A SNP-Based Molecular Barcode for Characterization of Common Wheat

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Abstract

Wheat is grown as a staple crop worldwide. It is important to develop an effective genotyping tool for this cereal grain both to identify germplasm diversity and to protect the rights of breeders. Single-nucleotide polymorphism (SNP) genotyping provides a means for developing a practical, rapid, inexpensive and high-throughput assay. Here, we investigated SNPs as robust markers of genetic variation for typing wheat cultivars. We identified SNPs from an array of 9000 across a collection of 429 well-known wheat cultivars grown in China, of which 43 SNP markers with high minor allele frequency and variations discriminated the selected wheat varieties and their wild ancestors. This SNP-based barcode will allow for the rapid and precise identification of wheat germplasm resources and newly released varieties and will further assist in the wheat breeding program.

Introduction

DNA fingerprinting is commonly used in crops to identify and characterize cultivars and to protect the rights of breeders [1]. Therefore, several marker systems have been developed for this purpose [2]. RFLPs (restriction fragment length polymorphisms) were the first molecular markers, though this marker system is no longer frequently employed due to the disadvantages of the complexity, low polymorphism rate, and high cost of this technique. SSRs (simple sequence repeats) are considered the second-generation molecular markers. Because they use a comparatively simple technique with a higher polymorphism rate and lower cost, SSR markers have been widely employed in the study of many crops, including wheat [3–5]. The third-generation molecular markers are single-nucleotide polymorphisms (SNPs). With the development of next-generation sequencing (NGS) technology and low-cost genome sequencing, a large number of SNPs are being identified and used to design arrays for major crops [6–11]. SNP arrays are utilized based on a standardized protocol, which makes the resulting data comparable between labs. In theory, SNP arrays comprise loci with unique positions along chromosomes or genomes, thereby largely avoiding the confusion associated with multiple sequence variants. This is especially important for common wheat, a hexaploid plant with three genomes (A, B and D). Recently, several SNP arrays produced by Illumina (9K and 90K) and Affymetrix (35K, 817K, 660K) have been used to evaluate population structure, genetic variation, selection and genome-wide association mapping for agronomic traits in wheat [6, 9, 12, 13]. However,
even using medium density SNP arrays to discriminate wheat germplasms or cultivars released every year is still not cost-effective. An economic and easily applied molecular barcode is required for determining cultivar characteristics. Here, we develop a minimal set of SNP markers that is robust enough to fingerprint a diverse collection of wheat genotypes. This SNP-based barcode is composed of 43 SNPs that can resolve 429 wheat accessions, which will facilitate its effective use in wheat germplasm identification and wheat breeding programs.

**Materials and Methods**

**Plant materials**

Two panels of materials were used in this study. Panel 1 was composed of 429 common wheat cultivars, including land races and modern varieties widely planted across the major wheat-growing areas of China from the 1930s to the 2010s. These cultivars confer important agronomic traits, such as resistance to diseases (stripe rust, sharp eyespot and Fusarium head blight), tolerance to stresses (drought, wet and salt), increased quality, larger grains, longer spikes, and dwarf height (S1 Table). In addition to these remarkable characteristics, some of these cultivars are founder parents from which many modern varieties were bred. The panel 1 samples were used to screen and develop the SNP barcode.

Panel 2 was composed of 193 bread wheat cultivars randomly selected from panel 1 and 96 pairs of wheat ancestor species including wild emmer wheat (*Triticum dicoccoides*) and goat grass (*Aegilops tauschii*) (S2 Table). Panel 2 was used to validate the resolution of the developed SNP barcode.

**DNA extraction and genotyping**

DNA was isolated from the leaves of two-week-old seedlings using a DNA extraction kit (CN. DP321, Tiangen Biotech Co., Ltd.). The DNA samples were genotyped using the Illumina wheat 9K Infinium Assay [6]. SNP clustering and genotype calling were performed using GenomeStudio v2011.1 software (Illumina). As described previously [6], the genotyping of polyploid wheat using the 9K SNP chip is complicated by the presence of homologous and paralogous gene copies. Therefore, we manually adjusted the clusters for each SNP using the GenomeStudio software. As suggested [6], when SNP clusters were too close together to allow the AB cluster to be correctly positioned between the AA and BB clusters, one of the HOM (homologous) clusters was defined with the AB cluster; actual HET (heterozygous) genotypes were not called. Then, recoding HOM genotypes was required (e.g., AB to AA). After adjustment of the data as previously described [6], the consistency of genotyping results between labs was largely ensured.

**Genetic analysis of SNP markers**

The SNP allele frequency and polymorphism information content (PIC) were estimated for each locus using PowerMarker v3.25 [14]. The PIC value is usually used to estimate the polymorphism for a marker locus among samples. The pairwise locus linkage disequilibrium (LD) was estimated by PowerMarker and Tassel 3.0 [15]. UPGMA trees based on Nei’s genetic distance (GS, Nei’s 1972) were constructed to confirm the resolution capacity of this barcoding system between materials using PowerMarker, and the UPGMA trees were visualized in Mega 5 [16].

**Generation of 2D barcode**

We used online software Caoliaoerweima (http://cli.im/) to generate the 2D barcode for each accession. The genotype based on the SNP barcode was entered, and the 2D barcode was automatically generated. Once the barcode was scanned, the genotype of each accession was shown.
Development of KASP primers
The SNP-based barcode was converted to Kompetitive Allele Specific PCR (KASP) primers, which are specific for SNP genotyping technology (LGC Genomics LLC, Beverly, MA, USA). For each KASP SNP, two allele-specific primers and one common primer were designed (S3 Table). Parameters for primer design were as follows: GC content was less than 60%, melting temperature (Tm) ranged from 55°C to 62°C, and PCR product size was no larger than 120 bp. There were only two choices of allele-specific primers, immediately up- or down-stream of the SNP site. Therefore, all the primers were manually selected, and GC content and Tm were calculated using DNASTar v7.0. Primers carrying standard FAM- or VIC-compatible tails (FAM tail: 5’-GAAGGTGACCAAGTTCATGCT-3’; VIC tail: 5’-GAAGGTCGGAGTCAACGGA TT-3’) with a targeted SNP in the 3’ end were synthesized by Invitrogen Trading (Shanghai). Primer mix was prepared as recommended by Kbioscience: 46 μl ddH2O, 30 μl common primer (100 μM), and 12 μl of each tailed primer (100 μM). The total reaction volume was 5 μl in a 384-well plate, composed of 2.43 μl of V3 2× Kaspar mix, 0.07 μl primer mix, and 2.5 μl template (10–20 ng of genomic DNA) as described previously [17]. Ten common wheat varieties were used to test the newly developed KASP primers. PCR was performed as follows: Hot start at 95°C for 15 min, followed by ten touchdown cycles (95°C for 20 s; Touchdown 61°C, -1°C per cycle, 25 s), then followed by 26 cycles of amplification (95°C 10 s; 55°C 60 s). Assays were performed in a QuantStudio 7 Flex Real-Time PCR system, and fluorescence was detected using QuantStudio™ Real-Time PCR software. As suggested by Trick et al. [17], if the signature genotyping groups had not formed after the initial amplification, additional amplification cycles (usually 5–10) were applied, and the samples were read again.

Results
SNP marker screening
Of the SNP loci with three typical clusters of AA, AB and BB genotypes, only those loci of the classical bi-allelic type were used for further analysis. According to suggestions on data conversion [6], if raw genotyping data were absent for the AA or BB genotype, AB genotypes were recoded to AA or BB, respectively. After the initial filtering, 3489 SNPs were retained for the wheat accessions in the panel 1.

Screening for the SNP barcode was conducted on panel 1 cultivars (Fig 1). First, monomorphic SNPs or those with more than 10% missing data were deleted using PowerMarker software. Then, the PIC value was calculated for each SNP, and the 50 SNP loci with the highest PIC values (hereafter called original SNP list) were retained. To evaluate the resolution, genotypes based on the 50 most variable SNP loci were used to construct UPGMA trees for the 429 accessions. Among them, 364 cultivars could be distinguished from each other, accounting for 84.8% of the panel 1 accessions. However, the remaining 15.2% cultivars were not distinguished due to the close relationships among the accessions. For example, Yangmai5 and Funo could not be clearly separated because Funo was a parent of Yangmai5 (Nanda2419/Triumph// Funo///St1472/506) [18]. Further analysis showed that a tiny difference was detected between Yangmai5 and Funo (GS = 0.0003) with 3231 SNP markers from the total 3489 SNPs with no missing data among all the parents of Yangmai5.

Using the UPGMA tree based on the original SNP list, to differentiate the closely related accessions, we identified the SNP loci with different alleles from the other 3439 SNP loci among these accessions. PIC values were calculated for these newly selected SNP markers based on panel 1 accessions. At least one SNP marker with a high PIC value was retained for each pair of closely related accessions. These newly screened SNP loci were sequentially added
to replace the SNP markers in the original list. A new UPGMA tree was constructed after each adjustment to the SNP list. In determining the optimal minimum numbers of SNPs used as the SNP barcode, the highest resolution of SNP markers and their distribution across chromosomes were all taken into account. Finally, 43 SNP loci were obtained from the panel 1 accessions to form a SNP-based barcode for hexaploid wheat (Table 1).

Characteristics of the SNP-based molecular barcode

The 43 SNP loci were disbursed throughout the 21 chromosomes (Fig 2). Relatively, more markers were selected from Chr1B and Chr3B (Table 1) due to the high polymorphism of the B genome between closely related accessions. These SNP loci were generally independent, with loose LD between loci (mean $R^2 = 0.1$), though pairs of SNP markers on Chr1B and Chr3B were linked (Fig 3). Of these 43 SNPs, 34 were transitions, 9 were transversions (Table 1), and 18 and 13 SNPs resulted in synonymous and nonsynonymous amino acid changes, respectively. The putative functions of these SNP loci are listed in S4 Table.

The minor allele frequency (MAF) ranged from 8% to 50%, with a mean MAF of approximately 37% across the 429 wheat cultivars. The genetic diversity (PIC) for the 43 SNP loci ranged from 0.14 to 0.38, with a mean PIC value of 0.34. Compared with the original polymorphic data (3489 SNPs), the 43 SNP markers selected showed increased diversity, by approximately 17%, for the panel 1 accessions (PIC values from 0.29 to 0.34) (Table 1). Based on the MAF
Table 1. Information about the 43-SNP-based molecular barcode and diversity among panel 1 accessions.

| SNP barcode | SNP id  | Chr | Position | SNP type | Type_of_change_I | Type_of_change_II | PIC | MAF |
|------------|---------|-----|----------|----------|------------------|------------------|------|-----|
| WB01_1A    | IWA530  | 1A  | 107.3    | T/C      | Transition       | Synonymous       | 0.35 | 0.34|
| WB02_1A    | IWA2783 | 1A  | 124.5    | A/C      | Transversion     | Nonsynonymous    | 0.35 | 0.35|
| WB03_1A    | IWA5405 | 1A  | 181.9    | T/C      | Transition       | Synonymous       | 0.37 | 0.44|
| WB04_1B    | IWA5186 | 1B  | 91.9     | A/G      | Transition       | Synonymous       | 0.37 | 0.47|
| WB05_1B    | IWA4153 | 1B  | 95.7     | T/C      | Transition       | Nonsynonymous    | 0.3  | 0.25|
| WB06_1B    | IWA4154 | 1B  | 95.8     | T/C      | Transition       | Nonsynonymous    | 0.3  | 0.25|
| WB07_1B    | IWA4155 | 1B  | 96.4     | T/C      | Transition       | Synonymous       | 0.3  | 0.25|
| WB08_1B    | IWA4031 | 1B  | 102.7    | A/G      | Transition       | Nonsynonymous    | 0.37 | 0.43|
| WB09_1B    | IWA695  | 1B  | 111.2    | T/C      | Transition       | -                | 0.37 | 0.48|
| WB10_1B    | IWA4525 | 1B  | 133.4    | A/G      | Transition       | -                | 0.37 | 0.48|
| WB11_1D    | IWA4884 | 1D  | 95.7     | T/C      | Transition       | Synonymous       | 0.22 | 0.15|
| WB12_2A    | IWA2938 | 2A  | 189.8    | A/C      | Transversion     | Nonsynonymous    | 0.37 | 0.49|
| WB13_2B    | IWA2442 | 2B  | 47.4     | A/C      | Transversion     | Nonsynonymous    | 0.36 | 0.39|
| WB14_2B    | IWA4323 | 2B  | 110.8    | T/G      | Transition       | Synonymous       | 0.26 | 0.19|
| WB15_2D    | IWA2961 | 2D  | 117.7    | A/G      | Transition       | Synonymous       | 0.36 | 0.39|
| WB16_3A    | IWA5285 | 3A  | 108.5    | T/C      | Transition       | Synonymous       | 0.37 | 0.5 |
| WB17_3B    | IWA5106 | 3B  | 13.8     | A/G      | Transition       | Synonymous       | 0.36 | 0.39|
| WB18_3B    | IWA2037 | 3B  | 33.2     | T/C      | Transition       | -                | 0.26 | 0.19|
| WB19_3B    | IWA8303 | 3B  | 37.2     | A/G      | Transition       | Synonymous       | 0.34 | 0.31|
| WB20_3B    | IWA662  | 3B  | 52.8     | T/C      | Transition       | -                | 0.37 | 0.47|
| WB21_3B    | IWA5618 | 3B  | 52.8     | T/C      | Transition       | Synonymous       | 0.37 | 0.45|
| WB22_3B    | IWA6279 | 3B  | 73.8     | A/G      | Transition       | Synonymous       | 0.14 | 0.08|
| WB23_3B    | IWA3710 | 3B  | 88.3     | A/G      | Transition       | -                | 0.37 | 0.41|
| WB24_3B    | IWA2124 | 3B  | 102      | A/G      | Transition       | -                | 0.37 | 0.43|
| WB25_3B    | IWA3835 | 3B  | 102.6    | T/G      | Transversion     | Nonsynonymous    | 0.37 | 0.41|
| WB26_3D3   | IWA3011 | 3D3 | 17.7     | T/C      | Transition       | -                | 0.35 | 0.34|
| WB27_4A    | IWA1691 | 4A  | 108.4    | A/G      | Transition       | Nonsynonymous    | 0.36 | 0.37|
| WB28_4B    | IWA4854 | 4B  | 41.4     | A/C      | Transversion     | Synonymous       | 0.34 | 0.31|
| WB29_4D    | IWA5375 | 4D  | 26.9     | T/G      | Transversion     | Nonsynonymous    | 0.35 | 0.36|
| WB30_5A    | IWA4805 | 5A  | 60.5     | T/G      | Transversion     | Synonymous       | 0.35 | 0.34|
| WB31_5A    | IWA5326 | 5A  | 123.1    | A/G      | Transition       | Nonsynonymous    | 0.2  | 0.13|
| WB32_5B    | IWA1755 | 5B  | 96.5     | A/G      | Transition       | Nonsynonymous    | 0.37 | 0.49|
| WB33_5B    | IWA2698 | 5B  | 111.6    | T/C      | Transition       | Nonsynonymous    | 0.37 | 0.46|
| WB34_5B    | IWA6526 | 5B  | 158.6    | A/G      | Transition       | Synonymous       | 0.37 | 0.44|
| WB35_5D3   | IWA7177 | 5D3cult| 18      | T/C      | Transition       | -                | 0.37 | 0.5 |
| WB36_6A    | IWA224  | 6A  | 114.5    | T/C      | Transition       | -                | 0.37 | 0.48|
| WB37_6B    | IWA8380 | 6B  | 37.2     | A/G      | Transition       | Synonymous       | 0.37 | 0.43|
| WB38_6B    | IWA3312 | 6B  | 73.7     | T/C      | Transition       | -                | 0.37 | 0.49|
| WB39_6D3   | IWA2476 | 6D3 | 6.4      | T/G      | Transversion     | Synonymous       | 0.35 | 0.34|
| WB40_7A    | IWA2506 | 7A  | 51.2     | T/C      | Transition       | -                | 0.35 | 0.35|
| WB41_7A    | IWA4187 | 7A  | 145.1    | A/G      | Transition       | Synonymous       | 0.36 | 0.39|
| WB42_7B    | IWA1437 | 7B  | 19.8     | T/C      | Transition       | Nonsynonymous    | 0.34 | 0.32|
| WB43_7D3   | IWA304  | 7D3 | 8.5      | A/G      | Transition       | -                | 0.37 | 0.5 |

Mean: 0.34, 0.37

* SNP id, chromosome position (Chr); SNP type, type of change I and II according to [6, 9]
* - unknown type of change II
* PIC, polymorphism information content; MAF, minor allele frequency

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and PIC values, we could deduce that the 43 SNP markers represent rich variation across wheat cultivars.

Resolution of the SNP-based molecular barcode

The overall predictive accuracy of the 43-SNP barcode was 100% for the 429 wheat cultivars, distinguishing all the accessions distinctively (Fig 4, S1 Fig). Each accession had its unique and special fingerprint. Among the 429 accessions, the genetic distances ranged from 0.0235 (Mianmai1403 and Mianmai23) to 1.6818 (Nongda311 and Taishan4), and UPGMA trees for the panel 1 accessions indicated that the 43-SNP-based molecular barcode was highly diagnostic. The fingerprints of these accessions demonstrated rich variation and were translated into a 2D barcode that was easily accessible by cell phone (Fig 5).
Usually, DNA barcodes discriminate samples both within and between species. Common wheat is hexaploid (*Triticum aestivum* L.), deriving from spontaneous hybridization of tetraploid *Triticum dicoccoides* (2N = 28, AABB) with diploid *Aegilops tauschii* (2N = 14, DD). The 43-SNP-based barcode was investigated to evaluate its utility for distinguishing accessions with different ploidy. The results indicated that this barcode not only separated common wheat but also distinguished common wheat from its ancestors, as shown by the UPGMA tree (S2 Fig). In addition, the 43 SNP markers discriminated Gansu96, a pedigree of synthetic wheat (durum wheat Lumillo as one parent), from other hexaploid wheat, demonstrating its capability in differentiating genome origin. Unexpectedly, this barcode was sufficiently robust to separate the wheat accession XJ1 from other Chinese landraces. Genetic distance showed that XJ1 was closer to its wheat wild ancestor than to common wheat. XJ1 was collected from the Xinjiang Autonomous Region, which has been suggested as one of the origin sites of common wheat [19]. These results indicated that the high capacity of this 43 SNP-based barcode is not limited to the identification and protection of new varieties.

![Pair-wise LD for the 43 SNP markers along chromosomes. The SNP information is listed in Table 1.](doi:10.1371/journal.pone.0150947.g003)
Conversion of the SNP markers into KASP probes

KASP technology is highly suitable for the validation of individual SNPs. In this study, we converted 41 of the 43 SNP fingerprinting markers into KASP markers (S3 Table). The remaining two SNPs (WB07 and WB19) could not be converted because the available DNA sequence flanking the SNP was too short or no optimal primer pairs could be designed. Thirty-three of the corresponding accessions for digital number are listed in S1 Table.

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![Fig 4. UPGMA trees showed high resolution of the SNP-based barcode. The corresponding accessions for digital number are listed in S1 Table.](doi:10.1371/journal.pone.0150947.g004)

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![Fig 5. 2D barcode for the panel 1 wheat accessions based on 43 SNP markers.](doi:10.1371/journal.pone.0150947.g005)
the 41 KASP primers (80%) could be used for the direct genotyping of varieties. Of the 429 common wheat varieties, 425 could be clearly distinguished by the 33 KASP primers (S3 Fig). The two pairs of accessions, Ganmai8 and Longchun8 (coded as 167 and 171, respectively, in S3 Fig) and Han6172 and Heng4399 (coded as 60 and 248, respectively, in S3 Fig), each have a close relationship in that the former is the parent of the latter.

Discussion

SNPs (single-nucleotide polymorphisms) are the third-generation molecular marker. Their advantages, including high frequency across the whole genome, ease of detection and cost efficiency, make SNP markers particularly popular. With the development of next-generation sequencing (NGS) technology, substantial numbers of SNPs are being discovered, and high diagnostic SNP arrays have been developed for several major crops [6–11]. NGS also provides the opportunity to extend DNA barcoding to new kinds of genomic data [20]. SNP-based barcodes have been developed for human diseases [21–23]. However, to date, no SNP-based barcode has been designed for crops. Here, for the first time, we screened a set of SNPs to develop a SNP-based barcode for wheat. The 43-SNP barcode is rich in variations and has high discriminatory power to discriminate the 429 hexaploid wheat accessions. On average, one SNP can identify ten accessions, a resolution capacity much higher than that reported for ISSRs [24] and SSRs [25], which have usually been used for the identification and evaluation of wheat accessions in the past.

As suggested, diagnostic SNP panels should be composed of the minimal number of SNPs required to differentiate all pairwise comparisons [26]. For SNP arrays with moderate density (i.e., the 90K SNP chip), the cost in consumables for each wheat sample is approximately $100 at current market rates. This is too expensive for breeders who have hundreds of wheat lines to benefit from marker-assisted selection (MAS) based on SNP arrays directly. Therefore, low-density SNP arrays specific for molecular breeding may serve as extended barcodes in the future. In addition to costs, the requirements of such a barcode are that it be stable and easy to operate. In this respect, SNP markers are more easily detectable than SSR markers at present. With the development of the KASP technique (http://www.lgcgroup.com/services/genotyping) specific for detecting single-nucleotide variations, SNP markers can be converted to KASP probes. Thus, SNP detection no longer relies on array technology, and the number of SNP markers depends instead on the requirements of the set of samples for comparison. Using the KASP system, the cost for one SNP per sample is approximately $0.12. In fact, the number of KASP probes or samples per PCR is flexible according to the aims of the experiment. The procedure of PCR amplification is quite simple. Within one to two hours, the genotypes of 384 samples for one SNP marker or one sample for 384 SNP markers will be available. More importantly, KASP probes are labeled by two fluorophores, eliminating the uncertainty of the final data. Any detection systems for Real-time PCR could, in theory, be used for signal capture of KASP probes. Thus, SNP markers detected using the KASP system are less expensive and more efficient than SSR markers [27].

A SNP barcode provides a tool to discriminate very closely related accessions or the origins of wheat subgenomes. In the present study, the wheat accessions used for generating the SNP-based barcode are collections grown in China from the 1930s to the 2010s with important agricultural characteristics. These accessions are rich in variants including landraces and modern cultivars. The 43-SNP-based barcode could discriminate closely related accessions, such as the parent Funo and pedigree Yangmai5. In addition, this SNP barcode could differentiate between genome donors of wheat. For example, the origin of the A and B genomes of Gansu96 is the durum wheat Lumillo, which was detected as disparate from those of other hexaploid wheat cultivars.
By 2006, more than 41,000 wheat accessions had been preserved in the Chinese GeneBank (personal communication). From 2001 to 2015, 340 new varieties were registered according to the national assessment standard, in addition to the varieties released by provinces in China. Therefore, major concerns for wheat researchers and breeders include how to classify the wheat collections in this GeneBank and how to ensure the authenticity and purity of seeds. This 43-SNP barcode may be highly useful in the above work. However, there is still some room to improve this SNP barcode. SNPs of important genes controlling functional divergence as well as SNPs in intergenic regions can be used for designing SNP barcodes in the future.

Supporting Information

S1 Table. Information for panel 1 of 429 wheat accessions: sample code, name, characterization, and genotyping by the 43 SNP markers.

S2 Table. Information for panel 2 of 193 common wheat and 96 wild relative pairs: sample code, name, and genotyping by the 43 SNP markers.

S3 Table. KASP primers for the SNP-based barcode.

S4 Table. Information about the 43-SNP-based barcode: chromosome position and annotation.

S1 Fig. Fingerprints of 429 wheat accessions. Each line represents one SNP locus, and each column represents one accession. The SNP and cultivar information is listed in S1 Table. Yellow, green, blue and purple colors represent nucleotides T, A, C and G, respectively. Missing data are indicated by grey color.

S2 Fig. UPGMA tree showing that the 43-SNP barcode can distinguish hexaploid wheat from its wild relatives. The black digital numbers are 193 elite cultivars, and the red ones are wild relatives.

S3 Fig. UPGMA trees based on 33 SNP markers with working KASP primers. The corresponding accessions for digital numbers are listed in S1 Table.

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Author Contributions

Conceived and designed the experiments: LFG XYK. Performed the experiments: LFG. Analyzed the data: LFG JZJ. Contributed reagents/materials/analysis tools: LFG. Wrote the paper: LFG JZJ XYK.
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