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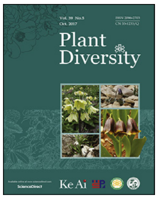

Identification of massive molecular markers in *Echinochloa phyllopogon* using a restriction-site associated DNA approach

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ABSTRACT

Echinochloa phyllopogon proliferation seriously threatens rice production worldwide. We combined a restriction-site associated DNA (RAD) approach with Illumina DNA sequencing for rapid and mass discovery of simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers for *E. phyllopogon*. RAD tags were generated from the genomic DNA of two *E. phyllopogon* plants, and sequenced to produce 5197.7 Mb and 5242.9 Mb high quality sequences, respectively. The GC content of *E. phyllopogon* was 45.8%, which is high for monocots. In total, 4710 putative SSRs were identified in 4132 contigs, which permitted the design of PCR primers for *E. phyllopogon*. Most repeat motifs among the SSRs identified were dinucleotide (>82%), and most of these SSRs were four motif-repeats (>75%). The most frequent motif was AT, accounting for 36.3%–37.2%, followed by AG and AC. In total, 78 putative polymorphic SSR loci were found. A total of 49,179 SNPs were discovered between the two samples of *E. phyllopogon*, 67.1% of which were transversions and 32.9% were transitions. We used eight SSRs to study the genetic diversity of four *E. phyllopogon* populations collected from rice fields in China and all eight loci tested were polymorphic.

1. Introduction

Echinochloa phyllopogon (= *Echinochloa oryzicola*) proliferation seriously threatens rice production worldwide. As a C_4-photosynthetic weed, *E. phyllopogon* is highly adapted to rice (C_3-photosynthesis type) planting environments, where it causes significant rice yield loss (Holm et al., 1979; Rao et al., 2007; Yamasue, 2001). Furthermore, *E. phyllopogon* has evolved resistance to various herbicides in different areas (Heap, 2015). Understanding the genetic diversity of agricultural pests, such as *E. phyllopogon*, is important for both evolutionary and population biology, and critical for agricultural management (Sun et al., 2015).

Microsatellite markers (simple sequence repeats, SSR) and single-nucleotide polymorphisms (SNP) are useful tools for studying genetic diversity and evolution (Zhang et al., 2011), and for developing high density genetic maps (Zhang et al., 2012). SSRs are short tandem repetitive sequences, which are co-dominant, abundant, multi-allelic, uniformly distributed, and can be detected by simple reproducible assays (Wang et al., 2015). SNPs are usually bi-allelic and characterized by low mutation rates; and thus, SNPS are stable from generation to generation across the genome (Kruglyak, 1997). This stability coupled with the abundance of SNPs makes them very useful both for linkage and genetic diversity studies (Talukder et al., 2014). To date, there are only eight SSR markers available for *E. phyllopogon* (Osuna et al., 2011; Lee et al., 2015), and an even more limited number of SNPs.

One promising approach to reduced-representation genomics is restriction site-associated DNA (RAD) sequencing, which sequences short DNA fragments flanking restriction enzyme cut sites, allowing orthologous sequences to be targeted across multiple samples to identify and score thousands of genetic markers (Miller et al., 2007). Therefore, a RAD sequencing approach can be successfully used to identify genome-wide SSRs (Gupta et al., 2015; Orjuela et al., 2010) and SNPs (Baird et al., 2008; Talukder et al., 2014; Vandepitte et al., 2013) in different species. In this study, we describe the generation of genomic RAD tags from *E. phyllopogon* plants. The RAD tags were sequenced using the Illumina platform and then annotated/categorized. These data allowed the discovery of a large number of SSR and SNP markers.

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2. Material and methods

2.1. DNA isolation

Seeds from *E. phyllopogon* individuals were collected and cultivated to fruiting stage in a greenhouse at Nanjing Agricultural University. Two *E. phyllopogon* plants with typical characteristics were used for SSR identification. Total genomic DNA was extracted from young leaves using DNeasy Plant Mini Kits (Qiagen, USA) according to the manufacturer's protocol.

2.2. RAD library preparation, sequencing and assembly

The RAD library was constructed at Hengchuang Inc. (China), according to the protocol described by Baird et al. (2008). Briefly, genomic DNA (300 ng) was digested for 60 min at 37 °C in a 50 μL reaction containing 20 U each of SgrAI and PstI (New England Biolabs, Beverly MA, USA). Reactions were stopped by incubating at 65 °C for 20 min. The P1 adapter (a modified Illumina adapter, see Baird et al., 2008) was ligated to the products of the restriction reaction, and the "barcoding" of the various samples was achieved with a set of index nucleotides in the P1 adapter sequence. A 2.5 μL aliquot of 100 nM P1 adapter was added to each sample, along with 1 μL 10 mM ATP (Promega), 1 μL 10× NEBBuffer4, 1 μL (equivalent to 1000 U) T4 DNA ligase (Enzymatics, Inc) and 5 μL water, then incubated at room temperature for 20 min, before heat-inactivated (20 min at 65 °C). The reactions were then pooled and the products randomly sheared to a mean size of 500 bp using a Bioruptor (Diagenode). The material was electrophoresed through a 1.5% agarose gel, and the DNA in the range 300–800 bp isolated using a MinElute Gel Extraction Kit (Qiagen). dsDNA ends were treated with end blunting enzymes (Enzymatics, Inc) to remove overhangs, and the samples purified using a MinElute column (Qiagen). 3′-adenine overhangs were then added by the addition of 15 U Klenow exo-(Enzymatics), followed by incubation at 37 °C for 10 min. Following repurification, 1 μL 10 μM P2 adapter (a modified Illumina adapter, see Baird et al., 2008) was ligated, as described above for P1. The samples were then purified as above, and eluted in a volume of 50 μL. Following quantification (Qubit fluorimeter), 20 ng were taken as the template for a 100 μL PCR containing 20 μL Phusion Master Mix (NEB), 5 μL 10 μM P1 adapter primer (Illumina), 5 μL 10 μM P2 adapter primer (Illumina) and water. The Phusion PCR settings followed product guidelines (NEB) over 18 cycles. The amplicons were gel purified, the size range 300–700 bp was excised from the gel, with the DNA content adjusted to 3 ng/μL. The constructed RAD libraries were sequenced on the NGS Illumina platform PE150 at Hengchuang Inc. (China), following the manufacturer's protocol.

To obtain clean, high quality reads, we discarded low quality raw sequences with adapter contamination or N content >10%. We used Stacks software for RAD tag clustering for each sample (ustacks). The Reads group (Read1 and Read2) at a same enzyme loci RAD were assembled by using the ABYSS software (Catchen et al., 2011).

2.3. SSR identification

SSR motifs were identified by SSRIT software (http://www.gramene.org/db/markers/ssrtool) using default parameters (Temnykh et al., 2001). Both perfect and imperfect di-, tri-, tetra-, penta- and hexa-nucleotide motifs were targeted. Di-nucleotide motifs with at least 4 repeats and other motifs with at least 3 repeats were selected. We used Primer3 software (http://sourceforge.net/projects/primer3/) to design primers in the flank regions of SSR sequences (SSR sequences were not contained in the primers), the replicated primers were removed and unique primers and relative loci were retained.

To analyze the frequency of SSR motifs, SSRs were first standardized (Wang et al., 2015). For example, SSRs with motifs of AT and TA were analyzed as AT, and motifs of ATG, TGA, GAT, TAC, ACT and CAT are analyzed as ATG.

2.4. Sequence annotation

For the contigs with SSR loci, sequence annotation and Gene Ontology analyses were further conducted. BlastN searches were performed against the Gene Ontology database (http://www.geneontology.org/), using 90% identity and a minimum alignment of 100 bp as cut-off parameters. A threshold E-value of e^{-15} was adopted for each annotation. The annotated sequences were assigned a function based on the Gene Ontology database (http://www.geneontology.org/); GO terms were determined with respect to cellular component, biological process and molecular function (Barchi et al., 2011).

2.5. SNP discovery

SNPs were detected by Stacks pipeline, ustacks software was used to build loci, cstacks software was used to create a catalog of loci, and sstacks software was used to match samples back against the catalog (Catchen et al., 2011). Default settings were used in Stacks.

2.6. Microsatellites amplification

To test the validity of the SSRs identified by RAD sequencing here, we used eight SSRs (Table 1) to study the genetic diversity of four *E. phyllopogon* populations collected from rice fields in China. We extracted total genomic DNA from four-leaf stage plants using a DNeasy Plant Mini Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. Isolated DNA concentration and relative purity were checked using Nanodrop ND-1000 (Thermo Scientific), and adjusted to 30–40 ng/μL. Forward primers of SSRs were labeled with fluorescent tags (Table 1). PCR amplification was conducted in a total volume of 10 μL. The PCR mixture contained 0.2 μL of DNA, 0.4 μL of each primer (10 μM), 5 μL of 2× PCR Taq Mix (Dongsheng Biotech, China), and ddH_2O to a final volume of 10 μL. The amplifications were performed using the following cycling program: initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, relative annealing temperatures for 30 s, and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. The amplification products were combined with formamide and a size standard GeneScan-500 LIZ (Applied Biosystems, Foster City, California, USA), and separated on a 3730 ABI automated sequencer (Applied Biosystems). Sample profiles were scored manually using GeneMarker v. 2.4 (Applied Biosystems).

2.7. Data analysis

The multilocus data were transformed to a binary matrix of presence/absence of each allele for each individual, which was used for further analysis with GenAlex 6.5 (Peakall and Smouse, 2012; Teixeira et al., 2014). Total number of alleles and the number of private alleles for each population were determined using GenAlex 6.5, and genetic diversity was determined using GenoDive2.0b23 (Teixeira et al., 2014), according to the tutorials (www.patrickmeirmans.com/software/GenoDive.html). GenoDive allows analyzing polyploids with unknown dosage of alleles (Meirmans and Van Tienderen, 2004).

3. Results

3.1. Sequencing and contig assembly

The sequencing procedure generated 71.45 million reads for the two *E. phyllopogon* samples (Table 2). After editing/trimming, 10,440.6 Mb of high quality sequences were available, which were assembled into 37,662 contigs. Average contig lengths for the two

Table 1
Characteristics of the eight primers tested for *E. phyllopogon* genotyping: locus name, forward (F) and reverse (R) primer sequences, motif, annealing temperature (Tm), fluorescent dye used (Fl. dye), allele size range (ASR), number of alleles amplified per sample, and number of alleles amplified among the plants of four populations sampled (Allele. total).

Marker	Sequence	Motif	ASR (bp)	Fl. dye	Tm (°C)	No. of alleles per sample			Allele. total
						Min.	Mean	Max.	
EG _1	F: GCTCCTGAACTGTGTACATTCTTGC R: TCGATTCACCCTTCAGCTTCTC	TG	123–153	TAM	49	0	0.7	2	5
EG_2	F: CATCGGATTCAGATTGAAAGGG R: GGTCGTAGGTCTATAGTCCGTAGAGTCA	TA	131–159	FAM	51.5	1	1.7	3	7
EG_301	F: GCGTCGTCAAGTCGTTCTTCTA R: TGTATTCAGCTGTCGTGCATGT	AT	147–173	TAM	57	0	2.4	3	8
EG_302	F: ATTCGAACACCCATCAACCAAC R: GAAACAGAAGGGAGGTGTGCTG	ATTT	133–293	FAM	57	1	2.8	5	12
EG_305	F: AGCCGTTCCTCTAGTCGGATTTCT R: TATTCAGCTGCCGTGCATGTAGTA	AT	100–162	ROX	57	3	4.1	6	14
EG_306	F: TAAAACAAAACGACCGGCGTAA R: TCAATCATTTCAGCCTTCGGAT	CT	146–167	HEX	57	1	1.25	2	7
EG_307	F: AACATTGTCATCACAAATATCATCATCA R: AATCAAGGAAGCCCCTTCACTC	ATC	108–134	TAM	57	2	3.5	5	8
EG_320	F: CAACTCATAAGACAATTCAAAGGGTTT R: GCATCATTTAAGCATCAAAATGACA	TA	136–153	FAM	57	2	3.0	4	5

FAM: 6-carboxyfluorescein, HEX: hexachloro-fluoresceine, ROX: carboxy-X-rhodamine, and TAM: 5-TAMRA (5-Carboxytetramethylrhodamine).

Table 2
Summary statistics of the RAD tags sequencing via Illumina for *E. phyllopogon.*

Feature	Total
Illumina reads (million)	71.45
Total base (million)	10,440.6
GC%	45.8%
Q20 (%)	94.0%
No. of contigs	37,662
Total length (bp)	12,789,629
Contig length range (bp)	200–588
Average contig length (bp)	339.5

samples were 334 and 346 bp. The GC content of *E. phyllopogon* was 45.8%.

3.2. Identification of SSRs

A screen of the dataset resulted in the identification of 4710 putative SSRs that permitted PCR primer design for *E. phyllopogon.* Tables S1 and S2 show motifs, number of repeats, sequence of 5′- and 3′-flanking, sequences and annealing temperatures of primers, sequence of PCR products and the potential relative genes for each SSR loci.

The majority of motifs among the RAD SSRs were dinucleotide (>82%) for both samples, and 14%–15% of the SSR motifs were trinucleotide (Table 3). The majority of SSRs were four motif-repeats. The abundance of SSRs decreased significantly ($P < 0.01$) with increasing motif-repeats for *E. phyllopogon* (Fig. 1).

Nearly all (97.3%) *E. phyllopogon* SSR motifs consisted of dinucleotide plus trinucleotide repeats. Thus, we further analyzed dinucleotide and trinucleotide motifs. Before the analysis, SSRs were standardized. For example, SSRs with motifs of AT and TA were analyzed as AT, and motifs of ATG, TGA, GAT, TAC, ACT and CAT were analyzed as ATG. AT was the most frequent, accounting for 36.3%–37.5%, followed by AG and AC (Table 4). Among the four kinds of dinucleotide motifs, CG dinucleotide repeats represented the lowest percentage of all SSRs (<6%). CCG was the most frequent

Table 3
Length distributions of SSR motifs identified for the two samples of *E. phyllopogon* tested.

Motif length	13	04
Dinucleotide	1908 (83.0%)	1998 (82.4%)
Trinucleotide	329 (14.3%)	360 (14.9%)
Tetranucleotide	40 (1.7%)	50 (2.1%)
Pentanucleotide	15 (0.7%)	13 (0.5%)
Hexanucleotide	6 (0.3%)	3 (0.1%)
Total	2298	2424

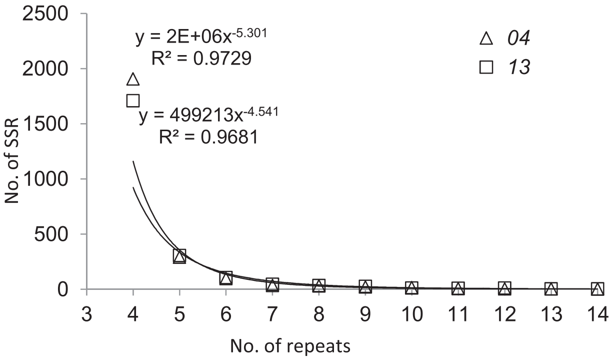

Fig. 1. SSR motifs with different repeat numbers for the two samples of *E. phyllopogon*.

Table 4
SSR motifs with a frequency > 0.5% and the ranges of PCR product length (mean length) of the relative motifs for the two samples tested for *E. phyllopogon*.

Motif	Count (% of total SSRs)		PCR product length (average length, bp)	
	13	04	13	04
AT	854 (37.2)	880 (36.3)	80–234 (133.5)	80–239 (131.0)
AG	562 (24.5)	617 (25.5)	80–208 (126.5)	80–208 (127.1)
AC	372 (16.2)	395 (16.3)	80–225 (130.3)	80–234 (126.6)
CG	120 (5.2)	106 (4.4)	80–204 (131.6)	80–237 (124.1)
CCG	99 (4.3)	103 (4.2)	80–172 (132.0)	80–160 (126.9)
AAG	45 (2.0)	43 (1.9)	85–159 (128.8)	81–153 (121.0)
AAT	28 (1.2)	30 (1.2)	80–160 (130.3)	80–160 (127.5)
ACC	27 (1.2)	14 (0.6)	80–157 (122.9)	80–220 (134.2)
AAC	25 (1.1)	47 (1.9)	85–155 (120.3)	122–155 (136.9)
AGG	24 (1.0)	25 (1.0)	81–188 (128.3)	80–159 (132.3)
AGC	23 (1.0)	29 (1.2)	80–157 (122.6)	89–159 (134.2)
ACG	22 (1.0)	15 (0.6)	86–160 (136.1)	83–159 (121.4)
AGT	22 (1.0)	32 (1.3)	80–160 (133.9)	81–160 (130.8)
ATG	14 (0.6)	22 (0.9)	91–160 (134.5)	87–159 (127.9)

Note: motifs with dinucleotide plus trinucleotide contributed to 97.3% of the total SSRs for both samples. Thus motifs with length >3 were not shown in this table.

kind of trinucleotide motif for both samples (Table 4), accounting for about 4% of the total SSRs for *E. phyllopogon*. The predicted length of PCR products amplified by SSR primers designed in this study are shown in Table 4.

In total, 78 putative polymorphic SSR loci were found by RAD sequencing (Table 5). These 78 SSRs include 65 SSRs with dinucleotide motifs, 10 SSRs with trinucleotide motifs, two with tetranucleotide motifs and one with a pentanucleotide motif. The AT dinucleotide repeat, which accounts for 49.4% of all motifs, was the most frequent kind.

To test the validity of the SSRs identified by RAD sequencing here, we used eight SSRs to study the genetic diversity of four *E. phyllopogon* populations collected from rice fields in China. We amplified 66 alleles from the eight microsatellite loci. The primer sequence EG_305 amplified 14 alleles, EG_302 amplified 12 alleles, and EG_320 and EG_1 amplified five alleles (Table 1). EG_305 amplified three to six alleles per sample, while EG_307 and EG_320 amplified two to five and two to four alleles per sample, respectively. Moreover, EG_305 amplified the most alleles on average (4.1). On average, 3.1–4.8 alleles were amplified from one locus per population (Table 6). All four populations showed private alleles, among which the populations EP13 and EP50 showed 13 and eight private alleles, respectively. The heterozygosity values of these populations ranged from 0.064 to 0.091, and their Shannon's information indices ranged from 0.087 to 0.381. Analysis of molecular variance (AMOVA) indicated that 39% of diversity occurs among populations, while 61% of diversity occurs within populations (Table 7).

Table 6
Diversity of four populations of *E. phyllopogon* using eight nuclear microsatellite loci.

Population	EP13	EP14	EP53	EP50	Total
No. of alleles	39	34	25	37	66
No. of alleles per locus	4.875	4.25	3.125	4.625	8.25
No. of private alleles	13	1	2	8	/
Heterozygosity	0.086	0.082	0.064	0.091	0.081
Shannon's information index	0.381	0.21	0.087	0.222	0.225

Table 7
Analysis of molecular variance (AMOVA) showing the partitioning of genetic variation within and between regions of *E. phyllopogon*.

Source	df	SS	MS	Est. var.	%	*P*
Among Pops	3	135.563	45.188	3.328	39	<0.01
Within Pops	44	231.250	5.256	5.256	61	<0.01

df = degree of freedom, SS = sum of squares, MS mean squares, Est. var. = estimate of variance, % = percentage of total variation, *P*-value is based on 9999 permutations.

3.3. Annotation of contigs with SSR loci

Using two *E. phyllopogon* individuals, we identified 4710 SSR loci in 4132 contigs, and annotated 643 contigs (Table S2). Among these 643 contigs, 8631 annotations, potentially referring to 2155 unigenes, were searched (a given gene product can be associated with more than one annotation). Annotated *E. phyllopogon* sequences with SSR loci were functionally assigned and arranged into Gene

Table 5
The 78 putative polymorphic SSR loci found by RAD sequencing.

Marker	Motif	Primer_F	Primer_R	Marker	Motif	Primer_F	Primer_R
EG_1	TG	gctcctgaactgtgtacattcttgc	tcgattcacccttcagcttctc	EG_40	GAA	aacagacaaaatacaaaagaaagcaca	gtttttcagcatcatcctgtgg
EG_2	TA	catcggattcagattgaaaggg	ggtcgtaggtctatagtccgtagagtca	EG_41	AT	tcactacgaaattatcgtttatggacaa	gcccgctccgtgtttagattat
EG_3	TA	ttgctttctgcaatgccaatta	gtccatgtggagtcagggagtt	EG_42	TA	atgggcgacaagcaagtatgat	gacggacgaaggtttgaagattt
EG_4	TA	ccgttgatgattaactcgttgattt	tgatggtagctacaagcgttgg	EG_43	GA	catcctctggctgcttctctct	gaatgtgagaatctccgctgct
EG_5	TA	ttcactatgctgaaccagcagc	ctgagtccggtatcgctcctta	EG_44	GA	acacctttctccatcctctggc	ccgctgctgctactactcttgg
EG_6	AT	ccatggtcaagtcactttgtctg	tctggatctcccaaattcatgtc	EG_45	TG	ttgtacaagcttctgagataacctga	atttcagaaactgtttgaattaggattt
EG_7	AAG	catttcttaccgtcccatctgc	cctttttcagggagaagccact	EG_46	TA	aaatggatatggcaaacgcatc	ccaagtccatcatgccaagttt
EG_8	AT	ttttgtaggcctaacctgttgtgg	tttttgctatgcatgtgtctactcg	EG_47	AT	tttgggattgtttatgaggtttga	cacacggcaaaatgaccaata
EG_9	TG	tataacatccctttcgttgccatc	tgcaatgaaattcagatattcggac	EG_48	AAG	tgctatgcatgaggagatgcag	ccttataccttggaggctcgct
EG_10	AAG	taaattgcccaaacaagaaagagg	atcggagtcccactcaacaaagta	EG_49	TA	aattctagtttgcgacgggttatt	ttgagtgaatgggatcgaaaaa
EG_11	TGCA	agccggtgcaggaagacag	aagaagggaaaaggtagtcgttgg	EG_50	TC	aaggacaaagtcgcagcgttt	atgggatttggttttggcttct
EG_12	CTCTC	tttgaagccttttcggtcttga	aacaagcagtggaagacgaagg	EG_51	GC	gccgggtgattaacggattagt	agactagctagccagcgggttg
EG_13	AT	ggcccaatataatatccatgcc	ctatcaagggcagctatttggg	EG_52	AT	aattcaacacaaccaaaggtaaaaa	tcaatgccatattgattctccc
EG_14	AT	ggtggtgtgtcctgatgtgtgt	tgtttccttttgtttttgttttgtttc	EG_53	TG	tcaaatggcaaagtatggaactca	tcattttctcaagaagcagtggtc
EG_15	TA	catgaactgttctgactccaacaac	aagcattgcagctctgtcttgt	EG_54	AT	aatattaacgtacccttgacaaatgaa	tttttgttggtacgtaagataaacaatc
EG_16	TA	tcagttgagctccatcatttgttt	tcactggctgttctttaccgtact	EG_55	AG	ccaagaaaccaactaagagccaaa	atttgtgcatgatgtgctttgc
EG_17	CGG	gatagcgactcgagcgtggt	tctcgagcatggggagagac	EG_56	AG	agcaagaaaccaactaagagccaa	aattcgtgcatgatgtgctttg
EG_18	ATG	agccatattgccttgtgaccaa	ttttccttgcgcaatttttcat	EG_57	TC	tgaaaagccagtggacagtcag	gagttcctcctgatggcaagaa
EG_19	AC	ccttcagctgatgtaatcttggtaag	tccatctctcagcacctgaaaa	EG_58	TC	tctccctccaaactttactattcacc	gctcaaaagatttgtctcgtcg
EG_20	AT	gaaggtcgtgcactatggtgag	agcaagttgaagcaatccaagg	EG_59	AT	cgtcaagtcgttcctctagtcg	tgtattcagctgtcgtgcatgt
EG_21	AT	cgccgtcaagtcattcctcta	tcagctgccgtgcatgtagta	EG_60	AT	tgccagacagtccaacaagcta	ggccgactctatattcatattagctgac
EG_22	CT	cacatgatacatccgttgcgtc	atcggaggagggggaagag	EG_61	TA	aatgcagtcaggcccttgttta	gcacgggcacatttcctagt
EG_23	AG	aaaacgccgcaaaaacaaaag	cccctctaggattctcgctgtt	EG_62	TC	cttcttcctcgcctccaattc	aaacaagttattacccggcgct
EG_24	TA	acgagcacccattatgttttgg	cgagatcccagagcaaagctac	EG_63	TA	cgattgcttaagggaataaatgg	caacattttactggtaatcctttcttg
EG_25	CT	atcaaacccccctcgaattcct	gagggagagaaagctgacaggc	EG_64	GA	tcttggctgaaaaatctatttggG	acctctcccacttgaagaagca
EG_26	TA	ttcaaaaattcgatctttgctgc	aacctttttccgtggcctacct	EG_65	AT	cccctgagcaaatttcaatcat	agggacagggaaggatcttgac
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EG_32	TA	gtgggctcataccttaatgccc	ggggagccatctctcttctcat	EG_71	GA	aggaagaaaagagaagtgaggcG	cgagcacctcctctaggaatca
EG_33	AT	gccgtcaagtcgttcctctagt	cagctgccgtgcatgtaatact	EG_72	TA	ctgcgggtgacatttgtacagt	gtctgaacacgttaccacaccg
EG_34	TCT	gatgatgatgcattcacttgagttg	tggatgatgtgagaggtgatgg	EG_301	AT	gcgtcgtcaagtcgttcttcta	tgtattcagctgtcgtgcatgt
EG_35	AT	tcctctagtcggatttcttaatttgc	tgtattcagctgtcgtgcatgt	EG_302	ATTT	attcgaacacccatcaaccaac	gaaacagaagggaggtgtgctg
EG_36	AG	catgaccatcaggcatcatctc	atgaagaagctactccgccgat	EG_305	AT	agccgttcctctagtcggatttct	tattcagctgccgtgcatgtagta
EG_37	TCT	tcagaaacaatatgttcctcatcatca	caaatgggtcacaagacgagaa	EG_306	CT	taaaacaaaacgaccggcgtaa	tcaatcatttcagccttcggat
EG_38	TG	ggagctggagaaactgaaggaag	cacttcgttgagggctcgatag	EG_307	ATC	aacattgtcatcacaaatatcatcatca	aatcaaggaagccccttcactc
EG_39	CA	gtggcatgtgaattgtttccct	caatcttacctcccaccttccc	EG_320	TA	caactcataagacaattcaaagggttt	gcatcatttaagcatcaaaatgaca

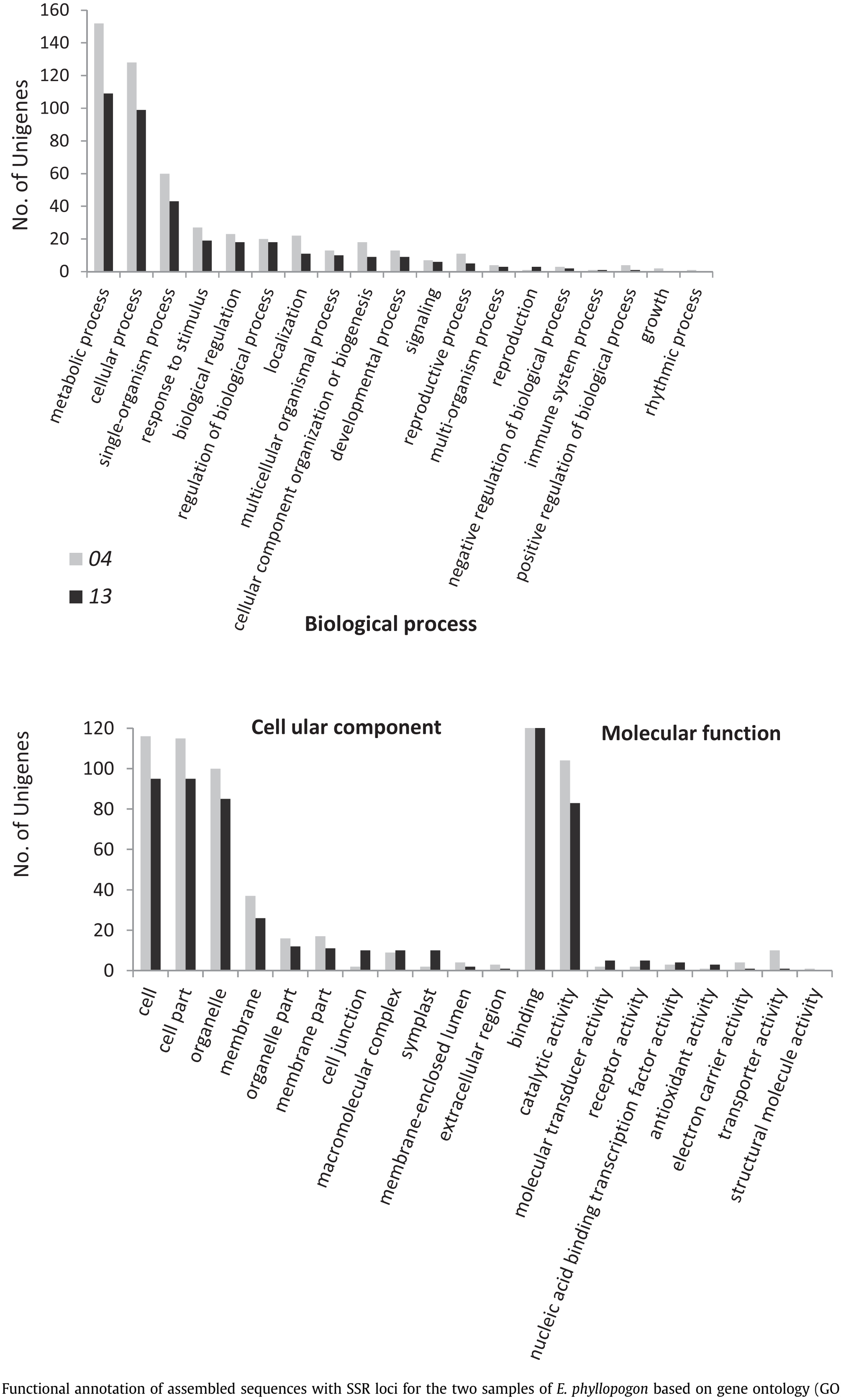

Fig. 2. Functional annotation of assembled sequences with SSR loci for the two samples of *E. phyllopogon* based on gene ontology (GO) terms.

Ontology (GO) slim categories (Fig. 2). GO analyses suggested that contigs with SSR loci were mostly related to metabolic processes (12.1% of the total 2155 unigenes) and cellular processes (10.5%) among biological processes; cell (9.8%), cell part (9.7%) and organelle (8.6%) among cellular components; and binding (12.6%) and catalytic activity (8.7%) among molecular functions.

3.4. SNP discovery

In total, 49,179 SNPs were discovered between the two samples of *E. phyllopogon*. Table S3 shows the kind, sequence and location of 49,179 SNPs discovered between two samples of *E. phyllopogon*. Among these SNPs, transversions (67.1% of total SNPs) were much more frequent than transitions (Fig. 3).

4. Discussion

4.1. High GC content of E. phyllopogon genome

Higher GC content in plant genomes possibly contributes to an increased ability to adapt to various arable lands that are mainly maintained and regulated by human disturbance. Šmarda et al. (2014) studied GC content in 239 different plant genomes, finding that the GC content of monocots varied between 33.6% and 48.9%, and increased GC content was documented in species able to grow in seasonally cold and/or dry climates, which possibly indicates GC-rich DNA may confer more stability during cell freezing and desiccation. The GC content of *E. phyllopogon* was higher than those of many monocots such as *Juncus inflexus* (33.7%), *Luzula badia* (33.6%), *Carex acutiformis* (35.6%), *Schoenoplectus lacustris* (35.8%), *Canna indica* (39.7%), *Oryza sativa* (43.6%) and *Triticum aestivum* (44.7%); and only lower than those of a few Poaceae species such as *Stipa calamagrostis* (47.5%) and *Zea mays* (47.4%) (Raats et al., 2013).

4.2. Characteristics on SSR motifs of E. phyllopogon

The majority of RAD SSR motifs were dinucleotide and with four motif-repeats. Gupta et al. (2015) identified SSR motifs in peanut (*Arachis hypogaea*) through RAD sequencing, and found that 67.6% of the motifs were dinucleotide, 14.6% were trinucleotide, 12.5% were tetranucleotide, 3.2% were pentanucleotide and 2.2% were hexanucleotide. Nevertheless, in eggplant (*Solanum melongena*), the percentages among total motifs with two to six nucleotides of dinucleotide, trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide were 20.4%, 37.9%, 12.8%, 18.1% and 10.9% (Barchi et al., 2011). Using RAD sequencing in eggplant, Barchi et al. (2011) found that AAC was the most frequent kind of motif, accounting for 19.0% of the total SSRs, followed by AT (9.6%). Wang et al. (2015) analyzed the genomes of nine plant species from the Poaceae family, and found that among the genome SSRs of *O. sativa* ssp. *indica*, *O. sativa* ssp. *japonica*, *Phyllostachys heterocycla*, *Sorghum bicolor* and *Z. mays*, AT was the most frequent motif, and also very frequent in other Poaceae plants.

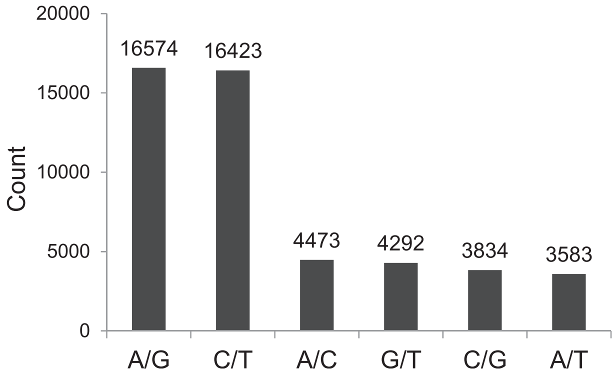

Fig. 3. Transitions and transversions occurring within a set of 49,179 *E. phyllopogon* SNPs.

To test the validity of the SSRs identified by RAD sequencing here, we used eight SSRs to study the genetic diversity of four *E. phyllopogon* populations collected from rice fields in China. All eight loci were polymorphic, particularly when compared with the five SSRs that have been used for *Echinochloa* since 2002 (Danquah et al., 2002; Nozawa et al., 2006; Lee et al., 2015).

4.3. Potential usage of the SSRs and SNPs identified

A great number of *Echinochloa* species are aggressive invaders and managing crop lands requires unique strategies for each (Holm et al., 1979; Tabacchi et al., 2006). Thus, correctly identifying Echinochloa spp. is of agronomical and economic importance. The genus *Echinochloa* contains about 35 species that are widespread in both tropical and temperate regions and in dry or water-flooded soils (Flora of China, 2015). The taxonomy of this genus is complex, and *Echinochloa* species show wide variability in morphological, biological and physiological features (Danquah et al., 2002; Tabacchi et al., 2006; Vidotto et al., 2007). Conventionally, the identification of *Echinochloa* species has been attempted taxonomically using morphological assessment of plants, which has frequently been found to be difficult and uncertain (Tabacchi et al., 2006). Moreover, there are different taxonomic key systems for *Echinochloa* species, which may lead to misidentification (Flora of China, 2015; Tabacchi et al., 2006). Molecular identification of the *Echinochloa* species is not yet reliable and requires further study (Danquah et al., 2002; Kaya et al., 2014; Tabacchi et al., 2006). In addition, molecular markers may be very useful in studying the origin and distribution of herbicide-resistant populations (Okada et al., 2013; Osuna et al., 2011). SNPs and SSRs are ideal molecular tools for gene location and molecular breeding (Danquah et al., 2002; Gupta et al., 2015; Vandepitte et al., 2013; Zhang et al., 2011).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.pld.2017.08.004.

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