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Phylogenetic Analyses of Four Chinese Endemic Wheat Landraces Based on Two Single Copy Genes

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Chinese endemic wheat landraces possess unique morphological features and desirable traits, useful for wheat breeding. It is important to clarify the relationship among these landraces. In this study, 21 accessions of the four Chinese endemic wheat landrace species were investigated using single-copy genes encoding plastid Acetyl-CoA carboxylase (*Acc-1*) and 3-phosphoglycerate kinase (*Pgk-1*) in order to estimate their phylogenetic relationship. Phylogenetic trees were constructed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian, and TCS network and gene flow values. The A and B genome sequences from the *Pgk-1* loci indicated that three accessions of *Triticum petropavlovskyi* were clustered into the same subclade, and the *T. aestivum* ssp. *tibetanum* and the Sichuan white wheat accessions were grouped into a separate subclade. Based on the *Acc-1* gene, *T. aestivum* ssp. *tibetanum* and *T. aestivum* ssp. *yunnanense* were grouped into one subclade in the A genome; the B genome from *T. petropavlovskyi* and *T. aestivum* ssp. *tibetanum*, and the Sichuan white wheat complex and *T. aestivum* ssp. *tibetanum* were grouped in the same clades. The D genome of *T. aestivum* ssp. *yunnanense* clustered with *T. petropavlovskyi*. Our findings suggested that (1) *T. petropavlovskyi* is distantly related to the Sichuan white wheat complex; (2) *T. petropavlovskyi*, *T. aestivum* ssp. *tibetanum* and *T. aestivum* ssp. *yunnanense* are closely related; (3) *T. aestivum* ssp. *tibetanum* is closely related to *T. aestivum* ssp. *yunnanense* and the Sichuan white wheat complex; and (4) *T. aestivum* ssp. *tibetanum* may be an ancestor of Chinese endemic wheat landraces.

Keywords: *Acc-1*, *Pgk-1*, Chinese endemic wheat landraces, phylogenetic relationships

Introduction

Landraces cultivated around the world in the past are important genetic resources (Harlan 1975). China was proposed as a center of diversity for wheat (Yen et al. 1988). Four unique Chinese wheat landrace species described as Xinjiang rice wheat (*Triticum petropavlovskyi* Udacz. et Migusch.), Tibetan weedrace (*T. aestivum* ssp. *tibetanum* Shao), Yunnan hulled wheat (*T. aestivum* ssp. *yunnanense* King) and Sichuan white wheat com-

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plex (*T. aestivum* L.) were discovered in Yunnan, Tibet, the Xinjiang Autonomous Region, and Sichuan, respectively (Dong and Zheng 2000). Cytological studies suggested that the Chinese endemic wheat landraces harbor primitive and stable AABBDD chromosomal constitutions (Shao et al. 1980; Dong et al. 1981; Chen et al. 1988; Huang et al. 1989; Chen et al. 1991), and possess unique morphological features, such as spike fragility and glume tenacity. These four unique wheat landrace species also possess desirable traits for wheat breeding, such as resistance to preharvest sprouting and tolerance to cold or heat (Chen et al. 1988; Dong et al. 1981; Shao et al. 1980). Thus, it is important to investigate the phylogenetic relationships of the four unique taxa as primary gene pools and their potentially useful traits for common wheat improvement.

Numerous studies have been conducted to elucidate the phylogenetic relationships among the four groups. Based on morphology and geographical distribution, *T. petropavlovskyi* was considered to be different from the other three landrace groups (Shao et al. 1980; Yen et al. 1988). In RFLP-based genetic analysis, *T. petropavlovskyi* was distinct from the other three Chinese landrace groups (Ward et al. 1998). *T. aestivum* ssp. *tibetanum* has a naturally broken rachis with wedge type disarticulation (Shao et al. 1980; Tsunewaki et al. 1990), the same as *T. aestivu* ssp. *yunnanense* (Shao et al. 1980; Tsunewaki et al. 1990). The Sichuan white wheat complex is composed of cultivated common wheats characterized by multifloret spikelets and rounded glumes (Yen et al. 1988). Based on distribution, morphological traits, and RFLP clustering, *T. aestivum* ssp. *tibetanum* was considered the ancestor of *T. aestivum* ssp. *yunnanense* and the Sichuan white wheat complex (Ward et al. 1998).

Despite intensive research the relationships of the four Chinese endemic landrace species remain uncertain, and few attempts have been made to study the phylogenetic relationships based on DNA sequences. The plastid acetyl-CoA carboxylase (ACCase) (*Acc-1*) and the plastid 3-phosphoglycerate kinase (PGK) (*Pgk-1*) genes are single-copy nuclear genes that are less susceptible to coevolution (Sang 2002; Smith et al. 2006; Chalupska et al. 2008), and therefore, useful in phylogenetic studies (Huang et al. 2002a,b; Fan et al. 2007; Golovnina et al. 2007; Yan and Sun 2011; Fan et al. 2012).

In the present research, we sequenced and analyzed the single copy *Pgk-1* and *Acc-1* genes, in 21 accessions representing the four endemic wheat landraces, to elucidate their phylogenetic relationships.

Materials and methods

Plant materials

The materials used in this study included 21 accessions of Chinese endemic wheat landrace species (Table S1*). The *Acc-1* sequences of accessions with EU numbers were obtained from previously reported data (Kang et al. 2010). The *Pgk-1* sequences of accessions with JQ numbers were obtained from our published data (Chen et al. 2013). The rest of the sequences were initially obtained in this study.

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

DNA amplification, cloning, and sequencing

DNA extraction followed a standard CTAB protocol (Doyle and Doyle 1987). The single-copy gene *Pgk-1* was amplified by polymerase chain reaction (PCR) using the primer pair PgkF1 and PgkF1 (Huang et al. 2002ab). Primers AccF1 and AccF2 (Fan et al. 2009) were used to amplify *Acc-1*.

PCR products were cloned into the pMD19-T vector (Takara, Dalian) following the manufacturer's instructions. Cloning of PCR amplifications from a single copy gene in an allopolyploid species should enable isolation of homoeologous sequences from each genome (Doyle and Doyle 1999; Fan et al. 2009). The cloned PCR products were sequenced by the Beijing Genomics Institute.

Phylogenetic reconstruction

Multiple sequence alignments of *Pgk-1* and *Acc-1* were conducted using Clustal X (Thompson et al. 1999), with manual adjustment. To reduce the size of the matrices and possible effects of PCR artifacts (Cronn et al. 2002), unique substitutions in single clones were ignored. Sequences generated in this study were submitted to GenBank and the accession numbers are provided in Table S1.

The sequence statistics, including nucleotide substitutions and transition/transversion (TS/TV) ratio were calculated using MEGA 6 (Tamura et al. 2011; Tamura et al. 2013). Nucleotide diversity, gene flow and genetic differentiation (G_{ST}) in each accession were analyzed using DnaSP (Librado and Rozas 2009). Tests of neutrality were performed as described by Tajima (1989).

Phylogenetic analysis using maximum parismony (MP) was performed for *Pgk-1* using the computer program PAUP* beta version 4.0b10 (Swofford D L, Sinauer Associates, http://www.sinauer.com). All characters were specified as unweighted and unordered, and gaps were excluded from the analyses. The most parsimonious trees were obtained by performing a heuristic search using the Tree Bisection-Reconnection (TBR) option with MulTrees, and 10 replications of random addition sequences with the stepwise addition option. Multiple parsimonious trees were combined to form a strict consensus tree. The overall character congruence was estimated by consistency index (CI), and retention index (RI). To infer the robustness of clades, bootstrap values with 1,000 replications (Felsenstein 1985) were calculated by performing a heuristic search using the TBR option with Multrees.

Maximum likelihood (ML) and Bayesian analyses were performed for *Pgk-1* and *Acc-1* using PhyML v3.0 for ML (Guindon et al. 2010; Guindon and Gascuel 2003) and MrBayes v3.2.2 for Bayesian (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), respectively. The nucleotide substitution models used in the ML method were estimated by Modeltest v3.7 using the Akaike Information Criterion (AIC) (Posada and Crandall 1998). The HKY + I + G model produced the highest log-likelihood value for *Acc-1* data, and the best-fit model was identified as GTR + G for *Pgk-1* data. ML heuristic searches were performed with 100 random addition sequence replications and TBR

branch swapping algorithm. The robustness of the trees was estimated by bootstrap supports (BS) (Felsenstein 1985).

For Bayesian analysis, MrModeltest v2.3 was used to test different models of sequence evolution, and the HKY + I + G model was chosen by AIC for *Acc-1* and GTR+G for *Pgk-1*. Analyses were performed with 2,000,000 generations of one cold and three heated Markov Chain Monte Carlo chains, sampling trees every 100 generations. The first 500 trees were discarded as "burn-in" and the remaining trees were used to construct the 50% majority rule consensus tree. Statistical confidence in nodes was evaluated by posterior probabilities (PP).

TCS analyses

The Templeton, Crandall, Sing (TCS) reconstruction method is an effective method to reveal specific progenitor descendant relationships in perennial *Triticeae* (Chen et al. 2013; Luo et al. 2012; Yan and Sun 2011), and thus it was employed to elucidate the number of halotypes of the *Acc-1* and *Pgk-1* sequences, and their relatedness. Before reconstructing the TCS networks, a test of recombination was performed using the Phi (pairwise homoplasy index) method with Splits Tree (Huson and Bryant 2006). Building upon this test, the *Acc-1* (P = 0.9019) and *Pgk-1* (P = 0.9838) sequences were used to generate the TCS networks. The TCS haplotype network was tested to evaluate possible genetic relationships between haplotypes with the computer program TCS 1.2.1 (Clement et al. 2000).

Results

Sequence characteristics

The characteristics of the *Acc-1* gene are shown in Table S2. Complete alignment of *Acc-1* sequences confirmed a 46 bp insertion/deletion (indel) at position 1160–1207. This 46 bp deletion occurred in *Acc-1* alleles in A and D genomes in all accessions.

The characteristics of the *Pgk-1* gene are shown in Table S2. Many gaps resulting from indels were found in alignment of the *Pgk-1* sequence data. In particular, apart from single nucleotide substitutions and deletions, two insertions and one deletion occurred in the A genome. Firstly, a 6 bp insertion (TCCACT) was found at position 63-70, and a 2 bp TA insertion was present at position 487-490. In addition, a 4 bp deletion (AACC) occurred at the position 569-574 in the A genome.

Gene flow and genetic differentiation (G_{ST}) of the four endemic wheat landrace species

Based on the *Acc-1* gene, the G_{ST} values among *T. aestivum* ssp. *tibetanum*, *T. aestivum* ssp. *yunnanense* and *T. petropavlovskyi* were less than zero. The G_{ST} values between the Sichuan white wheat complex and each of the other three landrace groups were greater than zero (Table S3). Moreover, the G_{ST} values for *Pgk-1* sequences among the four groups were greater than zero (Table S3).

Phylogenetic analyses of Acc-1 sequences

The aligned *Acc-1* sequences contained 143 variable sites, of which 23 were parsimony-informative. ML analysis resulted in a single phylogenetic tree (-Lnlikelihood = 3039.30) with assumed nucleotide frequencies A: 0.2438, C: 0.1936, G: 0.2200, and T: 0.3427, and gamma shape parameter = 0.8410. Bayesian analysis inferred the same topology as the ML analysis. The tree illustrated in Fig. S1 is the ML tree with BS above and PP below branches.

Phylogenetic analyses clearly separated the sequences of the *Acc-1* gene into two major clades, i.e., Clades I and II. Clade I included sequences of the A and B genomes, and Clade II included genome D. In Clade I, A genome-specific sequences from *T. petropavlovskyi* accessions (except AS362), *T. aestivum* ssp. *tibetanum*, *T. aestivum* ssp. *yunnanense* (except AS311), and the Sichuan white wheat complex (*T. aestivum* cv. Chinese Spring and *T. aestivum* cv. J-11) formed a group with 99% PP support, and one *T. aestivum* ssp. *tibetanum* accession (AS1026) together with one accession of *T. aestivum* ssp. *yunnanense* (AS343) formed a subclade. The sequences from the B genome mapped one major subclade, with 54% BS and 100% PP. In this subclade, one accession of *T. aestivum* ssp. *tibetanum* (AS330) and *T. aestivum* cv. J-11 were grouped into the same subclade (97% PP). In Clade II, two *T. aestivum* ssp. *yunnanense* accessions (AS331 and AS332) together with three accessions of *T. petropavlovskyi* (AS356, AS358, and AS360) were placed in the same group.

Phylogenetic analyses of Pgk-1 sequences

Maximum parismony analysis using *Hordeum vulgare* as the outgroup was conducted (CI = 0.6935; RI = 0.9718). A separate ML analysis using the GTR + G model resulted in a single tree with a mean log-likelihood value of –3159.59, and assumed nucleotide frequencies A: 0.2592, C: 0.1967, G: 0.2375, and T: 0.3067, and gamma shape parameter = 0.4490. The tree topologies were almost identical in both ML and Bayesian trees, and were similar to those generated by MP with minor differences. One of the most parsimonious trees with BS (above) and Bayesian PP (below) is shown in Fig. S2.

Phylogenetic analyses clearly separated sequences (A, B, and D genomes) into Clades I and II. Clade I contained all of the A genome sequences, except for *T. aestivum* cv. Chinese Spring, which clustered into one subclade with high statistical support (98% BS and 100% PP) (subclade A). Within Subclade A, three accessions of *T. petropavlovskyi* (AS356, AS358, and AS363) formed a group with 92% BS and 96% PP, and *T. aestivum* cv. J-11 and *T. aestivum* ssp. *tibetanum* (AS330) grouped into the same group with 80% BS. Clade II included the B and D genome sequences. Two subclades (subclades B and D) with high statistical support were included in this clade. Subclade B included all the B genome sequences, among which *T. aestivum* cv. Chinese Spring, *T. aestivum* cv. J-11 and *T. aestivum* ssp. *tibetanum* (AS1027) clustered into the same clade with 79% BS; and three *T. petropavlovskyi* (AS358, AS359 and AS360) formed a group with 95% BS and 100% PP. Subclade D consisted of sequences from the D genome with no clustering.

TCS analyses

The TCS procedure used to analyze haplotype relationships among the *Triticum* accessions in this study (Sun et al. 2009) defined a 95% parismony connection limit of 13 steps for exon alignment of the *Acc-1* (Fig. S3) and *Pgk-1* genes (Fig. S4). The TCS network consisted of three major haplotype groups corresponding to the A, B, and D genomes (Fig. S3 and S4). The length of the branches between two nodes was meaningless here and each one implied one nucleotide difference. There were several differences among the TCS haplotype networks, the ML tree for *Acc-1*, and the Bayesian tree for *Pgk-1*. Figure S3 shows that the difference in the A genome haplotype network was that *T. petropavlovskyi* (AS362) exhibited a similar haplotype to *T. aestivum* ssp. *yunnanense* (AS331). Two differences between the TCS and ML tree of the B genome were observed. Firstly, *T. petropavlovskyi* (AS362) connected with *T. aestivum* ssp. *yunnanense* (AS343); secondly, the *T. aestivum* cv. J-11 and *T. aestivum* ssp. *tibetanum* (AS330) group appeared to be related to the group including *T. petropavlovskyi* (AS359) and *T. aestivum* ssp. *tibetanum* (AS1026). The network of the D genome was similar to the ML tree that included two *T. petropavlovskyi* accessions (AS356 and AS360) and had a similar haplotype to *T. aestivum* ssp. *yunnanense* (AS332).

In Figure S4, haplotype groups (A, B, and D) were similar in the Bayesian tree from the *Pgk-1* sequences. The network of the A genome indicated a much closer relationship between *T. aestivum* cv. J-11 and *T. aestivum* ssp. *tibetanum*, similar to the Bayesian analysis. However, a group including three accessions of *T. petropavlovskyi* (AS356, AS358, and AS363) was unavailable in the haplotype network of the A genome. For the B genome, the haplotype of *T. aestivum* cv. Chinese Spring, *T. aestivum* cv. J-11, and *T. aestivum* ssp. *tibetanum* (AS1027) appeared to be related in the phylogenetic tree. The difference between the Bayesian analysis and TCS was a cluster with three *T. petropavlovskyi* (AS358, AS359, and AS360), which was not detected in the TCS network.

Discussion

Genetic diversity of four Chinese endemic wheat landrace species

Several studies of genetic diversity among Chinese endemic wheat landrace sepcies have been reported. Wei et al. (2001, 2002) analyzed genetic diversity using A-PAGE, SDS-PAGE, STS-PCR, and SSR markers in 32 accessions of unique Chinese endemic wheat landrace species. The results indicated higher genetic diversity for *T. petropavlovskyi* and *T. aestivum* ssp. *tibetanum* than for *T. aestivum* ssp. *yunnanense*. Based on RFLP analysis, Ward et al. (1998) found higher genetic diversities for *T. petropavlovskyi* and *T. aestivum* ssp. *tibetanum* than for *T. aestivum* ssp. *yunnanense*. However, analysis of an esterase isozyme revealed that *T. aestivum* ssp. *yunnanense* had high genetic diversity (Cui and Ma 1991). Akond et al. (2005) indicated that genetic polymorphism of *T. petropavlovskyi* was low. Furthermore, Wang et al. (2007) considered that *T. petropavlovskyi* had relatively low SSR variation. In the present study, many indels (nucleotide substitutions, insertions and deletions) were detected in the *Acc-1* and *Pgk-1* sequences among the four

Chinese endemic wheat landraces, confirming high nucleotide diversity. The different results concerning genetic diversity of Chinese endemic wheat landrace species might be influenced by sample size, different accessions, and different types of molecular markers used in each study.

Relationships between T. petropavlovskyi and the other three Chinese endemic wheat landrace species

Based on morphological traits, chromosome pairing behavior, and geographical trait, *T. petropavlovskyi* was considered to be distinct from the other taxa (Shao et al. 1980; Chen et al. 1988; Yen et al. 1988). Previous studies indicated that differences between *T. petropavlovskyi* and the Sichuan white wheat complex were related to the B genome (Yao et al. 1983; Chen et al. 1985; Chen et al. 1988). Based on HMW-glutenin and gliadin variations, Wei et al. (2002) suggested that *Glu-B1* differed between *T. petropavlovskyi* and the Sichuan white wheat. Furthermore, using RFLP, Ward et al. (1998) inferred that *T. petropavlovskyi* differed from the other Chinese wheat landraces. The results of SSR and EST-SSR markers indicated that *T. petropavlovskyi* was significantly different from the other three landrace groups in the A, B, and D genomes (Yang et al. 2005).

In the present study, *Pgk-1* sequences of the A and B genomes of *T. petropavlovskyi* clustered into the same subclade, indicating that *T. petropavlovskyi* is distantly related to the other three groups. However, the phylogenetic tree of *Acc-1* showed that *T. petropavlovskyi* was clustered with *T. aestivum* ssp. *tibetanum* and *T. aestivum* ssp. *yunnanense* in the B and D genomes, respectively. The results of G_{ST} among *T. petropavlovskyi*, *T. aestivum* ssp. *tibetanum* and *T. aestivum* ssp. *yunnanense* were less than zero indicating that these three landrace groups had a close genetic relationship. Overall, our results revealed a closer relationship among *T. petropavlovskyi*, *T. aestivum* ssp. *tibetanum* and *T. aestivum* ssp. *yunnanense* than between *T. petropavlovskyi* and the Sichuan white wheat complex.

Relationship among T. aestivum ssp. tibetanum, T. aestivum ssp. yunnanense, and the Sichuan white wheat complex

Because of semi-wild traits, geographical distribution, and morphological similarities, *T. aestivum* ssp. *tibetanum* was presumed to be the ancestor of *T. aestivum* ssp. *yunnanense* and the Sichuan white wheat complex (Chen et al. 1988; Yen et al. 1988; Yang et al. 1992). RFLP analysis revealed that *T. aestivum* ssp. *tibetanum* was genetically close to *T. aestivum* ssp. *yunnanense* and the Sichuan white wheat complex (Ward et al. 1998). Cluster analysis based on genetic distance (*GD*) indicated a closer relationship between *T. aestivum* ssp. *tibetanum* and *T. aestivum* ssp. *yunnanense* (Wang et al. 2007).

In the present research, the G_{ST} values of the *Acc-1* and *Pgk-1* sequences between *T. aestivum* ssp. *yunnanense* and *T. aestivum* ssp. *tibetanum* were low, indicating a close genetic relationship. Phylogenetic analyses of the *Pgk-1* gene showed that *T. aestivum* ssp. *tibetanum* and the Sichuan white wheat complex were clustered in one group based on the A and B genomes. The phylogenetic tree of *Acc-1* showed that *T. aestivum* ssp.

yunnanense and *T. aestivum* ssp. *tibetanum* were grouped together in the A genome, and *T. aestivum* ssp. *tibetanum* and the Sichuan white wheat complex were grouped in the B genome, a result that was congruent with the TCS analysis. These results suggested that the relationships among *T. aestivum* ssp. *tibetanum*, *T. aestivum* ssp. *yunnanense* and the Sichuan white wheat complex are close.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1*. Plants used in this study

Electronic Supplementary *Table S2*. Characteristics derived from *Acc-1* and *Pgk-1* sequences

Electronic Supplementary *Figure S1*. Maximum-likelihood tree from the *Acc-1* gene of Chinese endemic wheat landraces. Numbers above nodes are bootstrap values ≥50%; numbers below nodes are posterior probability values ≥90%; Genome composition, species name and accession number/cultivar name are indicated for each taxon

Electronic Supplementary *Figure S2*. Maximum parismony tree from the *Pgk-1* gene of Chinese endemic wheat landraces. Numbers above nodes are bootstrap values ≥50%; numbers below nodes are posterior probability values ≥90%; Genome composition, species name and accession number/cultivar name are indicated for each taxon

Electronic Supplementary *Figure S3*. TCS network inferred from the *Acc-1* gene of Chinese endemic wheat landraces. Accession numbers of each species are listed in Table 1. Haplotypes in the network are represented by circles of different color corresponding to the different Chinese endemic wheat landraces; the small circle in the lineage chains represents SNP changes

Electronic Supplementary *Figure S4*. TCS network inferred from the *Pgk-1* gene of Chinese endemic wheat landraces. Accession numbers of each species are listed in Table 1. Haplotypes in the network are represented by circles of different color corresponding to the different Chinese endemic wheat landraces; the small circle in the lineage chains represents SNP changes