



# Identification and expression analysis of the *WRKY* gene family during different developmental stages in *Lycium ruthenicum* Murr. fruit

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## ABSTRACT

**Background.** The *WRKY* gene family, one of the major transcription factor families in plants, plays crucial regulatory roles in physiological and biological developmental processes, and the adaptation of plants to the environment. However, the systematic study of *WRKY* structure, expression profiling, and regulatory functions has not been extensively reported in *Lycium ruthenicum*, although these aspects have been comprehensively studied in most plant species.

**Methods.** In this study, the *WRKY* genes were identified from a *L. ruthenicum* transcriptome database by using bioinformatics. The identification, phylogenetic analysis, zinc-finger structures, and conserved motif prediction were extensively explored. Moreover, the expression levels of 23 selected genes with fragments per kilobase of exons per million mapped reads (FPKM) > 5 were assayed during different fruit developmental stages with real-time quantitative polymerase chain reaction (RT-qPCR).

**Results.** A total of 73 putative *WRKY* proteins in the *L. ruthenicum* transcriptome database were identified and examined. Forty-four proteins with the *WRKY* domain were identified and divided into three major groups with several subgroups, in accordance with those in other plant species. All 44 *LrWRKY* proteins contained one or two conserved *WRKY* domains and a zinc-finger structure. Conserved motif prediction revealed conservation of the *WRKY* DNA-binding domain in *L. ruthenicum* proteins. The selected *LrWRKY* genes exhibited discrete expression patterns during different fruit developmental stages. Interestingly, five *LrWRKYs* (-20, -21, -28, -30, and -31) were expressed remarkably throughout the fruit developmental stages.

**Discussion.** Our results reveal the characteristics of the *LrWRKY* gene family, thus laying a foundation for further functional analysis of the *WRKY* family in *L. ruthenicum*.

**Subjects** Agricultural Science, Bioinformatics, Genomics, Plant Science, Forestry

**Keywords** *Lycium ruthenicum*, *WRKY* gene, Fruit developmental stages, Gene expression, Conserved motif, Transcriptome data

## INTRODUCTION

*WRKY* transcription factors (TFs) are the 7th largest family of regulatory genes screened from higher plants (*Marchise et al., 2007; Chen et al., 2018a; Chen et al., 2018b*). The first

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cDNA encoding a WRKY protein, *SPF1*, was cloned from *Ipomoea batatas* (Ulker & Somssich, 2004; Ling et al., 2011). Since then, several members of the WRKY proteins have been identified from several plant species (Liu & Ekramoddoullah, 2009; Liu et al., 2012). To date, only two WRKY homologs in non-plant species have been described, in *Giardia lamblia* and *Dictyostelium discoideum* (Wu et al., 2005). Notably, 72 WRKY members in *Arabidopsis thaliana* (Dong, Chen & Chen, 2003), 182 in *Glycine max* (Bencke-Malato et al., 2014), 59 in *Fragaria vesca* (Zhou et al., 2016), 127 in *Malus domestica* (Meng et al., 2016), 55 in *Cucumis sativus* (Ling et al., 2011), 80 in *Vitis vinifera* (Zhang & Feng, 2014), 86 in *Brachypodium distachyon* (Wen et al., 2014), 85 in *Manihot esculenta* (Wei et al., 2016), and 95 in *Daucus carota* (Li et al., 2016) have been identified. The name of the WRKY family is derived from the most common characteristic of these proteins, the WRKY domain, which is characterized by approximately 60 amino acid residues that often exhibit sequence-specific DNA-binding (Chen et al., 2018a; Chen et al., 2018b; Zhou et al., 2016). In the WRKY domain, the highly conserved heptapeptide sequence WRKYGQK is followed by a CX<sub>4-5</sub>X<sub>4</sub>-CX<sub>22-23</sub>-HXH (C<sub>2</sub>H<sub>2</sub>) or CX<sub>7</sub>-CX<sub>23</sub>X<sub>23</sub>-HXC (C<sub>2</sub>HC-) type zinc-finger motif (Eulgem et al., 2000). The conserved WRKY domain may have a longer sequence, for example, WRKYGKK or WEKYGQK. It may also be replaced by WRRY, WKKY, WIKY, WSKY, WKRY, WRIC, WVKY, or WRMC. The conserved WRKY domain helps in binding of the protein to W-box elements or sugar-responsive cis-elements (SURE) in the promoter regions of target genes (Sun et al., 2003; Rushton et al., 2010). Depending on the number of WRKY domains and the zinc-finger motif, the WRKY proteins can be categorized into three main groups (1, 2, and 3) (Eulgem et al., 2000). Group 1 proteins have two WRKY domains: an N-terminal WRKY domain and a C-terminal WRKY domain, followed by a C<sub>2</sub>H<sub>2</sub> zinc-finger motif (CX<sub>4</sub>X<sub>4</sub>-CX<sub>22-23</sub>HXH). Group 2 proteins have only one WRKY domain and a C<sub>2</sub>H<sub>2</sub> zinc-finger motif (CX<sub>4-5</sub>X<sub>4</sub>-CX<sub>23</sub>-HXH); this group is further divided into five subgroups (2a to 2e) according to the phylogeny of the WRKY domain. Group 3 proteins also have a single WRKY domain and a C<sub>2</sub>HC zinc-finger motif (CX<sub>7</sub>-CX<sub>23</sub>X<sub>23</sub>-HXC) (Eulgem et al., 2000; Rushton et al., 2010).

The WRKY gene family has recently gained attention for its involvement in a wide range of biological and physiological developmental processes in plants, including diverse biotic/abiotic stress responses (Li et al., 2016; Jiang et al., 2017; Yang et al., 2016). The WRKY gene family also plays a crucial role in the developmental and ripening processes of fruits; for example, *FvWRKY4*, -46, and -48 in group 2c are involved in fruit development and ripening in *F. vesca* (Zhou et al., 2016). In *Ziziphus jujube* *ZjWRKY8*, -26, -47, and -48 are involved in developmental process in younger fruits (Xue et al., 2019). In addition to the involvement in seed or fruit development, the WRKY genes also control seed pigmentation. *AtWRKY44* (transparent testa glabra, *TTG2*) controls the epidermal color of *Arabidopsis* seeds by contributing to transcriptional regulation. It directly binds the upstream regulatory region of *TT12*. *TTG1*, *TT2*, and *TT8* are involved in the biosynthesis of proanthocyanidins and pigmentation in *Arabidopsis* (Gonzalez et al., 2016). Moreover, *OsWRKY11* in *O. sativa* is involved in controlling flower development (Cai et al., 2014). The roles of WRKYs in plant responses to abiotic stresses such as drought, cold, salinity, heat, ultraviolet B (UV-B), osmotic stress, and ABA have been extensively documented

(Luo et al., 2013; Jiang, Liang & Yu, 2012; Ren et al., 2010; Tao et al., 2011; Li et al., 2010; Liu & Guan, 2012).

*Lycium ruthenicum* Murr. is a typical native halophyte and perennial shrub distributed across arid and semi-arid regions of Asia, America, and Africa (Liu et al., 2012; Dai, Wang & Zhang, 2016). Its excellent physiological features conferring drought resistance, coupled with its salt tolerance, make it a relevant plant for land desertification control and soil salinity improvement (alkalinity) (Zheng et al., 2011). Ripe *L. ruthenicum* fruits are considered the highest and richest wild source of anthocyanins and are unique from a pharmaceutical point of view (Chen et al., 2013). For instance, the fruits are used to treat health conditions, including early-onset diabetes, anemia, vision problems, impotency, and lung disorders. Recently evidence indicates that these fruits can improve liver and kidney function, boost the immune system, and promote longevity (Zheng et al., 2011; Dhar et al., 2011). Consequently, worldwide economic interest in the production of *L. ruthenicum* has recently grown. *L. ruthenicum* is not only an economically and ecologically important plant but could also be a model organism for research. The WRKY gene family has been comprehensively studied in many plant species (Jiang, Liang & Yu, 2012; Ren et al., 2010; Tao et al., 2011; Liu & Guan, 2012), but no previous studies have described the WRKY genes from *L. ruthenicum*. Because of the importance of the WRKY genes in various physiological and biological plant processes, systematic study of the identification, classification, expression, and functions of WRKY family in the fruit development of *L. ruthenicum* is of interest.

In the present study, we identified a total of 73 potential *LrWRKY* members in *L. ruthenicum* according to a transcriptome database. Forty-four *LrWRKYs* identified with WRKY domains were selected, and the identification, classification, phylogenetic analysis, conserved motif determination, and gene expression profiling were extensively examined. The expression levels of WRKY genes in different fruit developmental stages were further validated using RT-qPCR. This study reveals clues to aid in the functional characterization of *LrWRKY* genes and provides a basis for further genomic studies in *L. ruthenicum*.

## MATERIALS & METHODS

### Sequence database search and identification of the *LrWRKY* family in *L. ruthenicum*

*A. thaliana* and *O. sativa* WRKY protein sequences were obtained from The Arabidopsis Information Resource (TAIR) database (<http://www.arabidopsis.org/>) and the Joint Genome Institute (JGI) database (<https://phytozome.jgi.doe.gov>) separately and were used for comparative analysis. To obtain a comprehensive list of *LrWRKY* proteins, we obtained transcriptome data from the public Sequence Read Archive database of NCBI (<https://www.ncbi.nlm.nih.gov/sra>) under accession number SRR7700825 (Ma et al., 2018). To obtain the transcriptome sequence encoding WRKY proteins, we performed a Blastx search in the NCBI database. *A. thaliana* WRKY domain sequences were used as query sequences, and Blast default settings were used. The nucleotide sequences

of the possible *LrWRKYs* were translated into amino acid sequences in DNAMAN 8.0 (LynnonBioSoft, Quebec, Canada) software, to examine WRKY proteins encoded by the obtained transcriptome sequences (Data S1). The Protein families database (Pfam; <http://pfam.xfam.org/>) was used to verify whether the putative proteins had the distinctive features of WRKY proteins. Additionally, manual examination of the conserved WRKYGQK amino acid structure at the N-terminus and the zinc-finger structure at the C-terminus of the putative WRKY domain was conducted for further confirmation (Zhang & Wang, 2005).

### Multiple sequence alignment, phylogenetic analysis, and conserved motif construction

All 44 *LrWRKY* proteins with WRKY domains and several selected *AtWRKY* proteins (*AtWRKY6*, -14, -25, -26, -31, -40, and -51) were used to generate multiple protein sequence alignments by using the default settings in ClustalW (Larkin *et al.*, 2007). The 44 *LrWRKY* proteins with WRKY proteins from two model plant species, *A. thaliana* (72) and *O. sativa* (73) (Data S2), were used for phylogenetic tree construction in MEGA 5.0 software (Tamura *et al.*, 2011) with the maximum likelihood method and 1000 bootstrap replications. The conserved motifs in *LrWRKY* proteins were determined with Multiple Expectation Maximization for Motif Elicitation (MEME) suite version 5.1.1 (<http://meme-suite.org/doc/cite.html>), and five motifs were searched.

### Plant materials

*L. ruthenicum* fruit samples were collected from Shizuishan in Ningxia, China (38°56.799' N, 106°24.711' E, 1088 H) in three developmental stages, Rs1 (green ripeness stage), Rs2 (veraison ripeness stage), and Rs3 (complete ripeness stage), on the basis of the descriptions of Ma *et al.* (2018) and Guillaumie *et al.* (2011). Biological replicates of the material comprised three shrubs in each developmental stage. The fruit samples from the three shrubs were collected, immediately frozen in liquid nitrogen, and stored at -80 °C until total RNA isolation.

### RNA extraction, cDNA synthesis, and RT-qPCR analysis

We extracted total RNA from nine samples (three biological replicates per treatment) by using a TransZol Up Plus RNA Kit (Lot# M31018) conferring to the manufacturer's instructions. The RNA quantity and quality were determined with a TGen Spectrophotometer (TianGen), on the basis of the A260 nm/A280 nm and A260 nm/A230 nm ratios. An *Evo* M-MLV RT Kit AG11705 (Accurate Biotechnology) was used to reverse transcribe the total RNA into cDNA and remove any genomic DNA from the cDNA before RT-qPCR analysis, following the manufacturer's instructions.

For the RT-qPCR analysis, primers were designed on the basis of the RNA sequences obtained from the NCBI SRA (Ma *et al.*, 2018) in Primer 5 software and synthesized by TsingKe Biological Technology Co., Ltd. (Xi'an, China) (Data S3). To better understand the *LrWRKY* gene expression during different fruit developmental stages, we selected 23 genes with FPKM values >5 from the 44 genes with WRKY domains for RT-qPCR analysis. The *LrEF1-a* gene (JX427553) was used as a reference gene. We examined three biological

replicates and performed triplicate quantitative analyses; each replicate was analyzed on the basis of 0.5  $\mu$ l of each cDNA dilution with a Heff<sup>®</sup> q-PCR SYBR<sup>®</sup> Green Master Mix kit (Yeasen Biotech Co., Ltd) according to the manufacturer's protocol. The RT-qPCR analysis was performed with a QuantStudio<sup>™</sup> 5 Real-Time PCR Instrument (ABI) (Data S4). The *LrWRKY* gene relative expression was calculated according to Livak & Schmittgen (2001).

A heatmap of all 44 *LrWRKY* gene expression patterns was constructed during fruit development and ripening stages, on the basis of FPKM values (Data S5) in TBtools software (<https://github.com/CJ-Chen/TBtools>) (Chen et al., 2018a; Chen et al., 2018b).

### Data analysis

All reported gene expression levels are presented as means  $\pm$  SE ( $n = 3$ ). The significance level among means was analyzed with Duncan's multiple range tests ( $p \leq 0.05$ ) after one-way ANOVA analysis in SPSS statistical software (Ver. 22.0, SPSS Inc., Chicago, IL, USA).

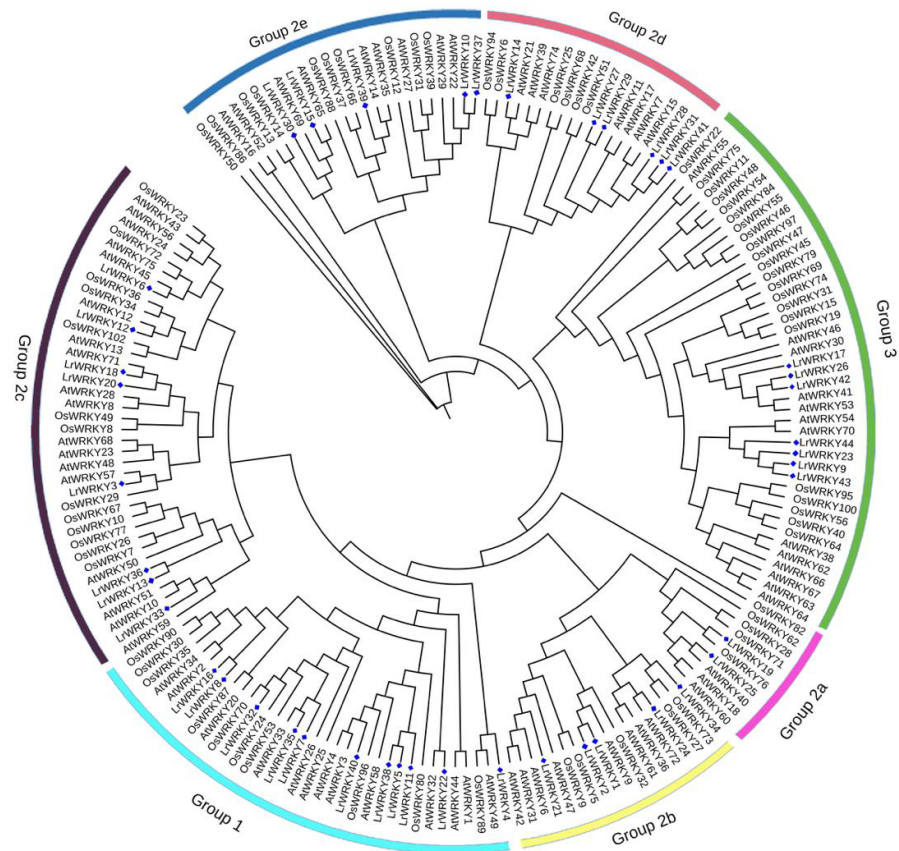
## RESULTS

### Identification of *LrWRKY* genes

We searched 43,573 unigenes for all possible *LrWRKY* genes from the transcriptome data of *L. ruthenicum* and obtained a total of 73 best hits, which were validated by BLAST via the NCBI database. We use the prefix "Lr" for *L. ruthenicum* and assigned the WRKY numbering according to the order of the annotated sequences from the database, as CL10207Contig1 (*LrWRKY1*) to CL8621Contig1 (*LrWRKY73*), respectively (Data S6). The lengths of members of the WRKY family in *L. ruthenicum* ranged from 5207 aa (*LrWRKY3*) to 485 aa (*LrWRKY67*). The open reading frames (ORF) of all members could not be predicted, due to the limitations of the sequencing technique.

### Phylogenetic analysis of *LrWRKY* genes

Phylogenetic analysis of plant WRKY genes is an effective way to understand the functions of uncharacterized WRKY members according to their evolutionary history and sequence similarity. Therefore, the 44 *LrWRKYs* identified to have WRKY domains were used to construct a phylogenetic tree. The phylogenetic analysis revealed that the *LrWRKY* proteins could be categorized into three main groups corresponding to groups 1, 2, and 3 in *A. thaliana* (Eulgem et al., 2000). Among the 44 *LrWRKY* proteins, 10 were classified into group 1 (representing 23%) consisting of N-terminal and C-terminal subgroups, 26 *LrWRKY* proteins were assigned to group 2 (representing 59%), and the remaining 8 *LrWRKY* proteins were classified in group 3 (representing 18%) (Fig. 1). Group 1 WRKY family members usually have two WRKY domains and a C<sub>2</sub>H<sub>2</sub>-type zinc-finger motif (CX<sub>4</sub>X<sub>4</sub>-CX<sub>22-23</sub>-HXH), but few *LrWRKYs* from group 1 contained only one WRKY domain (*LrWRKY8*, -11, -32, -35, and -38). *LrWRKY* proteins in group 2 had a single WRKY domain and a C<sub>2</sub>H<sub>2</sub>-type zinc-finger motif (CX<sub>4-5</sub> X<sub>4</sub>-CX<sub>23</sub>-HXH), which was further subdivided into five distinct subgroups (2a to 2e): two *LrWRKYs* belonged to 2a, four *LrWRKYs* belonged to group 2b, eight belonged to group 2c, six belonged to group 2d, and five belonged to group 2e, according to their phylogeny. *LrWRKYs* from group 3 were characterized by a C<sub>2</sub>HC-type zinc-finger motif (CX<sub>7</sub>-CX<sub>23</sub> X<sub>23</sub>-HXC) (Table 1).



**Figure 1** Phylogenetic tree according to WRKY domain sequences from *L. ruthenicum*, *O. sativa* and *A. thaliana*. Consistency of the predicted tree was tested using the Maximum Likelihood method and bootstrapping with 1,000 replicates in MEGA 5. The different colored arcs indicate different groups or subgroups and the blue diamonds represent LrWRKYs.

Full-size [DOI: 10.7717/peerj.10207/fig-1](https://doi.org/10.7717/peerj.10207/fig-1)

### Multiple sequence alignment of *LrWRKY* genes

The evolutionary relationship of the *LrWRKY* genes was analyzed through multiple sequence alignment of their conserved WRKY domains. For examination of the core domains, WRKY domains of AtWRKY proteins were selected (AtWRKY6, -14, -21, -25, -30, -40, and -51) from each group or subgroup as representatives for further comparison. The 44 *LrWRKY* proteins contained the highly conserved WRKY domains, and 41 were discovered to have the conserved heptapeptide sequence of WRKYGQK, whereas three members of group 2c (*LrWRKY*13, -33 and -36) differed by one amino acid, with the glutamine (Q) substituted by a lysine (K), thus forming WRKYGKK (Fig. 2). For group 1 members, the two WRKY domains, which were denoted group 1 N-terminal and group 1 C-terminal, clustered into two separate groups because of divergence in their sequences. We discovered a CX<sub>4</sub>-CX<sub>22</sub>-HXH zinc-finger structure in the N-terminal subgroup and a CX<sub>4</sub>-CX<sub>23</sub>-HXH structure in the C-terminal subgroup.

**Table 1** LrWRKYs grouping, zinc-finger structure and domain pattern.

Gene name	Group classification	Zinc finger	Domain pattern	Gene name	Group classification	Zinc finger	Domain pattern
LrWRKY1	2b	C <sub>2</sub> H <sub>2</sub>	N	LrWRKY23	3	C <sub>2</sub> HC	CX <sub>7</sub> -CX <sub>23</sub> X <sub>23</sub> -HXC
LrWRKY2	2b	C <sub>2</sub> H <sub>2</sub>	N	LrWRKY24	2b	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> -CX <sub>23</sub> -HXH
LrWRKY3	2c	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> -CX <sub>23</sub> -HXH	LrWRKY25	2a	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> -CX <sub>23</sub> -HXH
LrWRKY4	3	C <sub>2</sub> H <sub>2</sub>	CX <sub>7</sub> -CX <sub>23</sub> X <sub>23</sub> -HXC	LrWRKY26	3	C <sub>2</sub> HC	CX <sub>7</sub> -CX <sub>23</sub> X <sub>23</sub> -HXC
LrWRKY5	1	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> X <sub>4</sub> -CX <sub>22-23</sub> -HXH	LrWRKY27	2d	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> -CX <sub>23</sub> -HXH
LrWRKY6	2c	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> -CX <sub>23</sub> -HXH	LrWRKY28	2d	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> -CX <sub>23</sub> -HXH
LrWRKY7	1	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> X <sub>4</sub> -CX <sub>22-23</sub> -HXH	LrWRKY29	2d	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> -CX <sub>23</sub> -HXH
LrWRKY8	1	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> X <sub>4</sub> -CX <sub>22-23</sub> -HXH	LrWRKY30	2e	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> -CX <sub>23</sub> -HXH
LrWRKY9	3	C <sub>2</sub> HC	CX <sub>7</sub> -CX <sub>23</sub> X <sub>23</sub> -HXC	LrWRKY31	2d	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> -CX <sub>23</sub> -HXH
LrWRKY10	2e	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> X <sub>4</sub> -CX <sub>23</sub> -HXH	LrWRKY32	1	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> -CX <sub>22-23</sub> -HXH
LrWRKY11	1	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> X <sub>4</sub> -CX <sub>22-23</sub> -HXH	LrWRKY33	2c	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> -CX <sub>23</sub> -HXH
LrWRKY12	2c	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> -CX <sub>23</sub> -HXH	LrWRKY34	2b	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> -CX <sub>23</sub> -HXH
LrWRKY13	2c	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> -CX <sub>23</sub> -HXH	LrWRKY35	1	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> -CX <sub>22-23</sub> -HXH
LrWRKY14	2d	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> X <sub>4</sub> -CX <sub>23</sub> -HXH	LrWRKY36	2c	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> -CX <sub>23</sub> -HXH
LrWRKY15	2e	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> X <sub>4</sub> -CX <sub>23</sub> -HXH	LrWRKY37	2e	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> -CX <sub>23</sub> -HXH
LrWRKY16	1	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> X <sub>4</sub> -CX <sub>22-23</sub> -HXH X <sub>22-23</sub>	LrWRKY38	1	C <sub>2</sub> H <sub>2</sub>	N
LrWRKY17	3	C <sub>2</sub> HC	CX <sub>7</sub> -CX <sub>23</sub> X <sub>23</sub> -HXC	LrWRKY39	2e	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> -CX <sub>23</sub> -HXH
LrWRKY18	2c	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> -CX <sub>23</sub> -HXH	LrWRKY40	1	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> -CX <sub>22-23</sub> -HXH
LrWRKY19	2a	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> X <sub>4</sub> -CX <sub>23</sub> -HXH	LrWRKY41	2d	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> -CX <sub>23</sub> -HXH
LrWRKY20	2c	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> -CX <sub>23</sub> -HXH	LrWRKY42	3	C <sub>2</sub> HC	CX <sub>7</sub> -CX <sub>23</sub> X <sub>23</sub> -HXC
LrWRKY21	2b	C <sub>2</sub> H <sub>2</sub>	CX <sub>5</sub> X <sub>4</sub> -CX <sub>23</sub> -HXH	LrWRKY43	3	C <sub>2</sub> HC	CX <sub>7</sub> -CX <sub>23</sub> X <sub>23</sub> -HXC
LrWRKY22	1	C <sub>2</sub> H <sub>2</sub>	CX <sub>4</sub> X <sub>4</sub> -CX <sub>22-23</sub> -HXH	LrWRKY44	3	C <sub>2</sub> HC	CX <sub>7</sub> -CX <sub>23</sub> X <sub>23</sub> -HXC

**Notes.**

N, missen domain pattern.

**Conserved motif analysis of LrWRKYs proteins**

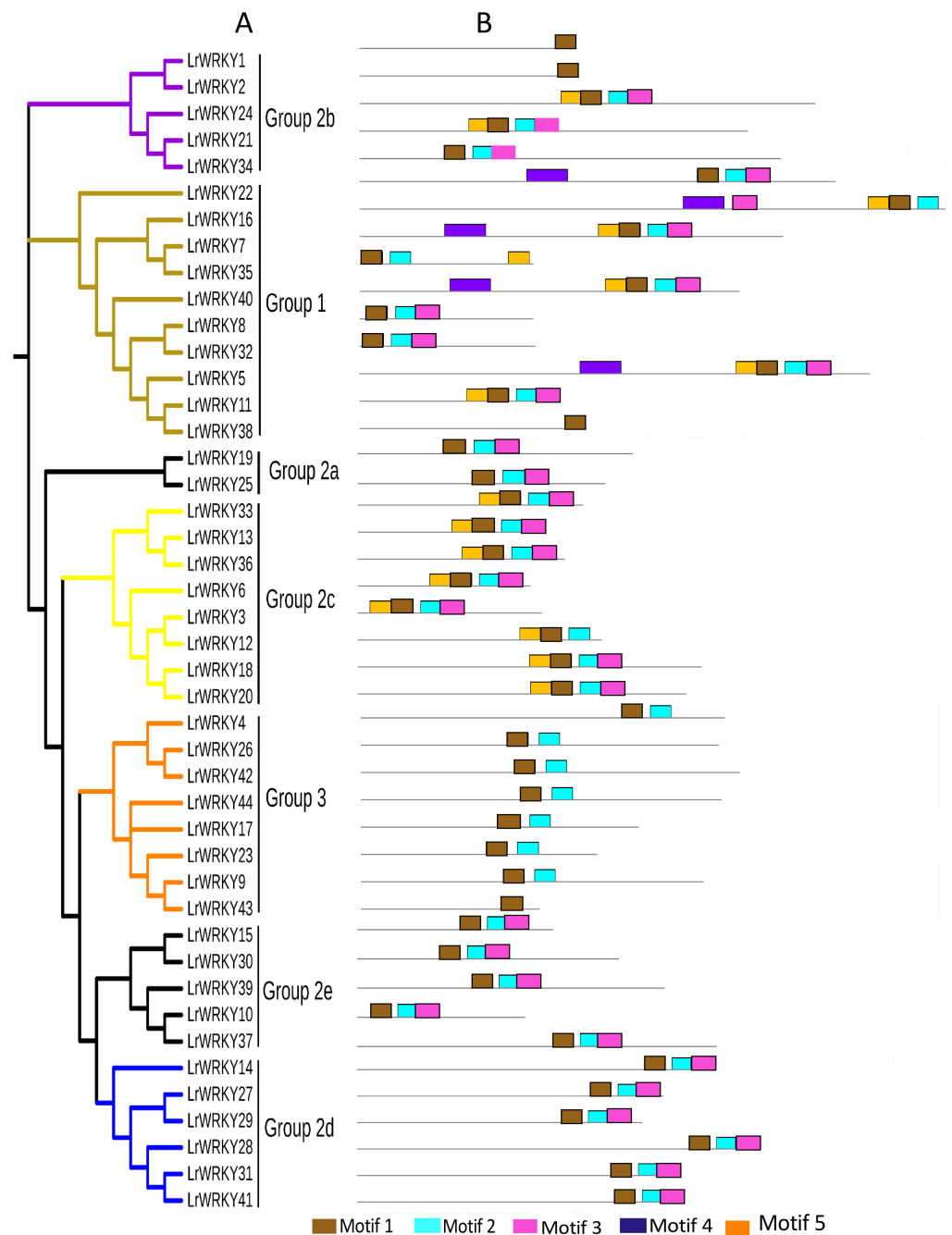
The conserved motifs of *L. ruthenicum* were examined with MEME, as described in the methods, to better understand the relationship and diversity of LrWRKY protein motif compositions. The results revealed that motifs 1 and 4 were WRKY DNA-binding motifs that encompassed the conserved heptapeptide WRKYGQK domain. The motif 4 WRKY domain belonged to the N-terminal domain, whereas motif 1 belonged to the C-terminal domain (Fig. 3, Table 2). Interestingly, motif 5 had the largest number of amino acid sequences and was present in groups 1, 2b, and 2c, whereas motif 3 had the smallest number of protein sequences but was present in all groups/subgroups except group 3. Most of the LrWRKY proteins contained at least two motifs. LrWRKY members belonging to the same group were discovered to share analogous motif compositions; for instance, motif 4 was exceptional to group 1 members. Groups 2a, 2e, and 2d contained three motifs (-1, -2, and -3) whereas group 2b and 2c contained four motifs (-1, -2, -3, and -5), except for LrWRKY1 and -2 which contained only motif 1. In addition, LrWRKY12 and -34 belonged to group 2b and 2c but did not contain motif 5. Group 3 contained motifs 1 and -2, except for LrWRKY43, which contained only motif 1. Comparisons between randomly selected AtWRKY members with their LrWRKY orthologs revealed that AtWRKY6, -14,

Group 1:	AtWRKY25N	NDGYWRKYGQKQVKSENPRSYFKT---YPDVSKKIVETASDG-QITEI IYKGGINPKP
	LrWRKY22N	RDGYNWRKYGQKQVKSPQGSRSYYRT---YSESAKKIECSHTN-SVMEI IYRSQINDPP
	LrWRKY16N	EDGYNWRKYGQKQVKGSEFPRSYYKT---HPNFPVKKKVERSQEG-HITEI IYKGAINDPKP
	LrWRKY7N	DDGYNWRKYGQKQVKSENPRSYFKT---YFNPCTKKKVERSLDG-QITEI IYKGNINDPKP
	LrWRKY35N	XXGYNWRKYGQKQVKGSENPRSYFKT---FPNCP TKKKVERNLDG-HITEI IYKGSINDPKP
	LrWRKY40N	DDGYNWRKYGQKHVKGSEYPRSYYKT---HPNCPVKKKVERS LDG-QVTEI IYKGGINDLPP
	LrWRKY38N	CDGYKWRKYGQKVKSECPRSYYT-----
	LrWRKY5N	SDGYNWRKYGQKMKVASECPRSYYKT---HLKFPVKKKVERS IDG-HITEI IYKGHINDPLP
	AtWRKY25C	IDGFRWRKYGQKVVKGNTNPRSYFKT---FQGCVKKQVERSAADERAVLITTYEGRINDIP
	LrWRKY22C	SDGYRWRKYGQKMKVKGPHPRNYYRT---SAGCPVKKHIERATDGTSAITTYKGVINDDMP
	LrWRKY16C	DDGYRWRKYGQKVVKGNTNPRSYFKT---SAGCNVRKHVERASHDLKSVITTYEGRIND--
	LrWRKY7C	DDGYRWRKYGQKVVKGNTNPRSYFKT---SPGCPVKKHVERASQDIRSVITTYEGRINDVDP
	LrWRKY40C	DDGYRWRKYGQKVVKGNTNPRSYFKT---SQGCNVRKHVERAATDPKAVITTYEGRINDVDP
	LrWRKY5C	DDGFKWRKYGQKVVKGNTNPRSYFKT---YPGCNVRKHVERASADPKSVITTYEGRINDIP
LrWRKY8C	DDGYKWRKYGQKVVKGNTNPRSYFKT---NAGCPVKKHVERASHDPAVITTYEGRINDVDP	
LrWRKY11C	DDGYKWRKYGQKLVKGSQHPRSYFKT---YGGCNVRKQVERASTDPKAVITTYEGRINDIP	
LrWRKY32C	DDGYRWRKYGQKVVKGNTNPRSYFKT---FTGCPVKKHVERASHDLRAVITTYEGRINDVDP	
Group 2a:	AtWRKY40	KDGYQWRKYGQKVTRDNPSPRAYFKTA---CAPSSVKKKQVRSVEDQSVLVATYEGEIND-PM
	LrWRKY25	KDGYQWRKYGQKVTRDNPSPRAYFKTS---YAPTFPVKKKQVRSVEDQSILVATYEGEINDSKM
	LrWRKY19	KDGYHWRKYGQKVTRDNPFPRAYFKTS---FAPTFPVKKKQVRSVQDPSVLVATYEGEIND-LH
Group 2b:	AtWRKY6	SDGCQWRKYGQKMAKGNPCPRAYRT---MATGCPVKKQVQRCAEDRSILITTYEGNINDPLP
	LrWRKY1	NDGCQWRKYGQKIAKGNPCPRAY-----
	LrWRKY2	NDGCQWRKYGQKIAKGNPCPRAY-----
	LrWRKY24	NDGCQWRKYGQKISRGNPCPRSYFKS---VAPLCPVKKQVQRCLDMSVLITTYEGTINDSLP
	LrWRKY21	TDGCQWRKYGQKMAKGNPC- RAYYRT---MAAGCPVKKQVQRCAEDRTILITTYEGTINDPLP
LrWRKY34	NDGCQWRKYGQKIAKGNPCPRAYRT---CTVAPNCPVKKQVQRCAEDMSILITTYEGTINDPLP	
Group 2c:	AtWRKY51	DDGFKWRKYGKKSVKNNINKRNYYKS---SEGCSVKKRVERDGDAAAYVITTYEGVINDIESL
	LrWRKY33	DDGYKWRKYGKKVKSNNTYLNRNYYKS---SGDKVKKKVERDGTDSRYLITTYEGQINDHERP
	LrWRKY13	DDGYKWRKYGKKMVKNSFNPRNYYKS---VEGCPVKKRVERDKEDSRVITTYEGVINDS--
	LrWRKY36	DDGFKWRKYGKKMVKNSFNPRHYYKS---SGGCNVKKRVERDNADSSVIVITTYVGIINDIESP
	LrWRKY6	DDGYRWRKYGQKAVKNNNYPNPRSYFKT---QEGCNVKKQVQRSLKDEGVVVITTYEGMTINDPID
	LrWRKY3	EDGYRWRKYGQKAVKNSPYNPRSYFKT---NTKCTVKKRVERSEDSSIVITTYEGQINDHTV
	LrWRKY12	DDGYKWRKYGQKVVKNSLHPRSYFKT---HSNCRVKKRVERLSEDCRMVITTYEGRIND---
	LrWRKY18	EDGYRWRKYGQKAVKNSPYNPRSYFKT---SQKCPVKKKVERSYQDPSIVITTYEGQINDHLP
	LrWRKY20	EDGYRWRKYGQKAVKNSFNPRNYYRT---TQKCPVKKKVERSYEDPSIVITTYEGQINDHCP
Group 2d:	AtWRKY21	PDDYSWRKYGQKPIKGSPPRGGYYKS---SMRGPARKHVERCLEDPAMLIVTYEAEINDPKL
	LrWRKY14	PDEYSWRKYGQKPIKGSPPRGGYYKS---SMRGPARKHVERCLEEPSMLIVTYEAGEINDPRL
	LrWRKY27	GDEFYSWRKYGQKPIKGSPPRGGYYKS---SVRGPARKHVERAMDDPAMLIVTYEAGEINDLTQV
	LrWRKY29	ADEYSWRKYGQKPIKGSPPRGGYYKS---SVKGCPRKHVERATDDPGMLIVTYEAGEINDRYVQ
	LrWRKY28	PDDYSWRKYGQKPIKGSPPRGGYYKS---SVRGPARKHVERAFDDAAMLIVTYEAGEINDSHS
	LrWRKY31	PDDYSWRKYGQKPIKGSPPRGGYYKS---SVRGPARKHVERALDEPTMLIVTYEAGEINDSVS
	LrWRKY41	PDDYSWRKYGQKPIKGSPPRGGYYKS---SVRGPARKHVERAMDDPTMLIVTYEAGEINDSLS
Group 2e:	AtWRKY14	SDLWANWRKYGQKPIKGSPPRGGYYKS---SSKGC SARKQVERSRTPNMLVITYTSEINDPWP
	LrWRKY15	SDSWANWRKYGQKPIKGSPPRGGYYKS---SSKGC PARKQVERSRADPSMLVITYTSEINDPWP
	LrWRKY30	-DSWSWRKYGQKPIKGSPPRGGYYKS---SSKGC PARKQVERSRDPTLLITYTSEINDQIP
	LrWRKY39	SDLWANWRKYGQKPIKGSPPRGGYYKS---SSKGC SARKQVERSRTPNMLVITYTSEINDPWP
	LrWRKY37	SDTWSWRKYGQKPIKGSPPRGGYYKS---TSKGLARKQVERNRSDPKMFIVITYTAEINDPMP
	LrWRKY10	SDMWSWRKYGQKPIKGSPPRGGYYKS---TSKGLARKQVERNRSDPNMFIVITYTAEINDPMP
Group 3:	AtWRKY30	DDGFSWRKYGQKDLGAKFPRGGYYRTYRKSQGEATKQVQRSDENQMLLEISYRGIINDSQA
	LrWRKY4	DDGYSWRKYGQKLVKGSSEFPRSYYKT---YFNEVKKIFERSPDG-HITEI IYKGSINDPKP
	LrWRKY17	EDGYSWRKYGQKDLGANHPRAYYRTHRHTQGLATKQVQRSDGNSSIFEVTYKGRINDSKVA
	LrWRKY26	DDGYSWRKYGQKDLGAKYPRSYFKTYRAMQNWATKQVQRSDDDPALFEITYKGSINDENLA
	LrWRKY42	DDGYSWRKYGQKDLGAKYPRSYFKTYRHMQNWATKQVQRSDDDPTVFEITYYRGTINDHQA
	LrWRKY44	DDGCANWRKYGQKNILNSKYPRCYRSHKYDKE RATKQVQRIDEP IYRITYFDHINDV KCS
	LrWRKY43	DDGHANWRKYGQKQILLHATYPR-----FDFTIYPPFFNQE-
	LrWRKY23	DDGHANWRKYGQKQILLNAKYPRHYFRTHKFDQGEKANKQVQRIQENPPLFRITTYGHINDTKT
	LrWRKY9	DDGHVWRKYGQKQILLDAPFRSYFKTYKFDQGEQATKQEQRIQANPPFRFTMYQGHINDTTN

**Figure 2** Multiple sequence alignment of the 44 LrWRKY proteins and selected *A. thaliana* proteins. 'N' and 'C' indicate the N-terminal and C-terminal WRKY domains. The conserved WRKY amino acid signature and the amino acids forming the zinc-finger motifs are highlighted in yellow and red, missing amino acids are represented by dashes.

Full-size  DOI: 10.7717/peerj.10207/fig-2





**Figure 3** Phylogenetic relations and conserved protein motifs in *L. ruthenicum*. (A) The unrooted phylogenetic tree was constructed according to WRKY proteins sequences of *L. ruthenicum* with 1,000 bootstrap replicates. Details of groups are shown in different colors. (B) The motif composition of LrWRKY protein were identified using MEME. The different colored boxes represent different motifs and their position in each WRKY sequence. Each motif is indicated by a colored box indicated in the key at the bottom.

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**Table 2** Analysis and conserved motifs determination in *LrWRKY* proteins.

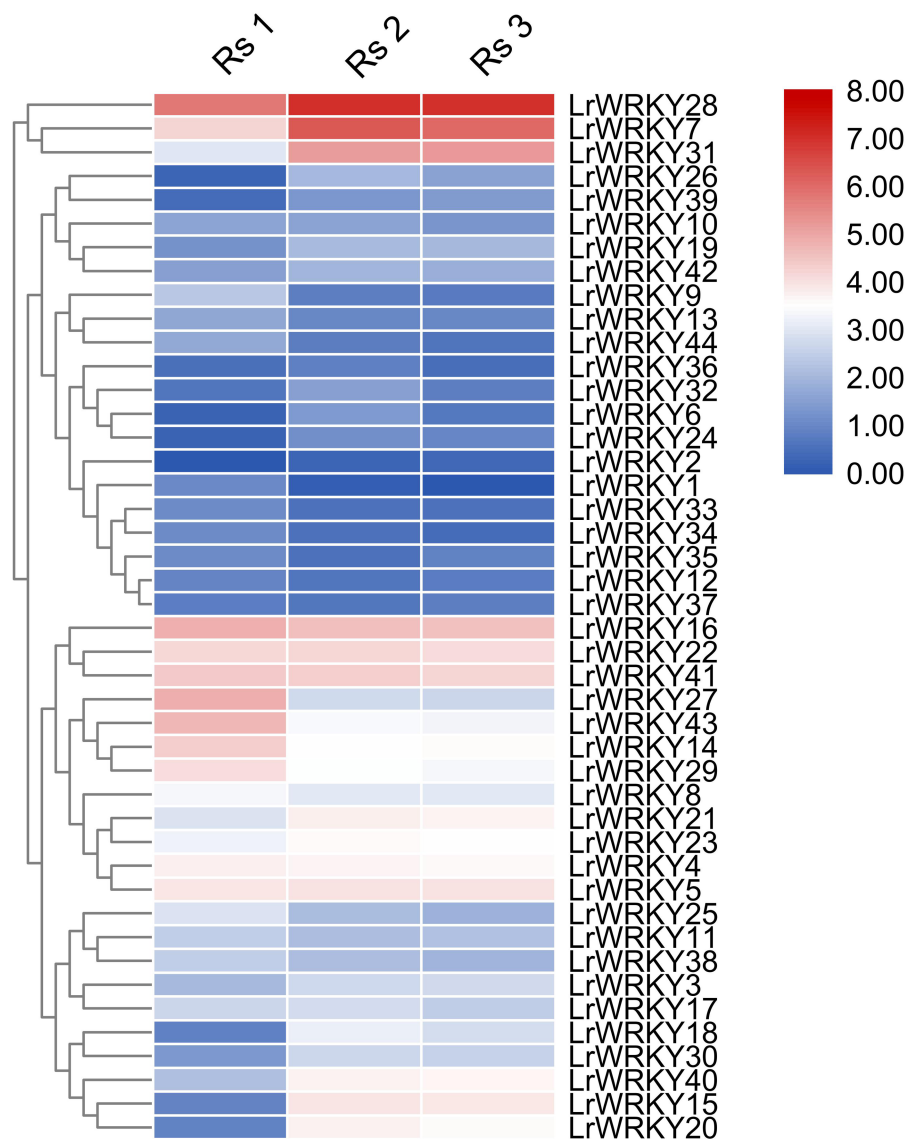
Motif	E value	Width	Motif sequence
1	8.9e-762	21	GYSWRKYGQKPVKGSPPYPRSY
2	2.9e-500	21	CPVRKQVERSEDPSMLITTY
3	5.8e-114	11	EGEHNHPVPAA
4	2.0e-112	21	KKVRKPRVAVRTRSEVDILDD
5	8.4e-104	41	DGYNWRKYGQKQVKGSEYPRSYKCTHPNCP VKKKVERSLD

-21, -25, -30, -40, and -51 shared the same motif compositions with their prospective *LrWRKY* group members (Data S7). Only *LrWRKY*1, -2, -38, and -43 motif compositions differed from the selected *AtWRKY* members.

### Expression analysis of *LrWRKYs* in different developmental stages of fruits

Expression profiles of the 44 *LrWRKY* genes were evaluated in three different fruit developmental stages (Rs 1, Rs 2, and Rs 3). The heatmap illustration of expression profiles of *LrWRKY* genes is shown in Fig. 4. The expression profiles revealed that all 44 *LrWRKYs* exhibited discrete expression during fruit development. Twenty-one *LrWRKYs* showed insignificant expression in all developmental stages of the fruit, and the FPKM values were <5 in all three stages. The remaining 23 members of *LrWRKYs* showed relatively higher expression, with FPKM >5 in at least one of the fruit developmental stages. Six *LrWRKY* genes exhibited markedly high expression patterns across all fruit developmental stages, with FPKM values >20. Interestingly, we also discovered that some *WRKY* genes classified in the same groups/subgroups had differential expression. For example, group 1, group 2d, *LrWRKY*3, and -20 from group 2c exhibited substantial expression with high FPKM values. However, group 2b members were weakly expressed in almost all developmental stages. The lowest FPKM value recorded in group 2d was 5.3, whereas some genes had FPKM values as high as 130.1 (Fig. 4, Data S4).

To gain a deeper knowledge of the gene expression patterns and to validate the *LrWRKY* genes, we performed RT-qPCR analysis of 23 selected genes. *LrWRKYs* exhibited different expression patterns in Rs 1, Rs 2, and Rs 3 (Fig. 5). Some *LrWRKYs* members maintained stable expression under different ripening stages, for example, *LrWRKY*3, -5, -17, -22, and -41. A total of nine *LrWRKYs* (-4, -8, -14, -23, -25, -27, -29, -40, and -43) decreased significantly from Rs 1 to Rs 3, among which *LrWRKY* 8, -23, and -27 maintained stable expression in Rs 2 and Rs 3. However, five *LrWRKYs* (-20, -21, -28, -30, and -31) continuously increased from Rs 1 to Rs 3, except for *LrWRKY* 30, which maintained stable expression in RS 1 and RS 2. In addition, three *LrWRKYs* increased first and peaked in RS 2, as compared with RS 1, and then decreased in RS 3.

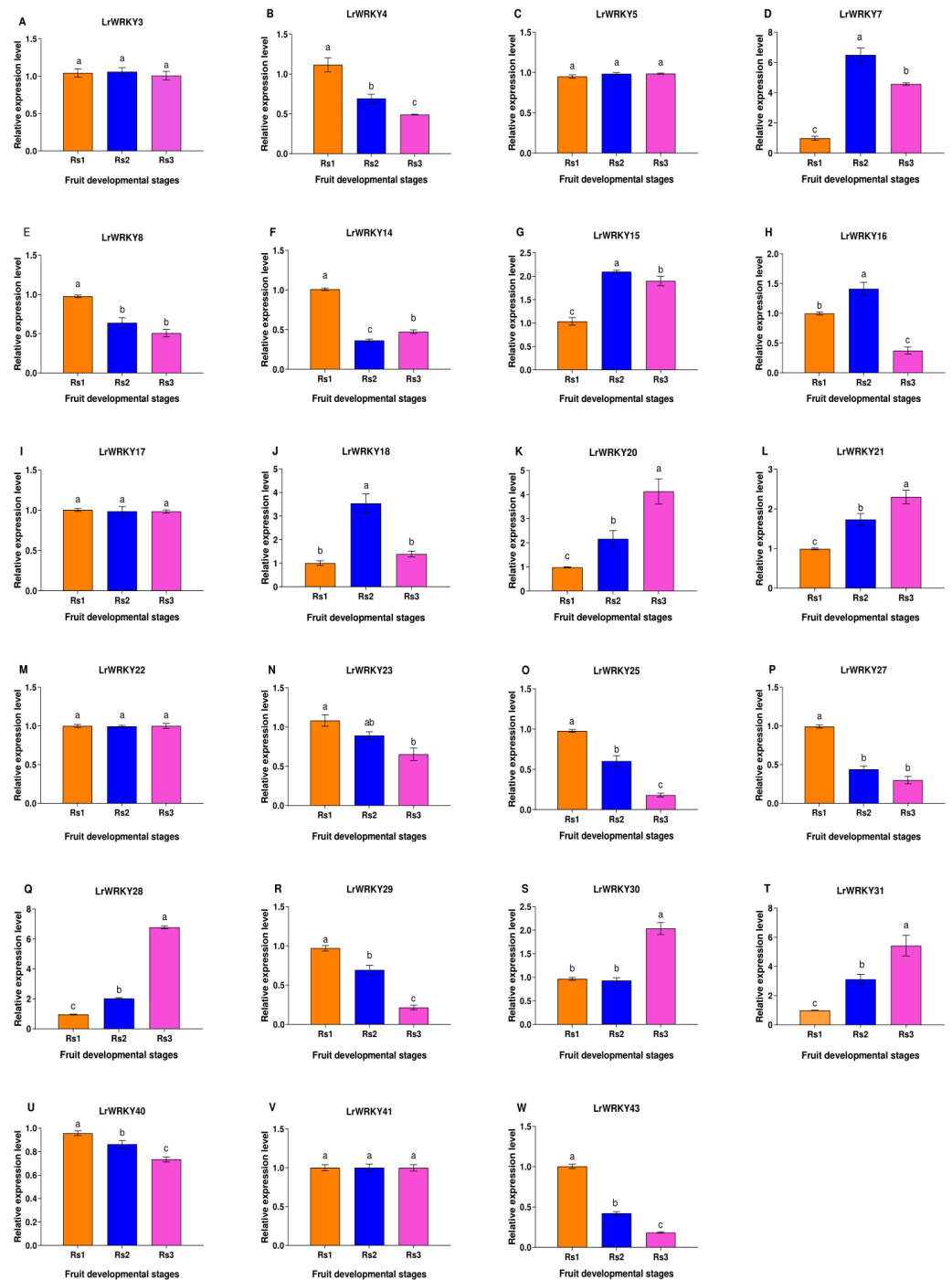


**Figure 4** Heatmap representation of the *LrWRKY* genes during fruit developmental stages of *L. ruthenicum*. The expression profiles of the 44 *LrWRKY* genes were based on FPKM-values. Red and blue boxes indicate high and low expression levels for each gene.

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## DISCUSSION

The WRKY gene family is one of the major families of TFs with crucial roles in various plant physiological processes (Huang *et al.*, 2012; Huang *et al.*, 2015; He *et al.*, 2012). Genome-wide analysis, including identification and expression profiling of WRKY genes in *V. vinifera* (Wang *et al.*, 2014), *F. vesca* (Zhou *et al.*, 2016), and *A. thaliana* (Miao *et al.*, 2004), has been broadly investigated. Because of technical limitations, the reference genome database of *L. ruthenicum* is not available. In this present study, the WRKY family analysis in *L. ruthenicum* was mainly based on transcriptome data, and a total of 73 possible WRKY



**Figure 5** RT-qPCR validation of twenty-three selected *LrWRKYs* expression levels during fruit developmental stages. The Rs1 represent green ripeness stage, Rs2 represent veraison ripeness stage, and Rs3 represent complete ripeness stage. The name of the gene is written on top of each bar diagram; (A) *LrWRKY3*, (B) *LrWRKY4*, (C) *LrWRKY5*, (D) *LrWRKY7*, (E) *LrWRKY8*, (F) *LrWRKY14*, (G) *LrWRKY15*, (H) *LrWRKY16*, (I) *LrWRKY17*, (J) *LrWRKY18*, (K) *LrWRKY20*, (L) *LrWRKY21*, (M) *LrWRKY22*, (N) *LrWRKY23*, (O) *LrWRKY25*, (P) *LrWRKY27*, (Q) *LrWRKY28*, (R) *LrWRKY29*, (S) *LrWRKY30*, (T) *LrWRKY31*, (U) *LrWRKY40*, (V) *LrWRKY41*, (W) *LrWRKY43*. The different letters indicate significant differences among developmental stages ( $p < 0.05$ ).

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gene members were identified. Forty-four *LrWRKY* genes with the conserved WRKY domain were identified. Our results provided the first systematically identified set of WRKY family members in *L. ruthenicum*.

After construction of the evolutionary tree and multiple sequence alignment based on *L. ruthenicum* WRKY conserved domains, we discovered that the 44 *LrWRKYs* could be categorized into three major groups, as described earlier in *A. thaliana* by [Eulgem et al. \(2000\)](#). The N-terminal and C-terminal WRKY domains in group 1 were assembled into different groups, thus potentially revealing separate evolution of the two domains. Previous studies have described group 2 WRKY genes as the group with the most members, for example, *A. thaliana* ([Eulgem et al., 2000](#)), *B. distachyon* ([Wen et al., 2014](#)), *Caragana intermedia* ([Wan et al., 2018](#)), and *M. esculenta* ([Wei et al., 2016](#)). Our study also revealed that group 2 *LrWRKY* genes had the highest proportion of members (59%), thus suggesting the activity of group 2 during the evolutionary processes and indicating that this group might have regulatory functions in fruit development and ripening. Group 3 showed the smallest number of *LrWRKY* genes (18%) among the three groups, possibly because of transcriptome data limitations or underestimation during our analysis. The WRKY TFs have multiple diverse functions in plant growth and various stress responses, and WRKY genes within the same group or subgroup usually retain similar regulatory functions ([Chen et al., 2018a](#); [Chen et al., 2018b](#)). Group 1 WRKY genes have been reported to participate in a wide range of plant growth and development processes. For instance, *AtWRKY2*, -26, -34, and -44 in group 1 are involved in seed germination, post-germination growth, leaf senescence, and root hair growth in *Arabidopsis* ([Guan et al., 2014](#); [Li et al., 2017](#); [Gonzalez et al., 2016](#)), and they share a similar homologous relationship with their *L. ruthenicum* counterparts (*LrWRKY4*, -5, -7, -8, -11 -16, -22, -32, -35, -38, and -40), thus suggesting related developmental roles among the genes. Most of the group 2a subfamily genes are involved in stress signaling, and group 2a genes in *L. ruthenicum* may share similar functions. Some members of group 2c (*AtWRKY10*, -28, -57, -75, and *OsWRKY72*) in *A. thaliana* and *O. sativa* have been reported to be involved in salt stress, flowering, seed development, and coloration ([Chen et al., 2018a](#); [Chen et al., 2018b](#)); these members have homologous relationships with *LrWRKY* (-3, -6, -12, -13, -18, -20, -33, and -36) in group 2c. In addition, *ZjWRKY26*; *VvWRKY14*, -19, -52; and *FvWRKY4*, -46, and -48, belonging to group 2c, have been reported to be mainly involved in the developmental process of fruits in *Z. jujube* ([Xue et al., 2019](#)), *V. vinifera* ([Wang et al., 2014](#)), and *F. vesca* ([Zhou et al., 2016](#)) respectively. Similarly, the group 2d subfamily genes are involved in the regulation of growth and development. Among the WRKY groups, group 3 genes are considered to have evolved the most, and they appear to play a crucial role in plant adaptation and development ([Tao et al., 2018](#)). In *A. thaliana*, group 3 members (*AtWRKY46*, -54, and -70) are involved in brassinosteroid signaling and regulate both osmotic stress and plant development ([Chen et al., 2017](#)). Thus, the specific functions of WRKYs might provide clues regarding the same homologs in *LrWRKYs* for further functional identification.

Nevertheless, the conserved heptapeptide WRKY domain WRKYGQK is the most common characteristic of WRKY proteins, although this sequence is sometimes substituted by WRKYGKK through mutation ([Rushon et al., 2010](#)). Our analysis uncovered

substitution of some WRKYGQK sequences (*LrWRKY13*, -33, and -36) in group 2c with a WRKYGKK sequence, a result similar to findings describing the substitution of glutamine (Q) by lysine (K) in *A. thaliana* (*AtWRKY51*, -52, and -59) (Eulgem et al., 2000), *C. intermedia* (*CiWRKY2*, -41, -50, and -51) (Wan et al., 2018), and *Panicum miliaceum* (*PmWRKY2*, -15, -23, -24, and -28) (Yue et al., 2016). According to Van Verk et al. (2008), the deviation of the WRKYGQK motif might affect DNA binding activity; therefore, *LrWRKY13*, -33, and -36 genes are recommended for further investigation in studies of their functions and binding specificities. This study also established that the C<sub>2</sub>HC zinc-finger was present in only group 3 WRKY domains, whereas C<sub>2</sub>H<sub>2</sub> was found in group 1 and group 2 domains; this result confirmed similar reports in *A. comosus* (Tao et al., 2011), *C. sativus* (Ling et al., 2011), and *A. thaliana* (Eulgem et al., 2000). However, this finding differs from those from an earlier report in *O. sativa* (Wu et al., 2005), indicating that the zinc-finger structure C<sub>2</sub>HC and C<sub>2</sub>H<sub>2</sub> concomitantly present in the N-terminus in group 1N. Group 1 proteins usually contain two conserved WRKY domains, but *LrWRKY8*, -11, -32, -35, and -38 group 1 proteins with a single conserved WRKY domain. This finding may be a result of the limitations of the transcriptome data.

The analysis of motif composition revealed that *LrWRKYs* share a similar motif composition within the same group or subgroup. Of note, *AtWRKY6* -14 -21 -25 -30 -40, and -51 share similar motifs (Data S7) with their *L. ruthenicum* WRKY orthologs, thus presenting clues regarding their regulatory functions. Although the functions of the major motifs in *LrWRKYs* remain undefined, *LrWRKYs* with the same conserved motifs may have similar functions. The presence of all five motifs identified in *LrWRKYs* confirms their conserved nature in this study.

Many studies have shown that WRKY function in regulating plant growth and development, while also playing essential roles in plant adaptability to stress (Chen et al., 2018a; Chen et al., 2018b). *L. ruthenicum* is widely useful as an economically important shrub with fruits rich in anthocyanins. Because of the potential roles of WRKY genes in the developmental stages of fruit in *L. ruthenicum*, we analyzed the expression patterns of *LrWRKY* genes, on the basis of the available transcriptome data, and performed RT-qPCR validation. We identified 44 *LrWRKYs* showing different expression patterns in the heatmap and inferred their different roles in controlling fruit development. Some *LrWRKY* genes, for instance, *LrWRKYs* 3 -5 -17 -22, and -41, maintained stable expression under different developmental stages. Additionally, the expression of *LrWRKYs* -4 -8 -14 -23 -25 -27 -29 -40, and -43 decreased significantly during fruit development, similarly to observations in *RcWRKY21* and -32 in *Ricinus communis* (Li et al., 2012). Moreover, *LrWRKYs* -20 -21 -28 -30, and -31 showed continuous significant increases in expression from green ripening to complete ripening during fruit development. Comparatively, *AtWRKY10* and -44, and *OsWRKY7* (in *A. thaliana* and *O. sativa*, respectively) are also highly expressed during seed/fruit development, biosynthesis of proanthocyanidins, and seed pigmentation (Zhang & Feng, 2014; Gonzalez et al., 2016; Luo et al., 2005), thus implicating similar functions in *L. ruthenicum*. In addition, *Z. jujube* *WRKY8* -26 -47, and -48 and *F. vesca* *WRKY4* -46, and -48 are expressed significantly in fruits, and their expression increases continuously from younger fruits to mature fruits (Zhou et al., 2016; Wang et al., 2014). Finally, four

*LrWRKYs* (-7 -15 -16, and -18) increased first in Rs2 and then decreased in Rs3; likewise, in seed development in *Jatropha curcas*, three genes (*JcWRKY14* -22, and -30) are also expressed at higher levels in the maturation stage (S2) than in the early development stage (Xiong et al., 2013), thus suggesting a putative role in regulating fruit development in *L. ruthenicum*. Nevertheless, more in-depth study is necessary to comprehensively verify the functions of all the *LrWRKY* genes.

## CONCLUSIONS

In the present study, a total of 73 WRKY members were identified in *L. ruthenicum* according to the transcriptome data. Forty-four proteins were identified with the WRKY domain and, on the basis of their amino acid sequences, were divided into three major groups with several subgroups, in accordance with those in other plant species. All 44 *LrWRKY* proteins contained one or two conserved WRKY domains and a zinc-finger structure. The conserved motif prediction revealed that the WRKY DNA-binding domain has been conserved in *L. ruthenicum* proteins. Phylogenetic and gene expression analysis provided in-depth knowledge of the important functions of WRKYs during fruit development and ripening. These results may serve as a foundation enabling further genomic and functional analysis of the WRKY family in *L. ruthenicum*.

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### Competing Interests

The authors declare there are no competing interests.

## Author Contributions

- Richard John Tiika and Huirong Duan conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Jia Wei conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Rui Ma performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Hongshan Yang performed the experiments, prepared figures and/or tables, and approved the final draft.
- Guangxin Cui conceived and designed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Yanjun Ma conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.

## Data Availability

The following information was supplied regarding data availability:

Raw data is available in the [Supplemental Files](#).

## Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.10207#supplemental-information>.

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