Figure S1: Relative fluorescence intensity histogram of *K. parviflora* (Z1064) (left) and *Z. officinale* (Z2012-1) (right) obtained through flow cytometry analysis; Figure S2: A representative electropherogram showing amplicon size (Z1082-Kp10); Table S1: Passport information of all plant materials used in current study; Table S2: SSR markers and fluorescently labeled tail for PCR validation; Table S3: All designed SSR markers.

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