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Development of gene-based InDel markers on putative drought stress responsive genes and genetic diversity of durian (Durio zibethinus)

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Abstract

Insertion-deletion (InDel) markers are co-dominant, relatively abundant and practical for agarose gel genotyping. InDel polymorphism usually affects gene functions. Nucleotide sequences of durian (*Durio zibethinus*) are available, but InDel makers have not been well established. This study aimed to develop drought-related gene-based InDel markers for durian through bioinformatic analysis of RNA-Seq datasets. The polymorphism of the markers was verified in 24 durian genotypes local to Thailand. Bioinformatic analysis indicated 497 InDel loci having lengths more than 9 bp. To evaluate these InDel markers, 15 InDel loci were selected. Nine markers were successfully amplified a clear polymorphic band pattern on 2% agarose gel. The polymorphic information content (PIC) of these nine markers ranged from 0.1103 to 0.5808. The genetic distance between the 24 genotypes ranged from 0.222 to 0.889. The phylogeny based on the nine InDel loci distinguished the 24 genotypes, and divided samples into four groups. This set of gene-based InDel markers on putative drought responsive genes will be useful for genetic studies.

1. Introduction

Durian (*Durio zibethinus*; Family Bombacaceae) is a highly valued fruit in Southeast Asia, known for its rich, sweet, and creamy taste as well as its strong and distinctive aroma (Siriphanich, 2011) (Husin et al., 2018). In recent years, fresh and processed durians have gained popularity in both domestic and export markets. Thailand is the largest global producer and exporter of durians, with an estimated 941 rai of land, or about 150 hectares, dedicated to durian agriculture. In 2022, this area yielded 1,252 tons of durians with a production value of approximately 4 billion Baht (Office of agricultural economics, 2022).

As global warming continues to worsen, the incidence of drought is increasing common and, like many fruit plants, durian is particularly sensitive to drought, which can significantly reduce growth and productivity. As a result, the development of new durian varieties with improved drought-resistance should be a primary consideration for durian breeding programs (Husin et al., 2018). Moreover, farmer usually used local durian varieties as a sufficient rootstock due to their ability to adapt and tolerance to stress environment (Arisena et al., 2023). Choosing the tolerant rootstock is another good option for durian production. Using local varieties as a tolerant rootstock has successfully determined in Thongdaeng for Phytopthora tolerance (Srisink, 2023). In Thailand, there are over 300 varieties of durian named (Uy and Villaverde, 2021). Even the only a few varieties being grown in commercial cultivation, the plenty of local varieties have a value as effective rootstock and germplasm resources.

In breeding program or germplasm selection, molecular marker technology is a promising tool for verifying germplasm and effectively selecting desired plants. In order to develop markers, genetic variation between genotypes is required. Various types of molecular markers, such as restriction fragment length polymorphism (RFLP), random amplified polymorphism (RAPD), and microsatellites or simple sequence repeats (SSRs), have been used in genetic studies of durian (Nei and Takezaki, 1983). Recently, high-throughput sequencing has led to the identification of many single-nucleotide polymorphisms (SNPs), insertions and deletions (InDels), and short sequence repeats (SSRs) in several organisms (Teh et

al., 2017). InDels have several advantages over SNPs in terms of practicality for routine laboratory use, as they do not require expensive infrastructure for genotyping. Gene-based InDels can also result in different gene functions (Whankaew et al., 2020), and the genomic density of InDels is higher than that of SSRs (Liu et al., 2013). Despite these advantages, InDel markers have not received much attention in durian research, and although the draft genome has been established, InDel markers have not yet been identified.

In this study, we retrieved InDel loci from RNA sequencing data, in order to make it available for user. And identified drought stress-related genes as well as genotyped in local durian population. We believe that these user-friendly marker sets will help breeders develop a strategy for further improvements in durian production and help researchers with genetic assessments.

2. Materials and methods

2.1 Plant materials and sequence data sets

Twenty-four durian (*D. zibethinus*) genotypes were used to evaluate identified InDel marker. All were local cultivars in southern Thailand and included one each of Mohmavee, Tuzu, Kanpet, Namdam, Artadanga, Krongpinung, Mulae, Puangmanee, Ipuang, Iworn, Bintung, Bula, Tungkai, Lamoon, and 10 unnamed cultivars (Table 1). Seeds of each sample were collected and grown in a greenhouse at Prince of Songkla University, Hat Yai, Thailand.

Table 1
Details of 24 local durian samples used in the study.

Number	Sample name	Code	Collected location
1	Artadanga	ATD2	Bannang Sata District, Yala
2	Bintung	BT6	Bannang Sata District, Yala
3	Bula	BL1	Bannang Sata District, Yala
4	Ipuang	IP4	Than To District, Yala
5	Iworn	IW7	Than To District, Yala
6	Kanpet	KP5	Ban Ta Khun District, Surat Thani
7	Krongpinung	KPN8	Krongpinung District, Yala
8	Lamoon	LM10	Yaha District, Yala
9	Mohmavee	MW12	Yarang District, Pattani
10	Mulae	ML24	Bannang Sata District, Yala
11	Namdam	HD	Ban Ta Khun District, Surat Thani
12	Puangmanee	PN16	Yarang District, Pattani
13	Tungkai	TK6	Bannang Sata District, Yala
14	Tuzu	TS11	Bannang Sata District, Yala
15	Un1	Un1	Rattaphum District, Songkhla
16	Un2	Un2	Rattaphum District, Songkhla
17	Un3	Un3	Betong District, Yala
18	Un4	Un4	Betong District, Yala
19	Un5	Un5	Betong District, Yala
20	Un6	Un6	Betong District, Yala
21	Un7	Un7	Betong District, Yala
22	Un8	Un8	Betong District, Yala
23	Un9	Un9	Phunphin District, Surat Thani
24	Un10	Un10	Phunphin District, Surat Thani

RNA seq data of the *D. zibethinus* cultivars Monthong, Musang King, and Puang Manee derived from arils were retrieved from the NCBI database (Teh et al., 2017). Sequence read archive (SRA) accession numbers, total bases, and total reads are shown in Table S1.

2.2 Bioinformatics analysis for InDel identification

The quality of sequence reads was examined by fastQC (v0.11.9) software. The RNA-seq reads were mapped to the durian reference genome (RefSeq Accession No. GCF_002303985.1) using BWA (v0.7.17) with a default parameter. After sorting mapped files with samtools, variants were called using bcftools (v1.14) to generate genotype likelihoods at each genomic position with coverage from the BAM file. The variants, including SNPs and InDels, were filtered using bcftools with read depth and quality greater than 10 and 20, respectively. The filtered variants were then annotated by Annovar, using the gene-based annotation mode.

2.3 Functional annotation and drought-related gene selection

The nucleotide sequences corresponding to the filtered InDels were retrieved from genome data. The deduced amino acid sequences of those InDels were then functionally annotated by standalone EggNOG mapper (v5.0) with default parameters. Finally, drought responsive genes that contained an InDel size greater than or equal to nine nucleotides were chosen for evaluation in the durian population.

2.4 Validation of DNA polymorphism using annotated Indel markers

Fifteen candidate InDels were selected to validate the markers. Forward and reverse primers were manually designed for PCR amplification (annealing temperature 55°C and PCR product sizes 100–500 bp).

For DNA preparation and PCR analysis, genomic DNA was extracted from young leaves using DNA extraction with a modified CTAB method as described by Sathapondacha et al. (Sathapondecha et al., 2021). Briefly, leaves were frozen with liquid nitrogen and finely ground. The ground leaves were washed with a sorbitol buffer (100 mM Tris pH 8.0, 0.35 M sorbitol, 5 mM EDTA pH 8.0, 1% w/v PVPP, and 1% v/v beta-mercaptoethanol) five times, or until the solution was no longer viscous. The prepared leaf sample was added to CTAB lysis buffer (AppliChem), which included 1% w/v PVPP, 0.3% v/v beta-mercaptoethanol, and 20 µg/ml RNase A, and heated at 60°C for 30–60 min. After centrifugation, the supernatant was transferred to a new tube and subsequently added with 1 volume of 24:1 chloroform:isoamyl alcohol before mixing by inverting the tube. DNA in the aqueous phase was precipitated with 0.7 volume of isopropanol and washed with 70% ethanol. The obtained DNA pellet was dissolved in deionized water and kept at -20°C until used. The quality and quantity of DNA were measured using Nanodrop and agarose gel electrophoresis.

A 50 ng of DNA was used in a PCR reaction with the candidate InDel markers. The 20 µl total volume of PCR mixture contained 1X PCR buffer (Vivantis), 0.2 µM of each primer, 0.25 mM dNTP, 2.0 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Vivantis). The PCR temperature profile was initialized at 95°C for 2 min and followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C

for 7 min. PCR products were analyzed by 2% agarose gel electrophoresis. Each clear polymorphic fragment was scored. The number of polymorphic markers and heterozygosity among the accessions were tested. Polymorphic information content (PIC) and heterozygosity were calculated online at https://gene-calc.pl/pic. Descriptive statistics of the microsatellite data, such as the number of alleles and observed and expected heterozygosity, were determined using GenALex version 6.50 software. A phylogenetic tree was constructed using POPTREE2 from DA distance (Nei and Takezaki, 1983) by using the unweighted pair-group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973). The phylogenetic tree was built using a bootstrap value of 1,000.

3. Results and Discussion

Molecular markers enable the fast and effective improvement of crops. In this era of big data, nucleotide sequences for many economically valuable plants are available online. Gene-based InDel markers on putative drought responsive genes were identified from available RNA seq databases to provide PCR-gel-based allele-specific markers for durian genotyping.

3.1 Identification of InDel marker from RNA seq data and Functional annotation

In this study, a total of 1,123,854 variants were called from pooled durian aril RNA-Seq reads (Monthong, Musang king, and Puangmanee varieties). Among these, 179,435 variants were located on exonic regions, which corresponded to 26,381 mRNAs. These 26,381 mRNAs were functionally annotated. The results from COGs revealed that most of the InDels (7,119 mRNAs) were classified to the unknown function category, followed by signal transduction mechanism (2,677 mRNAs), post-translational modification protein turnover and chaperons (2,352 mRNAs), and transcription (2,303 mRNAs) (Figure S1). After filtering the results, 3,751 InDels were identified, distributed across many genomic features. Most InDels were located in intronic regions (37.56%) followed by intergenic and exonic regions at 14.22% and 3.10%, respectively (Fig. 1). Furthermore, the annotation of InDels in exonic regions revealed many classes of variation. Frameshift mutation accounted for 53.20% of exonic InDels, whereas stopgain mutation accounted for 1.10% equal to 41 InDels. Frameshift and stopgain mutation usually result in the loss or gain of gene functions. For example, a 26-bp deletion in the DREB transcription factor gene (*TaDTG6-B*) coding region could ameliorate drought tolerance in wheat by inducing a gain-of-function of the gene (Mei et al., 2022). A 42-bp InDel polymorphism in *FRIGIDA* gene in *Arabidopsis lyrate* corresponded to a 14-amino acid length difference at the protein level, which conferred a 15-day difference in flowering (Kuittinen et al., 2008). The details of InDel polymorphism verified in this study, including the effect and length of the insertion or deletion, scaffold location, name of mRNA, and information from annotation, were reported in Table S1. This is the first report of a large number of InDels of durian.

3.2 Drought-related genes selection

Climate change causes extreme weather events that could bring drought, high rainfall or high temperatures. Plants cannot escape these conditions, so need to fight them, which results in reduced productivity. Some plant genotypes can survive under stress conditions and still be productive. Genetics is the key to understanding this ability. In the last decade, functional genes that respond to drought stress have been identified and studied. Several physiological processes are associated with drought stress, including photosynthesis, respiration, metabolism, and osmotic regulation. These processes responded to the activation of several drought-induced and antioxidant genes in several signaling pathways, especially the abscisic acid (ABA) signaling pathway (dos Santos et al., 2022). In the present study, InDels from 15 drought-responsive genes were selected according to experimental evidence and the presence of insertion or deletion lengths greater than 9 bp (Table S1). The selected genes were known to be involved in the regulation of ABA signaling and salt or drought stress; for example, the pentatricopeptide repeat-containing protein (*DzPPR*, AT3G22470) (Jiang et al., 2015). The *aquaporin*, *GDSL esterase/ lipase*, and chaperon genes were differentially expressed in drought conditions in the opium poppy plant (Kundrátová et al., 2021). Differential expression of the *CASP-like* gene was found in Foxtail millet under drought stress (Guo et al., 2023). Villin-4 was demonstrated to regulate tolerance to both biotic and abiotic stresses including ABA and salt treatments (Ge et al., 2021) and response to drought in *Ammopiptanthus mongolicus* root (Sun et al., 2017). Protein S-acyltransferase could play a role in lipid post-translational modification which is involved in several development processes and drought (Li et al., 2022). The NRT1/ PTR family protein, a nitrate di/tri-peptide transporter, plays roles in the transportation of several plant hormones, including ABA and auxin (Chiba et al., 2015) and also responds to drought susceptibility (Guo et al., 2023). Several of the genes selected were involved in certain drought-response mechanisms such as lipase in lipid accumulation (Liang et al., 2019), calmodulin-binding protein in calcium ion changes (Zeng et al., 2015), and TBC1 domain family (Woldesemayat and Ntwasa, 2018).

3.3 InDel polymorphism validation

Choosing molecular markers requires the ability to detect polymorphisms. From 24 local durian genotypes, 15 InDel markers of drought-related genes were selected and validated. Nine InDel markers generated clear polymorphic band patterns (Fig. 2). InDels with insertion/deletion differences between 10–30 bp were used, indicated that 10 nucleotides difference are able to detected by 2% agarose gel electrophoresis. The InDel markers can separated between homozygous and heterozygous band pattern. Some markers showed heteroduplex band above homoduplex band, which cause three band in a lane. Twenty-five alleles were obtained from nine InDels with an average of 2.22 allele per locus. Number of alleles at each locus ranged from 2–3 alleles, which mostly related to the result from in silico. Allele number of two was commonly observed by InDel markers. Adedze et al, (2021) were chosen 39 InDel markers with nucleotide difference more than 30 bp and genotyped in cucumber. Of these, 38 InDel markers generated two alleles at a locus. Observed heterozygosity ranged from 0.120–0.669. The PIC of primers ranged from 0.1103 to 0.5808 (Table 2). The highest PIC value was obtained for DzPAT7, followed by DzTCB1, which produced most alleles (three). The degree of polymorphism can be estimated from heterozygosity. The usefulness of molecular markers is indicated by PIC values, which is a

parameter commonly used in the literature to assess the discriminatory power of molecular markers (Serrote et al., 2020).

Table 2
Characteristic of nine putative drought stress-related InDel loci from 24 durian genotypes.

Sequence name	primer name	Na	Ho	uHe	PIC
Lipase-like	DzLIpase	2	0.417	0.479	0.3589
Probable methyltransferase PMT23	DzPMT23	2	0.292	0.361	0.2909
Calmodulin-binding protein 60 D-like	DzCBP60D	2	0.625	0.467	0.3528
TBC1 domain family member 8B-like	DzTCB1	3	0.458	0.465	0.4104
Putative gamma-glutamylcyclotransferase	DzAIG2	2	0.042	0.120	0.1103
Probable protein S-acyltransferase 7	DzPAT7	3	0.583	0.669	0.5808
Protein NRT1/ PTR FAMILY 5.10-like	DzNPF5.10	2	0.250	0.284	0.2392
Trafficking protein particle complex subunit 12-like	DzTRAPPC12	2	0.167	0.156	0.1411
CASP-like protein 4D1	DzCASPL	2	0.417	0.496	0.368
Mean		2.22	0.36	0.39	0.32

Na: number of different alleles, Ho: observed heterozygosity, uHe: unbiased expected heterozygosity, PIC: polymorphic information content

3.4 Genetic diversity analysis

In order to established the application of InDel markers on putative drought stress-related genes, genetic diversity using this set of InDel was performed in 24 local durian genotypes. Pair-wise genetic distances were calculated and used for two-dimensional (2-D) PCoA with GenAlEx (Fig. 3). The first principal coordinate (Coord. 1) and the second principal coordinate (Coord. 2) accounted for 28.45% and 18.04% of total variation, respectively (Fig. 3). The PCoA plot did not group the 24 local durian genotypes based on geographical distribution. The genetic distance between the 24 genotypes ranged from 0.222 to 0.889. The phylogenetic analysis showed that the 24 genotypes were divided into four groups. This result supports the assumption that gene-based InDel markers on putative drought responsive genes can be useful for genetic studies.

In summary, InDel loci of durian from *In silico* determination were provided. A set of nine agarose-based InDel markers on putative drought stress-related genes were shown to provide a user-friendly marker for genetic analysis. InDels with insertion/deletion differences at least 10 bp were successfully detected by 2% agarose gel.

Declarations

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Competing interest

The authors declare no competing interests.

Author Contributions

Ponsit Sathapondecha and Sukhuman Whankaew contributed to the study conception and design. Material preparation, data collection and analysis were performed by all authors. The first draft of the manuscript was written by Ponsit Sathapondecha and Sukhuman Whankaew. All authors read and approved the final manuscript.

Corresponding author

Sukhuman Whankaew

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

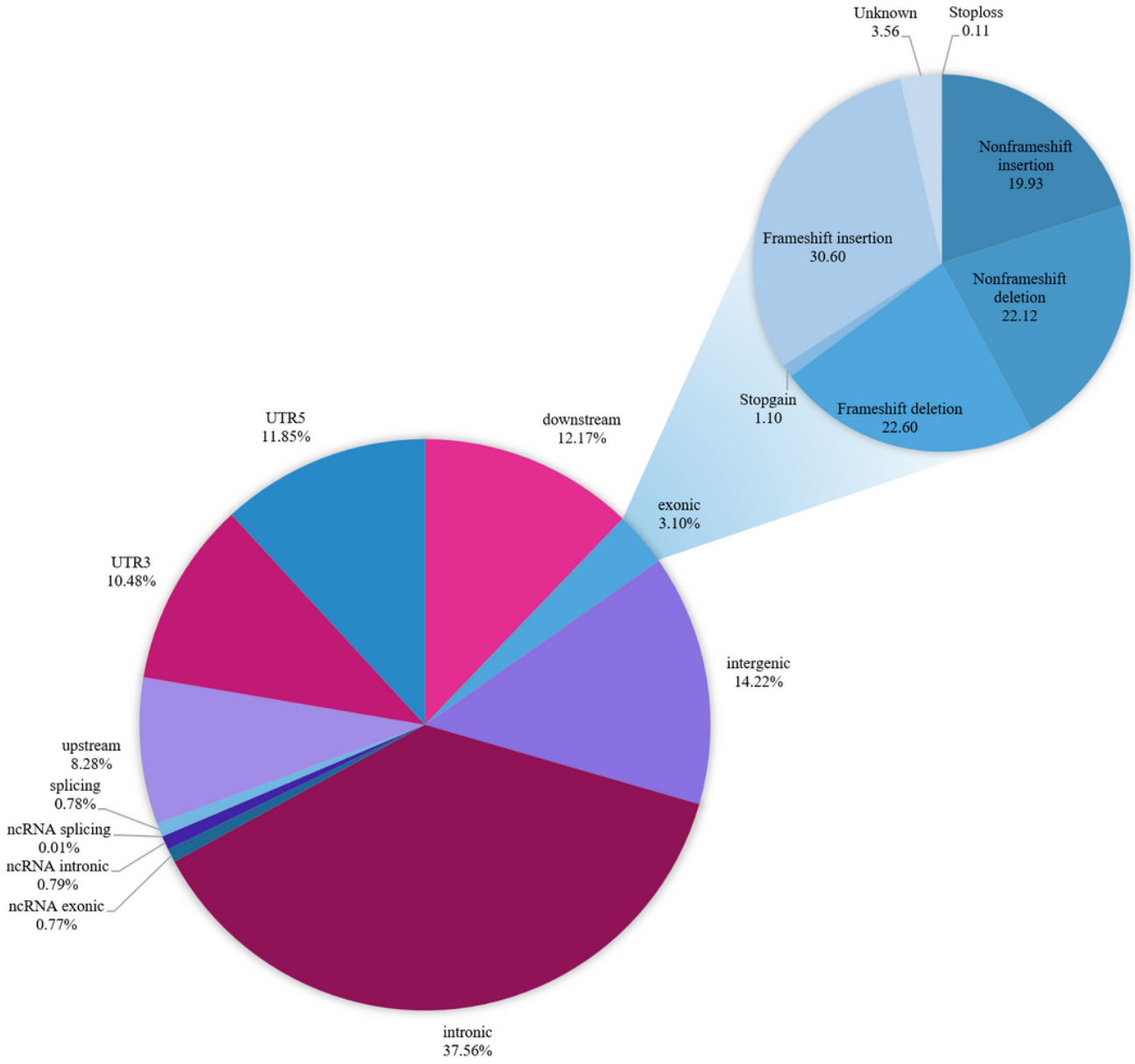

Figure 1

Total Indels. The blue pie chart indicates the percentage of total InDels identified in the genome, whereas the orange pie chart indicates the percentage of InDels identified in exonic regions.

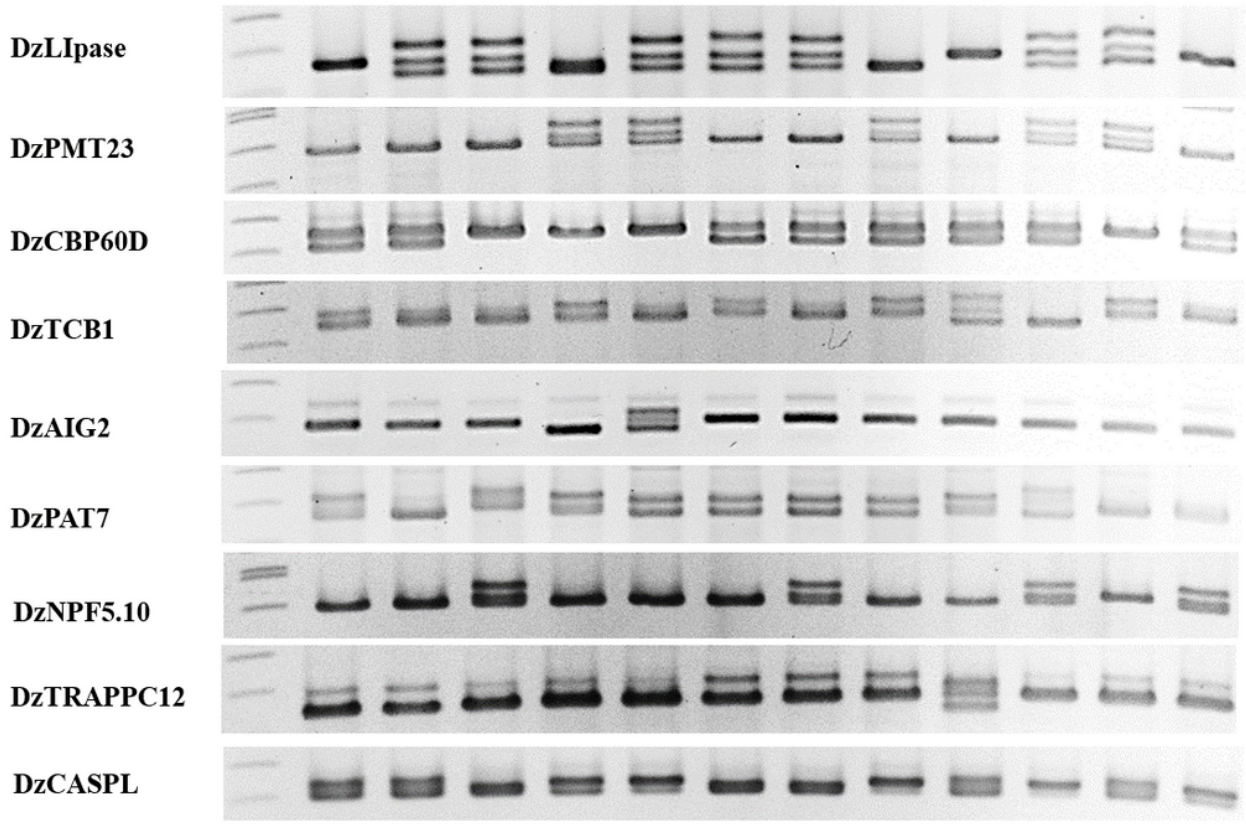

Figure 2

The band patterns obtained from polymorphic InDel markers on nine putative drought stress-related genes in 24 durian genotypes

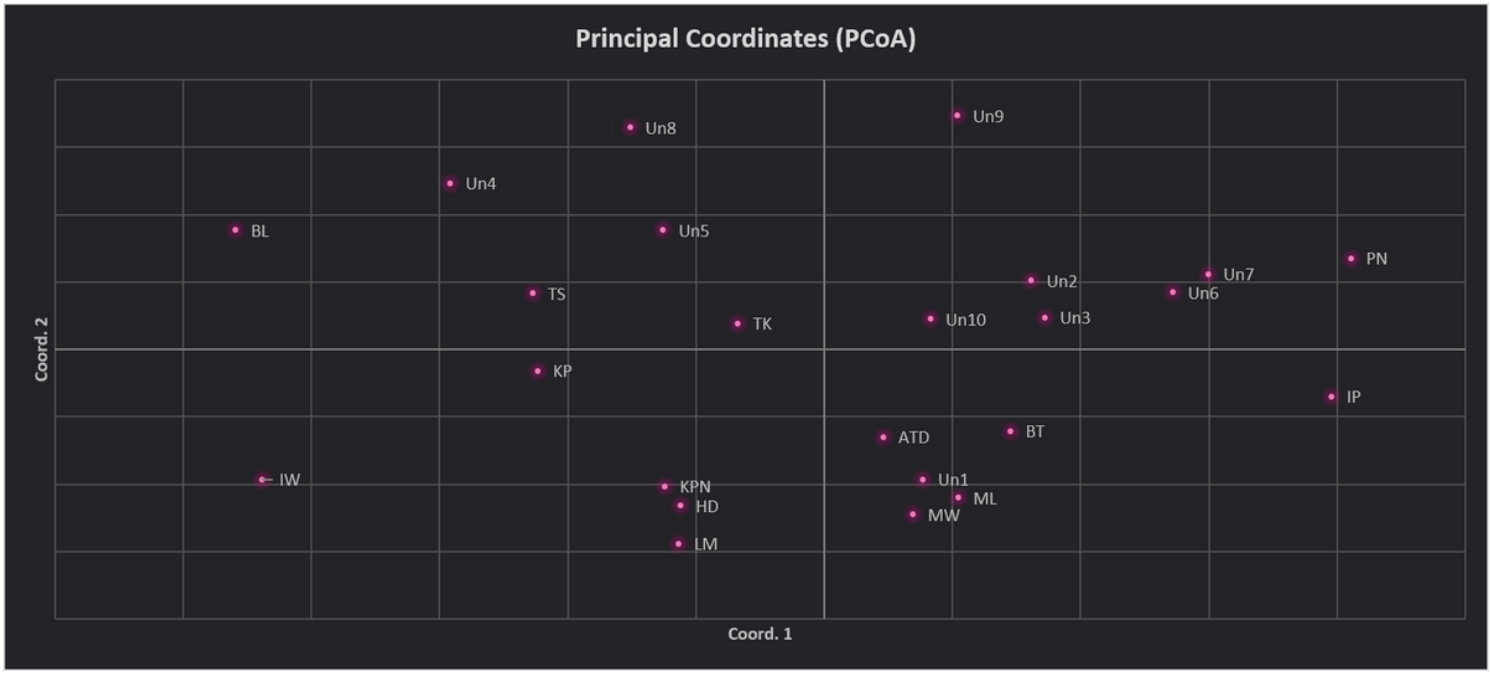

Figure 3

PCoA (principal coordinates analysis) plots based on genotypic data of nine gene based InDel loci.

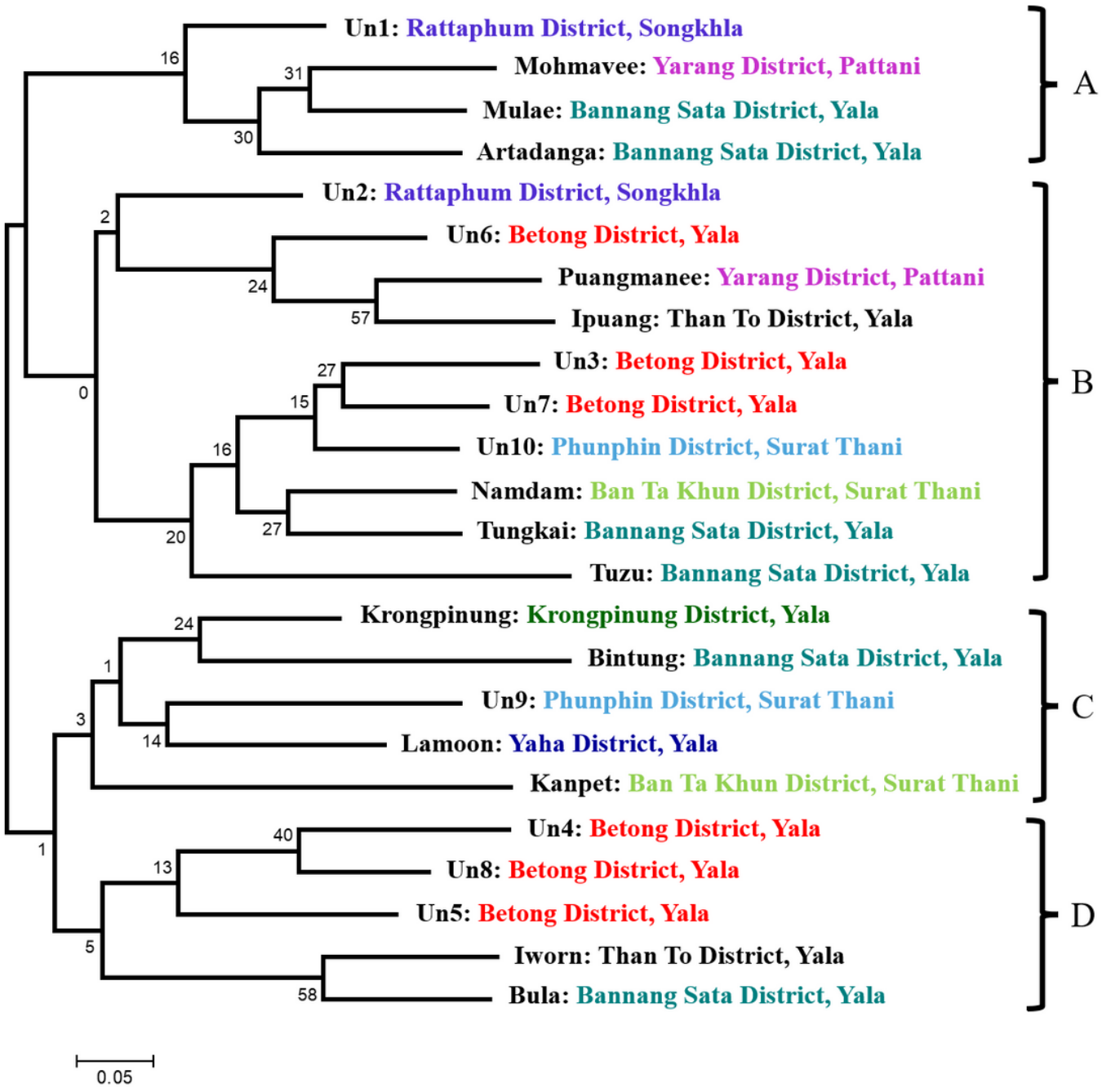

Figure 4

Phylogenetic relationships among 24 local durian genotypes. Nei's standard genetic distances with sample size correction (Dst) (Nei 1972) were calculated using genotypic data of nine gene based InDel loci. A consensus dendrogram was constructed using the unweighted pair-group method with arithmetic mean (UPGMA). Bootstrap values are indicated on the nodes as percentages tested with 1000 bootstrap replicates.

Supplementary Files

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- FigureS1.png