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RESEARCH ARTICLE

Mining and characterization of EST-SSR markers for *Zingiber officinale* Roscoe with transferability to other species of Zingiberaceae

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Abstract *Zingiber officinale* is a model spice herb, well known for its medicinal value. It is primarily a vegetatively propagated commercial crop. However, considerable diversity in its morphology, fiber content and chemoprofiles has been reported. The present study explores the utility of EST-derived markers in studying genetic diversity in different accessions of *Z. officinale* and their cross transferability within the Zingiberaceae family. A total of 38,115 ESTs sequences were assembled to generate 7850 contigs and 10,762 singletons. SSRs were searched in the unigenes and 515 SSR-containing ESTs were identified with a frequency of 1 SSR per 25.21 kb of the genome. These ESTs were also annotated using BLAST2GO. Primers were designed for 349 EST-SSRs and 25 primer pairs were randomly picked for EST SSR study. Out of these, 16 primer pairs could be optimized for amplification in different accessions of *Z. officinale* as well as other species belonging to Zingiberaceae. GES454, GES466, GES480 and GES486 markers were found to exhibit 100% cross-transferability among different members of Zingiberaceae.

Keywords Microsatellite · *Curcuma* · *Zingiber zerumbet* · *Hedychium spicatum* · Ginger

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Introduction

Zingiber officinale Roscoe (Zingiberaceae) is a perennial plant. It is native to tropical climates of India, Malaysia, Australia, China, Brazil, United States and several other parts of the world (Langner et al. 1998). Rhizome of *Z. officinale*, commonly called as ginger, is generally consumed as a spice for its flavor enhancing effects. Gingerols, shogaols, paradols and zingerone are the main phytoconstituents responsible for its pungency and flavor (Pour et al. 2014). Ginger is also an age old medicine used for its wide array of pharmacological activities. It has been reported to have carminative, gastroprotective, antiemetic, antitussive, antipyretic, spasmolytic, analgesic and peripheral circulatory stimulant effects (Suekawa et al. 1984; Ghosh et al. 2011; Marx et al. 2013; Haniadka et al. 2013; Pour et al. 2014). The extract of ginger was shown to posses anti-inflammatory and anticancer activities (Miyoshi et al. 2003; Habib et al. 2008). It has also shown prominent and effective glycaemic control properties in diabetes mellitus and related complications (Li et al. 2012).

Most commercially cultivated varieties of *Z. officinale* rarely flower and generally do not produce viable seeds (Inden et al. 1988). However, it is noteworthy that considerable diversity is known in terms of color of rhizome, its size, aroma, fiber content and chemical profiles, in various cultivated varieties (Pandotra et al. 2013a; Khan et al. 2016). Present study was planned to understand the genetic basis of such high morphological diversity observed in *Z. officinale*, despite being a vegetatively propagated plant. For this, expressed sequence tag (EST) databases offer a rich source of information and EST-SSRs have been widely used for diversity analysis and development of new molecular markers in plant species. Simple sequence repeats (SSRs) or microsatellites are short

repetitive DNA sequences which occur due to slipped strand mispairing (Leclercq et al. 2010). It has also been shown that SSRs found in coding sequences (EST-SSRs) are polymorphic across species which may be helpful in phylogenetic analysis (Gandhi et al. 2010; Awasthi et al. 2012) as well as plant breeding studies (Scott et al. 2000; Pashley et al. 2006).

Earlier, we have studied chemical diversity, differential ability to accumulate heavy metals, morphological diversity and genetic diversity on the basis of inter simple sequence repeat and retrotransposon based markers in various accession of *Z. officinale* (Pandotra et al. 2013a, b; Khan et al. 2016). Present study involves characterization of the ESTs of *Z. officinale* for the type of SSRs present, annotation of their putative function and roles in different biological processes. Apart from studying diversity in accession of *Z. officinale*, the EST-SSR markers developed in this study were also assessed for their ability to be cross-transferred to other species of Zingiberaceae family.

Materials and methods

Collection of plant material

25 accessions of *Z. officinale* were collected from different locations in India, as shown in Table 1. *Zingiber zerumbet* (L.) Roscoe ex Sm. (Zingiberaceae), *Hedychium spicatum* Sm. (Zingiberaceae), *Curcuma longa* L. (Zingiberaceae), *C. amada* Roxb. (Zingiberaceae), *C. aeruginosa* Roxb. (Zingiberaceae), *C. aromatica* Salisb. (Zingiberaceae) and *C. angustifolia* Roxb. (Zingiberaceae) plants were used to assess the cross species transferability of EST-SSR markers. These plants were grown in institutional experimental farms [Jammu, India 32°44′N:74°54′E], as described earlier (Pandotra et al. 2013b).

Data mining for EST-SSR markers and primer designing

38,115 EST sequences of *Z. officinale* were downloaded from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov). EST-SSRs were mined using MIcroSAtellite identification tool (MISA, http://pgrc.ipk-gatersleben.de/misa/). The parameters used for identification of SSRs were same as described in our previous study (Awasthi et al. 2012; Gandhi et al. 2010; Mahajan et al. 2015). Primers used for EST-SSR study were designed using PRIMER3 software (http://primer3.ut.ee/).

DNA isolation and amplification of genic microsatellite markers

Total DNA was isolated from young leaves, by CTAB method (Doyle and Doyle 1987), and analyzed using electrophoresis and NanoDrop 2000c spectrophotometer (Thermo Scientific, MA, USA). 20 µl polymerase chain reaction (PCR) was carried out in a PCR machine (Eppendorf, Germany). The reaction mixture comprises of 50–100 ng of genomic DNA, PCR buffer (10×; with $MgCl_2$), 2 µl dNTPs (2 mM), 2 µl of each primer (0.5 µM), 1U of *Taq* DNA polymerase (New England Biolabs, England, UK). The PCR was set at following conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, 1 min at T_m (for detail see Table 2), 1 min at 72 °C, and final extension at 72 °C for 10 min followed by hold at 4 °C. A control reaction was performed using 18S rDNA primers to ascertain the presence of amplifiable genomic DNA. PCR products were evaluated by polyacrylamide gel electrophoresis (PAGE) for changes in amplicon size. Amplicon size changes up to ± 15 base pairs were taken into consideration, remaining were discarded as possible indels and mispriming.

Statistical evaluations

EST-SSR bands were scored as discrete variables. Presence and absence of a band was scored as 1 and 0 respectively (binary data). Dice coefficient of similarity (Dice 1945) was calculated for the accessions, to examine the genetic relatedness. Further, for plotting of dendrogram, a similarity matrix generated using the Dice coefficient was clustered in 'SAHN' subroutine using UPGMA (Unweighted Pair Group Method with Arithmetic mean) method. NTSYSpc ver 2.2 tool was used for statistical evaluation.

Results

Identification and characterization of EST SSR marker from *Z. officinale*

We mined the EST resource of *Z. officinale* containing a total of 38,115 ESTs, and clustered them using MegaBLAST. Clusters were assembled using a CAP3 assembly to generate 7850 contigs and 10,762 singletons. SSRs were searched in these unigenes and 512 microsatellite containing ESTs were identified with 500 ESTs having single SSRs while 15 ESTs had more than 2 SSRs. 349 EST sequences were found appropriate for primer designing.

Table 1 Locations of different accessions of *Z. officinale* collected from various regions of India

S. no.	Accession code	Location	Longitude E (°)	Latitude N (°)
1	Zo51	Nainital	79.2700	29.2300
2	Zo63	Bhageshwar	79.7700	29.8500
3	Zo75	Tehri	78.4800	30.3800
4	Zo76	Tehri	78.4800	30.3800
5	Zo77	Dehradun	78.0290	30.3180
6	Zo78	Nainital	79.2700	29.2300
7	Zo91	Sagar	78.6667	23.8000
8	Zo142	R S Pura	74.7300	32.6300
9	Zo148	Jammu	74.8700	32.7500
10	Zo151	Sagar	78.6667	23.8000
11	Zo154	Sagar	78.6667	23.8000
12	Zo160	Calicut	75.7700	11.2500
13	Zo162	Calicut	75.7700	11.2500
14	Zo164	Calicut	75.7700	11.2500
15	Zo241	Barabanki	81.2000	26.9200
16	Zo254	Sagar	78.6667	23.8000
17	Zo256	Sagar	78.6667	23.8000
18	Zo257	Sagar	78.6667	23.8000
19	Zo287	Bhageshwar	79.7700	29.8500
20	Zo292	Tehri	78.4800	30.3800
21	Zo293	Tehri	78.4800	30.3800
22	Zo294	Bhageshwar	79.7700	29.8500
23	Zo197	Tehri	78.4800	30.3800
24	Zo308	Sirmour	77.2940	30.5594
25	Zo322	Sagar	78.6667	23.8000

The frequency of SSR was 1 per 25.21 kb. Five different repeat motifs were identified (di-, tri-tetra-, penta- and hexanucleotide). As expected, tri-repeats were the most abundant (39.74%) SSR in the ESTs followed by di- (24.48%), hexa-(13.75%), tetra-(12.24%) and penta (9.79%) as summarized in Supplementary Table 1. Among the di-nucleotide repeats, there was a distinct predominance of TA (24.6%, 32/130) and GA (21.5%, 28/130) repeats, with low frequencies of other di-nucleotide repeats. Amongst trinucleotide repeats, CGC (5.2%, 11/211), CTC (5.2%, 11/211), GAG (5.2%, 11/211), GGA (5.6%, 12/211) and TCC (7.1%, 15/211) repeats were found to be in majority. The most frequent tetra- and penta-nucleotide repeats were TTTG (6.1%, 4/65) and AATAT (7.6%, 4/52) respectively, but their frequencies were low. We also identified hexa-nucleotide repeats but most of them were found only once (Supplementary Table 2).

Functional annotation and classification of EST SSR sequences from *Z. officinale*

The annotations of 349 EST sequences were performed using Blast2GO tool (Conesa et al. 2005). The query sequence was aligned with the non-redundant protein sequences from NCBI database. A match with an E-value of 10^{-6} or less was considered as significant. Gene ontology analysis was performed to determine biological process, molecular functions and cellular component of these ESTs. Several ESTs were found to be involved in different biological processes like transport, protein modification, metabolic processes, response to different stresses while many had hydrolase, kinase activity and binding domains for nucleic acid, lipids and proteins (Supplementary Fig. 1).

EST SSR marker development and its cross transferability study within Zingiberaceae

25 accessions of *Z. officinale*, collected from different regions of India were used for wet lab validation of EST-SSR markers (Table 1). Prior to validation studies, cross-species transferability of these EST-SSR markers was assessed by carrying out in silico PCR against *Curcuma longa* unigenes. 12,565 ESTs from *C. longa* were assembled in the same way as described for *Z. officinale* into 2978 contigs and 4072 singletons. 116 primer pairs resulted

Table 2 Primers used in the study

Locus name	Primer sequence (5′ → 3′)	Repeat motif	Tm (°C)	Product size (bp)	Orientation
GES421	TGCTCCACTAGAAGAAGCCT	$(AT)_n$	55	478	Forward
	CACTTGAAGACCATGTCGAG				Reverse
GES 423	CTCATGCTCTCCGACATCT	$(TA)_n$	54	226	Forward
	CACTTACACGTACGGCACAT				Reverse
GES 425	GACTGTACCGACTGCAAGTG	$(AATAT)_n$	54	367	Forward
	AGAGACACACAGATGCATGG				Reverse
GES 426	GCAGAGACACCCTTTTGAAG	$(ATATT)_n$	53	369	Forward
	GACTGTACCGACTGCAAGTG				Reverse
GES 438	ACCTCCTCCTCCTCTTCAAC	$(GCCGAC)_n$	52	493	Forward
	GAACAGTCGAACATACAGCG				Reverse
GES 440	CCTCCTTCCAAACACAACAC	$(CAGC)_n$	55	243	Forward
	CAGTGATGTTAGCGTCGTCT				Reverse
GES 444	GGTCGAGACTGAAGACAAGG	$(TCTT)_n$	52	456	Forward
	AGGCACGACCTCAGATTAAC				Reverse
GES 449	TGAGCTGAGTCGAGTTGTGT	$(AAT)_n$	53	187	Forward
	ATCTGCCTCTCTTGGTCTTG				Reverse
GES 452	CTGGTACTGCAAGTACGTCG	$(GGATCC)_n$	51	287	Forward
	GTTCAATCTCCTGGAAGCAG				Reverse
GES 453	AGCCGAAATCTGTCCTGTAG	$(GGA)_n$	53	467	Forward
	TTGACACTGTATCACTGGGG				Reverse
GES 454	AGAGCTTTCTGCACTTCCAC	$(AAT)_n$	52	329	Forward
	GAAGTGTGCCCAAATAGCAC				Reverse
GES 456	CCTGAGAATAGCCAAGAGGA	$(TACCA)_n$	58	206	Forward
	CACATTCAAGTGCTATCCCC				Reverse
GES 457	ACACCTTGAAGACCATCCTC	$(TATC)_n$	50	243	Forward
	AGATGGAAGTTGGTAGGCAG				Reverse
GES 459	ACCCAATAGCACAACCTCAG	$(TGG)_n$	51	283	Forward
	CCTCAATGCTGCCACTAAC				Reverse
GES 464	CGAACACTACTGGATGAGCA	$(GGT)_n$	54	252	Forward
	CTCCGTACTACCATCAACCC				Reverse
GES 466	CTGTCTTCATCCACTTTCCC	$(CT)_n$	55	165	Forward
	TAGGCACAGACACGGACATA				Reverse
GES 472	TCACGTGACACAAGAGAAGC	$(AG)_n$	55	474	Forward
	CACTGTTCGACGCACTAATC				Reverse
GES 475	ATGTGCAGGCCATTGTTG	$(TA)_n$	54	357	Forward
	CAGGAGGTGCAAGAGAGAAT				Reverse
GES 480	GTCGTGGGTGCGAGATTA	$(TA)_n$	52	231	Forward
	AATATGGGATCCACTCTCCC				Reverse
GES 481	CAAGTGCTTCAGCTCCTACA	$(TATAT)_n$	55	410	Forward
	GCTAGCAGAAGGAAAAGTGC				Reverse
GES 486	ACTCACCGAGTCGGAAATAG	$(GATTG)_n$	53	308	Forward
	GTAGTGAGGCTTTGGCAGAT				Reverse
GES 495	GTGAGGGAGAAGGAGAAAGA	$(AGAAGG)_n$	51	135	Forward
	ATCGAGGTAGGTTTGGAGGT				Reverse
GES 497	AGCCCTTCCATGAGTTCTCT	$(CAG)_n$	53	237	Forward
	TCTGAACAGCTGGGTACTGA				Reverse
GES 511	GCAGGATAGGCTTCCTCTAT	$(TCT)_n$	53	305	Forward
	CGATAAGATGGAGAAGGAGG				Reverse
GES 512	GGGAGAGTAAACGGAAGTGA	$(GGC)_n$	51	268	Forward
	ATCCCTGTACTCCACGATGT				Reverse

in successful in silico amplification (data not shown). Out of 349 primer pairs that were designed from EST database of *Z. officinale*, PCR was carried for 25 primer pairs, selected in an arbitrary and random fashion for experiments. PCR products were run on agarose to check for gross amplification results, mispriming and null alleles. Null alleles were confirmed by using at least 5 times more template in PCR reactions. Out of 25, 16 primers sets were optimized for the EST-SSR study. PCR products were run on PAGE to test the change in amplicon size. Representative image showing amplification across 25 accessions using three EST SSR markers (GES440, GES452 and GES454) is shown in Fig. 1. The optimized EST-SSR markers were checked for cross species transferability among seven different species of Zingiberaceae: *Z. zerumbet*, *H. spicatum*, *C. longa*, *C. amada*, *C. aeruginosa*, *C. aromatica* and *C. angustifolia*. GES454, GES466, GES480, GES486 markers were transferable to all the accessions of *Zingiber, Curcuma* and *H. spicatum* while GES452, GES456 marker were transferable to all species under study except one (Supplementary Fig. 2a). Percentage transferability of EST-SSR markers was found to be 100% for GES454, GES466, GES480, GES486 (Supplementary Fig. 2b).

Discussion

Zingiber officinale is important medicinal spice, with wide range of pharmaceutical properties (Marx et al. 2013; Haniadka et al. 2013). Genetic variability observed in vegetatively propagated crops, such as *Z. officinale*, may be due to ancestral differences and/or spontaneous mutations (Elias et al. 2000; Jankowicz-Cieslak et al. 2012). Microsatellite expansion/contraction and retrotransposon mobility are amongst the key drivers of spontaneous mutations (Ellegren 2004). Here, we have made a systematic attempt to study the genic (protein coding) sequences of this plant for development of genetic markers. SSRs may be found in non-coding as well as protein coding (genic) DNA (Tóth et al. 2000; Zhao et al. 2014). However, variation in lengths of SSRs present in genic sequences may have profound effects on morphology, chemoprofile, fiber content, etc. Earlier we have reported differences in such parameters amongst the collected accessions of *Z. officinale* (Khan et al. 2016; Pandotra et al. 2013a, b). EST-SSR markers (genic microsatellites) have been widely used for gene mapping and diversity analysis in many plant species (Kantety et al. 2002; Chen et al. 2006). Earlier, we had developed EST-SSR markers for *Ocimum* genus and correlated with the essential oil composition (Mahajan et al. 2015).

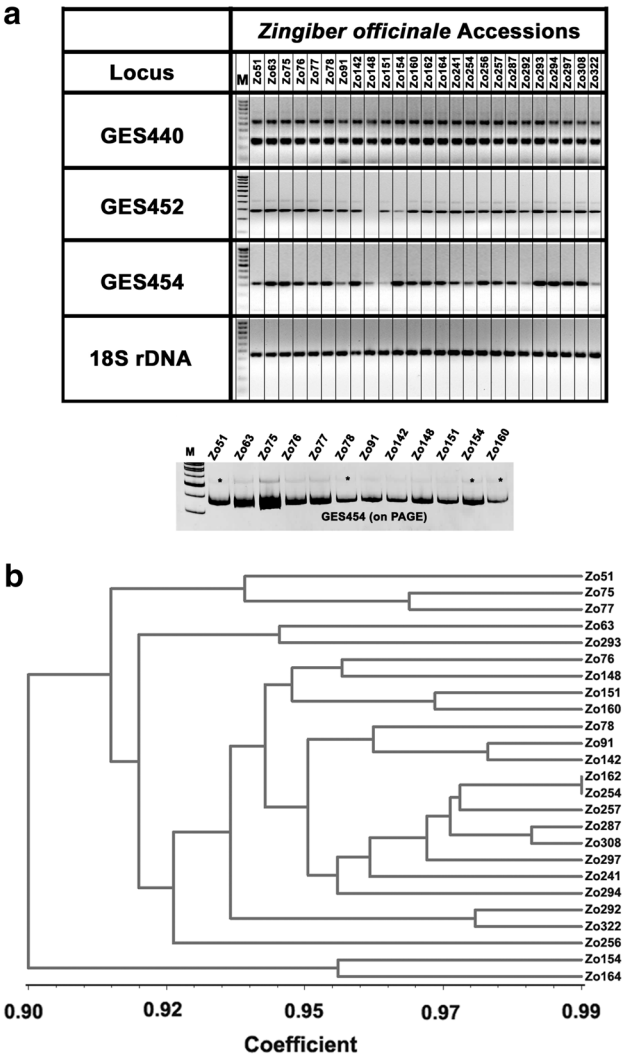

Fig. 1 Development of EST-SSR markers in different accessions of *Z. officinale* collected from various regions of India. **a** Representative gel image of three EST-SSR markers (out of 25) used in the study. 18S rDNA was used as control. Optimized PCRs were run on PAGE to test for changes in amplicon size. Amplicon size changes up to ± 15 base pairs were taken into consideration, **b** dendrogram generated using UPGMA analysis depicts phylogenetic relationships amongst different accessions

Abundance and distribution of microsatellites and EST-SSR primer development

In case of ginger, we found that 2.7% unigenes contained non-redundant microsatellites. In general, EST-SSR frequency was found to range between 2.65 and 16.82% for dicotyledonous species (Kumpatla and Mukhopadhyay 2005). While EST SSR frequency in monocots is generally low (1.5–4.7%) in comparison to dicots (Kumpatla and Mukhopadhyay 2005). Among dicots, computational analysis of EST data shows that there are significant differences in types and abundance of SSRs in various plants

(Kantety et al. 2002; Kumpatla and Mukhopadhyay 2005). The present study recorded a relatively lower abundance of genic SSRs as compared to other dicot plant species such as grapes, tea, coffee and turmeric (Scott et al. 2000; Poncet et al. 2006; Joshi et al. 2010; Huang et al. 2011; Backiyarani et al. 2013). The frequency of different SSR repeats in *Z. officinale* revealed that tri-nucleotide repeats were the most plentiful SSRs followed by di-, hexa-, tetra- and penta-nucleotide repeats. In general, genic SSRs with tri-repeats remained most common among the monocot and dicots (Kumpatla and Mukhopadhyay 2005).

Transferability of EST SSR marker within Zingiberaceae

16 optimized EST-SSR markers were found to be cross transferable among seven species (*Z zerumbet*, *H. spicatum*, *C. longa*, *C. amada*, *C. aeruginosa*, *C. aromatica* and *C. angustifolia*) of Zingiberaceae (Supplementary Fig. 2). Higher levels of transferability of these genic SSRs imitate the conserved nature of coding sequences in the microsatellite flanking region. This result suggested that these transferable genic microsatellite markers could be used for detection of markers associated with specific traits in other Zingiberaceae species and related genera. Similar results were seen among the other species of grapes, and pines (Decroocq et al. 2003; Chagn et al. 2004). In another study, 100% transferability of EST SSR (*C. longa*) marker was observed but among other species of *Curcuma* (Siju et al. 2010).

Conclusion

Ginger is a vegetatively propagated plant, however it is found in multiple varieties which display considerable diversity in morphological characters and chemoprofiles. Present study has isolated genic microsatellite markers from this commercially important plant. Since these markers originate from coding gene sequences, they can be utilized not only for studying the genetic diversity but also used for identification of candidate genes for particular traits. Further, many markers were found to exhibit a high degree of cross-genus and cross-species transferability which implies that they will be a precious resource for the comparative mapping by developing conserved ortholog set (COS) markers in evolutionary studies of different members of Zingiberaceae.

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Author contributions SuG and APG collected and maintained the accessions of *Z. officinale*. PA performed EST-SSR work. PA and AS carried out cross transferability studies. PA wrote the manuscript. VM and ShG helped in manuscript preparation. SGG designed the study, carried out bioinformatics analysis for discovery of EST-SSR markers and edited the manuscript and Figures. YSB provided critical inputs for the study as well as during preparation of the manuscript. Authors are thankful to Dr. Gandhi Ram and Mr. Pankaj Pandotra for maintaining the accessions in the field.

Compliance with ethical standards

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

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