Molecular cytogenetic characterization of natural hybrids of Roegneria stricta and Roegneria turczaninovii (Triticeae: Poaceae)

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May 11, 2021

Abstract

Hybridization is an important part of species evolution. The hybrid progeny population had rich genetic and phenotypic variation, which made the boundaries between them and their parents blurred and difficult to distinguish. There was little research on the origin of natural hybrids of Triticeae. In this study, we found a large number of putative hybrids of Roegneria in West Sichuan Plateau, China. The hybrid plants showed strong heterosis in plant height, tiller number and floret number. Morphologically, the putative hybrids showed intermediate of Roegneria stricta Keng and Roegneria turczaninovii (Drob.) Nevski. Hybrids had 28 chromosomes corresponding to that of R. stricta and R. turczaninovii (2n=4x=28). Meiotic pairing in hybrids were less regular than those of R. stricta and R. turczaninovii. GISH analysis showed that the hybrid plants had the same genome as that of R. stricta and R. turczaninovii (StY). Phylogenetic analysis based on the single copy nuclear gene DMC1 and chloroplast gene rps16 showed the plants were closely related to R. stricta and R. turczaninovii. This study indicated that the plants were hybrids of R. stricta and R. turczaninovii. The results provided data for the utilization of hybrid. This study provided a case study of natural hybrids.

Molecularcytogenetic characterization of natural hybrids of *Roegneria*

***stricta* and *Roegneria turczaninovii* (Triticeae: Poaceae)**

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Abstract Hybridization is an important part of species evolution. The hybrid progeny population had rich genetic and phenotypic variation, which made the boundaries between them and their parents blurred and difficult to distinguish. There was little research on the origin of natural hybrids of Triticeae. In this study, we found a large number of putative hybrids of *Roegneria* in West Sichuan Plateau, China. The hybrid plants showed strong heterosis in plant height, tiller number and floret number. Morphologically, the putative hybrids showed intermediate of *Roegneria stricta* Keng and *Roegneriaturczaninovii* (Drob.) Nevski. Hybrids had 28 chromosomes corresponding to that of*R. stricta* and *R. tu* rczaninovii (2n=4x=28). Meiotic pairing in hybrids were less regular than those of *R. stricta* and*R. turczaninovii* . GISH analysis showed that the hybrid plants had the same genome as that of *R. stricta* and *R. turczaninovii*(**StY**). Phylogenetic analysis based on the single copy nuclear gene *DMC* 1 and chloroplast gene *rps* 16 showed the plants were closely related to *R* . *stricta* and *R. turczaninovii.* This study indicated that the plants were hybrids of*R. stricta*and*R. turczaninovi* i. The results provided data for the utilization of hybrid. This study provided a case study of natural hybrids.

KEYWORDS Natural hybrids, GISH, phylogenetic analysis, *Roegneria* , genome, meiotic pairing.

Cover letter

Dear editor,

The submitted is the following manuscript for your consideration to Ecology and Evolution: "**Molecular cytogenetic characterization of natural hybrids of *Roegneria stricta* and*Roegneria turczaninovii* (Triticeae: Poaceae)** ", by Chen Chen, Zi-Lue Zheng, Dan-Dan Wu, Tan Lu, Cai-Rong Yang, Song-Qing Liu, Jia-Le Lu, Yong-Hong Zhou, Chang-Bing Zhang, Hai-Qin Zhang.

In the present study, to explore the origin and formation mechanism of natural hybrids, we performed morphological analysis, Karyotype analysis, chromosome pairing analysis, genomic *in situ*hybridization and phylogenetic analyses. The results confirmed that natural hybrids showed strong heterosis in plant height, tiller number and floret number. Morphologically, the hybrids showed intermediate of*Roegneria stricta* Keng and *Roegneria turczaninovii* (Drob.) Nevski. Meiotic pairing in hybrids were less regular than those of*R. stricta* and *R. turczaninovii* . GISH analysis showed that the hybrid plants had the same genome as that of *R. stricta* and*R. turczaninovii* (**StY**). Phylogenetic analysis based on the single copy nuclear gene *DMC* 1 and chloroplast gene*rps* 16 showed the plants were closely related to *R. stricta*and *R. turczaninovii* . This study indicated that the plants were hybrids of *R. stricta* and *R. turczaninovii* .

The work described has not been submitted elsewhere for publication, in whole or in part, and all authors listed have approved the manuscript that enclosed.

We hope that you will kindly consider this submission for publication.

If you have any questions, please contact me freely.

Sincerely,

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1 | INTRODUCTION

The tribe Triticeae (Poaceae) represented an important gene pool for genetic improvement of cereal crops and forage grasses. It included approximately 450 species that distributed in a wide range of ecological habitats over the temperate and subtropical and tropic alpine regions (Dewey, 1984). The majority of species in Triticeae were allopolyploids, with ploidy levels ranging from diploid (2n=2x) to dodecaploid (2n=12x). Natural hybridization between different genera or species oftenoccurred in the Triticeae. As early as 1926, the natural hybrid of*Triticum* - *Aegilops* - *Secale* was reported (Von Tschermak & Bleier, 1926). Stebbins et al. reported a series of natural hybrids in the Triticeae: natural hybrids of *E. condensatus* and*E. triticoides* (Stebbins & Walters, 1949); triploid hybrids of*Agropyron* and *Elymus* (Stebbins & Singh, 1950); natural hybrids of *Elymus* and *Sitanion* (Stebbins & Vaarama, 1954). At the same time, they performed artificial hybridization, artificial and natural hybrids for morphology, chromosome pairing, seed set and other aspects of comparison, speculated that the possible origin of natural hybrids (Stebbins & Walters, 1949; Stebbins & Singh, 1950; Stebbins & Vaarama, 1954).

Roegneria C. Koch was a relatively large perennial genus in Triticeae, and includes approximately 130 species, most of which were tetraploid with **StY** -genome, nearly 70 of which were found in China (Yang et al., 2008). *Roegneria* species not only provided genetic material for the improvement of forage crops but could also be used as potential contributors of genes for cereal crops (Keng, 1959). Predecessors have reported some studies on the hybrids of *Roegneria* , such as a hybrid of *Roegneria* and *Hordeum* (Zhou et al., 1995), a hybrid of *R. ciliaris* and *Leymus multicaulis* (Zhang et al., 2008). These hybrids were created by the artificial hybridization and could not replace the value of natural hybrids. In recent years, researchers had discovered some natural hybrids of *Roegneria* . For example, Zeng et al. (2012) had discovered the pentaploid natural hybrids (**StStYYP**) between *Roegneria* (**StY**) and*Kengyilia* (**StYP**).

In the process of plant system evolution, hybridization was the direct cause of the formation of diploid and polyploid species and the production of reticulate evolution (Rieseberg, 1995; Soltis & Soltis, 1993). However, it was not easy to accurately identify whether a species was a hybrid and to explore origin of hybrids (Rauscher et al., 2002). Early identification of hybridization was mainly based on morphological characteristics, and it was often based on the morphology intermediate of the parents to infer whether a plant came from a hybridization. However, the reliability of morphological markers was low, and morphology intermediate was not always related to hybridization. It may also be caused by convergent evolution or environment. Therefore, morphological markers could not be used alone to identify hybrids (Rieseberg, 1995). Cytological markers have been used as important criteria for hybridization, including karyotype analysis, meiotic pairing analysis, Genomic *in situ* hybridization (GISH) and Fluorescence *in situ* hybridization (FISH), which could be used to identify and analyze natural hybrids (Han et al., 2004; Mao et al., 2017). For example, Using FISH and GISH techniques, the two parents of*Elytrigia* ×*mucronata* could be studied through signal sites on chromosomes (Paštová et al., 2019). However, due to the high parental chromosome homology of interspecific hybrids, it was difficult to explore origin of hybrids by FISH and GISH. Therefore, even if a species has been determined from morphology or cytogenetics to be a hybrid or hybrid origin, it still needs to be verified with some other evidence (Soltis et al., 1992). Phylogenetic analysis could not only reflect the genetic relationship between hybrids and parents, but also overcome the shortcomings of non-dominance and insufficient repetitiveness of other molecular markers. The method was also the first choice for identifying natural hybrids (Quijada et al., 1997; Sang et al., 1995). Since genes at different sites in the genome of diploid or polyploid hybrids derived from different parent species, these genes have different evolutionary processes. This was the basis for detecting hybridization by phylogenetic analysis (Yu et al., 2011). Single- or low-copy nuclear genes, which were less susceptible to concerted evolution, could serve as useful markers for studies of phylogenetic relationships (Lei et al., 2018; Sha et al., 2010). Among the available nuclear sequences, DNA meiotic recombinase 1 (*DMC* 1) gene sequences have been used to examine hybridization events (Tang et al., 2017). The chloroplast DNA (cp DNA) is maternally inherited in grasses (Smith et al., 2006). Among the available chloroplast sequences, ribosomal protein S16 (*rps* 16) were used to identify the maternal donor of genera in Triticeae (Yan et al., 2014).

The experimental field of Sichuan Academy of Grassland Science (SAGS) located on Northwest Sichuan Plateau, China (Hongyuan county, Sichuan Province, 31°51' to 33deg33' N, 101deg51' to 103deg22' W)

at altitude 3500 m. Two species of*Roegneria* [*Roegneria stricta* Keng and *Roegneriaturczaninovii* (Drob.) Nevski] were planted very close in SAGS. We harvested the seeds of the two species and then individual planting. After three years growing, we found that 23 putative hybrids (5.75%, 23/400) randomly distributed in *R. stricta* field and 54 putative hybrids (16.36%, 54/330) randomly distributed in *R. turczaninovii* field (Figures 2 a-c). These putative hybrids grew stronger than around plants and their seed set was very low (0.23%-5.59%, Figure 3). They showed intermediate morphological characters of *R. stricta* and *R. turczaninovii* , such as pubescence of leaf, basal leaf sheath and stem node (Figures 1 d-o).

Both of *R. stricta* and *R. turczaninovii* were tetraploid perennial species (2n=4x=28) with the **StY** genome. *R. stricta* come from Luhuo County, Sichuan, China while *R. turczaninovii* origins from Linxi County, Inner Mongolia, China. Because these two species had the same ploidy and genome constitutions, natural hybridizations might occur between them if they were grown together for a long time. In the current study, we hypothesized that the sterile plants were hybrids of *R. stricta* and *R. turczaninovii* . To determine if this is indeed the case, we conducted different methods including morphological analysis, fertility analysis, karyotype, meiotic pairing analysis, *in situ* hybridization and DNA sequence analysis in these putative hybrids and their accompanying plants. The results provided useful resources for origin and formation mechanism of natural hybrids, species evolution in the Triticeae, and laid material foundation for breeding new varieties.

2 | METHODS AND MATERIALS

2.1 | Plant materials

57 putative nature hybrids including 17 hybrids (named RH1) found in*R. stricta* field and 40 hybrids (named RH2) appeared in *R. turczaninovii* field were used in this study (Table 1). The possible parents *R. stricta* and *R. turczaninovii* , and the other Triticeae species growing nearby were also obtained, including species of *Roegneria* and *Elymus* . All of them were collected from the experimental field of SAGS, Hongyuan County, Sichuan, China. 20 diploid species (representing the genomes **St** ,**H** , $\mathbf{E^e}$,$\mathbf{E^b}$, **W** , **P** , **Ta** ,**V** , **Ns** , **A** , **B** , and **D**), and species with different genomic combinations (**StY** , **StH**) from the tribe Triticeae were used for DNA sequences analysis. The names of the sampled taxa, abbreviations, accession numbers, ploidy level, genomic constitution and GenBank accession numbers were listed in Table S1. Materials with PI and W_6 were kindly provided by American National Plant Germplasm System (Pullman, WA, USA). The authors of the present study collected all other accessions, for which voucher specimens were deposited with the perennial nursery and herbarium of the Triticeae Research Institute, Sichuan Agricultural University, China (SAUTI).

2.2 | Morphological analysis

Morphological analysis was performed on *R. stricta* , *R. turczaninovii* and putative hybrids. Agronomic traits were observed including plant height, tiller number, floret number, length of flag leaves, width of flag leaves, length of spikes, etc. Each trait was measured 10 times and the average was calculated. The pubescence of leaf, basal leaf sheath and stem node were recorded by direct observation.

2.3 | Pollen fertility and seed set

The pollens grains from mature anthers were stained in an I_2 -KI solution for pollen fertility study. Seed set was estimated from a 10-spike sample per plant.

2.4 | Karyotype and meiotic pairing analysis

Karyotype analysis were followed by Gill et al. (1991). The procedures of fixation, staining and calculation of meiotic pairing followed Zhang and Zhou (2006).

2.5 | Chromosome preparation and *in situ*hybridization

Chromosomes were prepared for GISH analysis according to the method of Han et al. (2004). Total genomic DNA was extracted from fresh leaves by the CTAB method (Murray & Thompson, 1980). Plasmids (from

positive clones that are **St** genome) and the **StY** genome were labeled with fluorescein-12-dUTP or Texas-red-5-dCTP using the nick translation method. Hybridization procedure, detection, and visualization were performed according to the method of Wang et al (2017).

2.6 | Amplification and sequencing

The *DMC* 1 and *rps* 16 gene was amplified using the primers listed in Table S1 (Petersen & Seberg 2002; Shaw et al., 2005). All PCRs were conducted in a 50-μL reaction volume, with 1.5 U ExTaq polymerase (TaKaRa, Shiga, Japan). The PCR amplification protocols for the *DMC* 1 and *rps* 16 gene are presented in Table S1. PCR products were cloned into the pMD19-T vector (TaKaRa). At least 15 random independent clones were selected for sequencing by Shanghai Sangon Biological Engineering and Technology Service Ltd. (Shanghai, China).

2.7 | Data analysis

The phylogenetic analyses of *DMC* 1 and *rps* 16 data by using the maximum-likelihood (ML) method in PhyML 3.0 (Guindon et al., 2009). The best-fit evolutionary model for phylogenetic analysis was determined using ModelTest v3.7 with Akaike information criterion (Posada & Crandall, 1998). As a measurement of the robustness of tree clades, the bootstrap support (BS) values were calculated with 1000 replications and displayed in figure (above the branch) if the BS values were >50% (Felsenstein, 1985).

In addition to ML analysis, Bayesian analyses were also performed using MrBayes 3.1 (Ronquist & Huelsenbeck, 2003). The evolutionary model selected for Bayesian analyses was same as ML analysis.

3 | RESULTS

3.1 | Morphological characteristics

The 57 putative natural hybrids were perennial grasses, which were similar in morphology and phenology to *Roegneria* species, such as one spikelet per node and palea equaling lemma. Most of hybrids were stronger than their surrounding plants (Figures 1a-c). These natural hybrids combined some unique characteristics of *R. stricta* and*R. turczaninovii* , such as leaf pubescence, stem node pubescence and basal leaf sheath pubescence (Figures 1d-o).

The height of hybrids RH1 (128.90 ± 1.27 cm) and hybrids RH2 (132.68 ± 1.86 cm) was higher than that of *R. turczaninovii* (118.63 ± 1.11 cm) and *R. stricta* (118.70 ± 1.54 cm) (Figure 2a). Hybrids RH1 (284.18 ± 12.24) and hybrids RH2 (389.63±13.72) showed more tillers than*R. turczaninovii* (112.00 ± 9.72) and *R. stricta* (225.93 ± 15.35) (Figure 2b). The hybrids showed intermediate character between*R. stricta* and *R. turczaninovii* in the length of flag leaves, width of flag leaves, length of top second leaf, width of top second leaf, length of awn, and length of the spike (Figures 2c-2g, 2i).

3.2 | Evaluation of pollen fertility and seed set

The fertility, including pollen fertility and seed set, of*R. stricta* , *R. turczaninovii* and putative hybrids were shown in Figure 3. In *R. stricta* , the pollen fertilities were up to 92.05% and the seed sets were 90.02%. In *R. turczaninovii* , the pollen fertilities and seed set were high with 91.61% and 92.18%, respectively.

As for the hybrids RH1, the pollen fertilities varied from 1.01%-8.09%, and the seed sets were lower than those of their possible parents, varying from 0.41%-4.50% (Figure 3). As for the hybrids RH2, the pollen fertilities varied from 0.83%-13.63%, and seed set were lower, varying from 0.23%-5.59% (Figure 3). It could be seen that the pollen fertilities and seed sets of putative hybrids were very low, indicating that they were hybrids and not stable species.

3.3 | Karyotype analysis and chromosome pairing at metaphase I

Karyotype analysis showed that *R. stricta* , *R. turczaninovii* and putative hybrids were tetraploids (2n = 4x = 28) (Figure 4). The meiotic configurations of the possible parent and the putative hybrids were listed in Table S2.

Meiosis of *R. stricta* and *R. turczaninovii* were quite regular with 14 bivalents (Figures 5a-c, Table S2). Meiotic pairing in 17 hybrids RH1 were comparatively high, with an average of 0.98 univalents and 13.52 bivalents per cell with c-value of 0.89 (Figures 5d, e; Table S2). Chromosome pairing in 40 hybrids RH2 were comparatively high with an average of 0.85 univalents and 13.55 bivalents per cell with c-value of 0.90 (Figures 5g, h; Table S2). Except for hybrid RH2-31, all hybrids had univalent. In all hybrids, the c-value was higher than 0.81, suggesting that chromosome pairing of the hybrids was comparatively regular and two sets of genomes in hybrids were homology.

At the same time, some lagging chromosomes and chromosome bridges were observed at anaphase I. (Figures 5f, i).

3.4 | FISH and GISH analysis

To further explore the genomic constitutions of natural hybrids, we selected some hybrids for *in situ* hybridization. St_2-80 was a FISH marker for the **St** genome (Wang, et al., 2017). Signals produced by St_2-80 were present on the entire arm of the **St** genome chromosomes, except at the centromeric region and near centromeric region (Wang et al., 2017). This marker was used to detect if the **St** genome presented in the putative parents and hybrids.

FISH results showed that 14 chromosomes of putative parents and hybrids displayed fluorescent signals indicating that they contained one set of **St** genome (Figures 6a, c, e and 7a, c, e). This result was confirmed by GISH analysis, where 28 chromosomes of putative parents and hybrids were hybridized with the**StY** probe from *R. ciliaris* (Figures 6b, d, f and 7b, d, f). The results of FISH and GISH indicated that the genomic constitution of putative parents and 11 hybrids (RH1-3, RH1-8, RH1-11, RH1-14, RH2-2, RH2-10, RH2-12, RH2-15, RH2-17, RH2-37, RH2-39) were **StY** .

3.5 | Phylogenetic analyses of the nuclear gene*DMC*1 and the chloroplast gene *rps*16 sequences

In order to analyze the possible parents of the hybrids, we analyzed the nuclear gene *DMC*1 and the chloroplast gene *rps*16 sequences of the hybrids and their associated species of *Roegneria* and*Elymus*. The length of *DMC*1 sequences of hybrids ranged from 998 to 1004bp. The data matrix contained 1166 characters, of which 267 characters were variable and 235 were parsimony informative. A single phylogenetic tree generating by maximum likelihood analysis using the TPM1uf + G model (-Ln likelihood = 4547.37) was shown in Figure 8. The phylogenetic analyses of the *DMC*1 sequence were shown in Figure 8. In clade I (BS=54%, PP=0.97), the **St**-type sequences formed a strongly supported clade, which included diploid*Pseudoroegneria* (**St**) species, tetraploid *Elymus*(**StH**) and *Roegneria* (**StY**) species and hybrids. The **St**-type sequences of 15 hybrids and *R. turczaninovii*(**StY**) formed a subclade (BS=51%, PP=1.00). In clade II (BS=99%, PP=1.00), the **Y**-type sequences formed a strongly supported clade, which contained the tetraploid species of*Roegneria* (**StY**) species and hybrids. The **Y**-type sequences of 15 hybrids, *R. turczaninovii* (**StY**) and*R. stricta* (**StY**) formed a subclade (BS=64%, PP=0.83). In clade III (BS=96%, PP=1.00), the H-type subclade included diploid*Hordeum* species and tetraploid *Elymus* (**StH**) species. Clade IV (BS=96%, PP=1.00). other clade included 10 diploid species containing 10 different basic genomes ($\mathbf{E^e}$, $\mathbf{E^b}$,**W**, **P**, **Ta**, **V**, **Ns**,**A**, **B**, and **D**) In order to explore the maternal origin of the hybrids identified cytologically, *rps*16 sequence was selected for phylogenetic analysis. The length of hybrids *rps*16 sequences varied from 830 to 831bp. A lot of 28 sequences were selected for ML analysis.*Bromus sterilis* were used as the outgroup. The data matrix contained 881 characters, 30 were variable characters and 30 were parsimony informative. TIM1 + G as the best-fit model (-Ln likelihood = 1555.21) was used in phylogenetic analysis. The ML tree was displayed in Figure 9. The phylogenetic analyses of the *rps*16 sequence were shown in Figure 9. The *rps*16 sequences from hybrids RH1 were grouped with*R. stricta* (BS=64%, PP=0.97). The clade contained 5 hybrids RH1 sequences and *R. stricta*. The *rps*16 sequences from hybrids RH2 were grouped with *R. turczaninovii* (BS=88%, PP=1.00). The clade contained 10 hybrids RH2 sequences and *R. turczaninovii*. The above results showed that *R. stricta* was the maternal donor of the hybrids RH1, *R. turczaninovii* was the maternal donor of the hybrids RH2.

4 | DISCUSSION

4.1 | Identification of natural hybrids

R. stricta and *R. turczaninovii* were planted in the experimental base of SAGS. We collected the seeds of the two species and planted them individually. We found that there were randomly distributed sterile plants in the experiment field. These sterile plants grew stronger than around plants and their seed set is very low (Figures 1a-c and 3). They were morphologically intermediate between *R. stricta*and *R. turczaninovii* , such as pubescence of leaf, basal leaf sheath and stem node (Figures 1d-o). We hypothesized that the plants were hybrids of *R. stricta* and *R. turczaninovii* .

Karyotype analysis showed that 57 putative natural hybrids were tetraploids, 2n=4x=28 (Figure 4). Chromosomal pairing analysis showed that meiosis of *R. stricta* and *R. turczaninovii* were more regular than the putative hybrids (Figure 5). Except for hybrid RH2-31, all hybrids had univalent at MI. Univalent varying from 0.42 to 2.00 in hybrids RH1, whereas it varying from 0 to 3.45 in hybrids RH2 (Table S2). In all hybrids, the c-value was higher than 0.81, indicating that chromosome pairing of the hybrids was comparatively regular, and had two sets of homology genomes.

FISH analysis using St_2-80 showed that the 14 chromosomes of *R. stricta* , *R. turczaninovii* and the natural hybrids displayed **St** type (Figures 6a, c, e and 7a, c, e), indicating that they contained one set of the **St** genome. This result was confirmed by GISH, where 28 chromosomes were hybridized with the **StY** probe from *R. ciliaris* (Figures 6b, d, f and 7b, d, f). The results of *in situ* hybridization showed that the genomic constitution of the parents and hybrids was **StY** .

The above results indicated that these hybrids were tetraploids with the**StY** genome.

4.2 | Origin of hybrids RH1 and RH2

Single copy nuclear gene is inherited from both parents and is rarely affected by concerted evolution. Therefore, it is used as an ideal DNA sequence in the analysis of plant molecular phylogeny to study the relationship of related species, polyploid plant phylogenetic relationship, speciation history of allopolyploid, source of species genome donor, generation of hybridization events, and gene introgression of polyploid species have significant advantages (Sang, 2002; Tang et al., 2017). In the present study, we performed phylogenetic analyses of the hybrids, the associated species (*R. stricta*, *R. turczaninovii*, *R. grandis*, *E. sibiricus*) and other Triticeae species with different genomic compositions. Two distinct*DMC*1 copies of the sequences from hybrids, *R. stricta*,*R. turczaninovii* and the other Triticeae species growing nearby were divided into **St** and **Y** clades (Figure 8). In the**St** clade, 15 hybrids sequences and *R. turczaninovii*formed a subclade (BS=51%, PP=1.00). In the **Y** clade, 15 hybrids sequences, *R. turczaninovii* and *R. stricta* formed a subclade (BS=64%, PP=0.83). The results of phylogenetic analysis based on nuclear gene *DMC*1 showed that natural hybrids have close relationships with *R. stricta* and *R. turczaninovii*, and they all contained **St** and **Y** genomes. The results were consistent with cytological studies. Phylogenetic analyses based on *rps*16 sequence showed 5 hybrids RH1 sequences were grouped with *R. stricta* (BS=64%, PP=0.97), 10 hybrids RH2 sequences were grouped with *R. turczaninovii*(BS=87%, PP=1.00). Furthermore, hybrids RH1 were collected from*R. stricta*, hybrids RH2 were collected from *R. turczaninovii*. So, *R. stricta* was the female parent of the hybrids RH1, *R. stricta* was the female parent of the hybrids RH2.

Therefore, based on the results of cytological and phylogenetic analysis, we suggested that *R. stricta* and *R. turczaninovii* were the female and male parents of the hybrids RH1; *R. turczaninovii* and *R. stricta* were the female and male parents of the hybrids RH2.

4.3 | Formation process of natural hybrids

Under natural conditions, hybridization was one of the main evolutionary mechanisms of plants (Goulet et al., 2017; Soltis & Soltis 2009). Although some large families and genera were difficult to produce hybrids (Ellstrand et al., 1996), there were also frequent interspecific hybridizations of several genera, such as *Asplenium* (Lee et al., 2015), *Senecio* (Abbott & Lowe, 2004), *Betula* (Wang et al., 2014), *Rhododendron* (Yan et al., 2013), etc. Triticeae was a young group, there was a large possibility of random hybridization among the relative genera in the Triticeae (Barkwoth & Bothmer, 2009). The generation of natural hybrids

was affected by the genomic constitution of species, pollination habits and environmental conditions. In addition to the overlapping or very close distribution, the parents of natural hybridization also needed to be close in flowering stage. For example, the flowering stage of *Begonia crassirostris*and *Begonia hemsleyana* have lasted for several months, which increasing the possibility of flowering encounters with other species, so it was more likely to occur hybridization (Tian, 2017). The good open-air environment and close planting, favorable conditions were created for the occurrence of natural hybridization.

In this study, different genera species with different genome constitutions in Triticeae were planted in the experiment base of the SAGS, such as *Roegneria* (**StY**), *Elymus*(**StH**) etc. Due to these plants were perennials, they could be kept in the field for more than ten years. The two adjacent fields are planted with *R . stricta* and *R. turczaninovii* , respectively. After three years of planting, we collected their seeds respectively and individually planted them for expanding propagation. There were about 400 plants in the *R. stricta* field, and about 330 plants in the *R. turczaninovii* field. We found some putative natural hybrids randomly distributed in these fields. The natural hybrids morphologically combined some unique characteristics (Figure 1d-o) of the two species. *R. stricta* was similar to *R. turczaninovii* in flowering time and their distribution areas was also close, which provided conditions for the natural hybridization.

We selected 20 basic genomic species (representing the genomes**St** , **H** , $\mathbf{E^e}$,$\mathbf{E^b}$, **W** , **P** , **I** ,**Ta** , **V** , **Ns** , **A** , **B** , and**D**) of Triticeae, hybrids and associated species growing around hybrids for phylogenetic analysis. The results indicated that 15 natural hybrids have close relationships with *R . stricta* and*R. turczaninovii* . We speculated that the hybrids were produced by the hybridization between *R. stricta* and *R. turczaninovii*in this study. Their ploidy and the genomic constitution were the same, the distribution area was close, and the flowering time was synchronous, all of which form a favorable environment and factors for natural hybridization. In the process growing, their pollen pollinated each other and underwent natural hybridization to form natural hybrids.

From the perspective of hybridization rate, there were 23 hybrids out of the about 400 *R. stricta* plants, and the natural hybridization rate was about 5.75%, while among the about 330 *R. turczaninovii*plants, there were 54 hybrids, natural hybridization rate was about 16.36%. It can be seen that natural hybridization rate of *R. turczaninovii* was about 3 times that of *R. stricta* . The reason may be that the source of the *R. stricta* parents was single and the genetic diversity was low, while the *R. turczaninovii* parent has higher genetic diversity. Large morphological differences were observed in the field of *R. turczaninovii* , which leaded to a higher natural hybridization rate. The genetic diversity of the *R. stricta* parents and *R. turczaninovii* parents needed to be further verified by molecular markers or other methods.

4.4 | Hybrid speciation

Hybridization was increasingly recognized as an important component of species evolution (Mallet, 2005; Mallet et al., 2016; Stukenbrock, 2016). New species were produced by different ways of evolution, such as polyploidization, homoploid hybridization, and introgressive hybridization (Rieseberg & Carney, 1998; Wang, 2017). Due to the abundant genetic and phenotypic variation in the hybrid progeny population, the boundaries between them and their parents became blurred and difficult to distinguish (Abbott, 2003). Therefore, in the past, botanists only defined a new species according to morphological characteristics, which was easy to lead to the wrong understanding of species. In other words, some previously published species were likely to be hybrid or intermediate state of hybrid transition to new species. For example, in the Triticeae, Zeng (2011) through cytology and DNA sequence analysis showed that *Kengyilia stenachyra* is a natural hybrid of *K. rigidula* and *Elymus nutans* , rather than a real species. Hybrid progeny population was highly variable, which retained the important transition types in the evolution process, and was an indispensable part of the material system to explore the formation mechanism of hybrid speciation (Rieseberg, 1997). So, the natural hybrids found in this study provided a good research material for exploring the formation. Further research will be needed on the formation of hybrid species.

4.5 | Utilization of natural hybrids

Heterosis was a very important biological phenomenon that hybrids surpass parents in individual size, via-

bility, fertility, environmental adaptability, yield and quality (Shull, 1908). Breeders have long used heterosis as a means of genetic improvement (Keeble, 1910). In this study, we found some natural hybrids with good forage traits in plant height, tillers, and leaf, but the fertility was very low. If these natural hybrids could be genetically improved to create new forage varieties, it would have good ecological and economic benefits. We had found an increase in fertility in hybrid F_2(˜ 25%). We will continue to self-cross the hybrids to improve fertility.

ACKNOWLEDGMENTS

The authors are thankful to the National Natural Science Foundation of China (Grant Nos. 31870309 and 31670331) and the Science and Technology Bureau of Sichuan Province (2021NZZJ0010, 21YYJSYJ0083) for their financial supports.

AUTHOR CONTRIBUTION

Chen Chen : Conceptualization (Equal); Formal analysis (Lead); Investigation (Lead); Methodology (Equal); Project administration (Equal); Writing-original draft (Lead); Writing-review & editing (Equal). **Zilue Zheng** : Conceptualization (Equal); Data curation (Equal); Formal analysis (Equal); Investigation (Equal); Software (Lead); Writing-original draft (Equal); Writing-review & editing (Equal). **Dandan Wu** : Conceptualization (Equal); Data curation (Equal); Formal analysis (Equal); Resources (Supporting); Software (Equal); Supervision (Equal); Visualization (Equal). **Lu Tan** : Formal analysis (Equal); Investigation (Supporting); Software (Equal).**Cairong Yang** : Conceptualization (Supporting); Methodology (Supporting). **Songqing Liu** : Resources (Supporting).**Jiale Lu** : Project administration (Supporting); Supervision (Supporting); Validation (Supporting). **Xing Fan** : Software (Supporting). **Lina Sha** : Visualization (Supporting).**Houyang Kang** : Supervision (Supporting). **Yi Wang** : Validation (Supporting).**Yonghong Zhou** : Validation (Supporting).**Changbing Zhang** : Data curation (Equal); Project administration (Equal); Resources (Equal); Supervision (Equal). **Haiqin Zhang** : Conceptualization (Equal); Funding acquisition (Lead); Investigation (Equal); Project administration (Equal); Resources (Equal); Supervision (Lead); Writing-original draft (Equal); Writing-review & editing (Equal).

DATA ACCESSIBILITY

Data are available from the Dryad Digital Repository at https:// doi.org/10.5061 /dryad.v41ns1rw9. The haplotype sequences of our study involved are deposited in GenBank with accession numbers MZ130327-MZ130377.

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TABLE 1 The materials used in this study

Parents and hybris	Accession No.	Genomes	2n	Plants No.	Origins	Origins
Roegneria turczaninovii	ZY 11140	**StY**	28	6	6	Inner Mongolia, china
Roegneria stricta	Y 2102	**StY**	28	14	14	Sichuan, China
Hybrid	RH1-1 ˜RH1-17	–	–	17	17	Sichuan, China
Hybrid	RH2-1 ˜RH2-40	–	–	40	40	Sichuan, China

TABLE 2 Plant materials used in Phylogenetic analysis

Number	Species/Hybrids	Genome	2n	Accesssion	Locality	GenBank No.
						*DMC*1 *rps*16
1	hybrid 1	**StY**	4x	RH1-3	Sichuan, China	
2	hybrid 2	**StY**	4x	RH1-6	Sichuan, China	
3	hybrid 3	**StY**	4x	RH1-8	Sichuan, China	
4	hybrid 4	**StY**	4x	RH1-12	Sichuan, China	
5	hybrid 5	**StY**	4x	RH1-14	Sichuan, China	
6	hybrid 6	**StY**	4x	RH2-2	Sichuan, China	
7	hybrid 7	**StY**	4x	RH2-5	Sichuan, China	

Number	Species/Hybrids	Genome	2n	Accesssion	Locality	GenBank No.
8	hybrid 8	**StY**	4x	RH2-7	Sichuan, China	
9	hybrid 9	**StY**	4x	RH2-10	Sichuan, China	
10	hybrid 10	**StY**	4x	RH2-11	Sichuan, China	
11	hybrid 11	**StY**	4x	RH2-13	Sichuan, China	
12	hybrid 12	**StY**	4x	RH2-14	Sichuan, China	
13	hybrid 13	**StY**	4x	RH2-15	Sichuan, China	
14	hybrid 14	**StY**	4x	RH2-16	Sichuan, China	
15	hybrid 15	**StY**	4x	RH2-17	Sichuan, China	
16	hybrid 16	**StY**	4x	RH2-18	Sichuan, China	
17	hybrid 17	**StY**	4x	RH2-30	Sichuan, China	
18	hybrid 18	**StY**	4x	RH2-31	Sichuan, China	
19	hybrid 19	**StY**	4x	RH2-37	Sichuan, China	
20	hybrid 20	**StY**	4x	RH2-39	Sichuan, China	
21	Putative parent *Roegneria strictus* (Keng) S.L.Chen	**StY**	4x	Y 2102	Sichuan, China	
Number	Species/Hybrids	Genome	2n	Accesssion	Locality	GenBank NO. *DMC*1 *rps*16
22	Putative parent *Roegneria turczaninovii* (Drobow) Nevski	**StY**	4x	ZY 11140	Inner Mongolia, China	
23	*Elymus sibiricus* L.	**StH**	4x	PI 619579	Xinjiang, China	EU366409* GQ855198*
24	*Elymus caninus* L.	**StH**	4x	PI 314621	Former Soviet Union	EU366407* EU366408*
25	*Elymus elymoides* (Raf.) Swezey	**StH**	4x	PI 628684	United States	FJ695161* FJ695160*

Number	Species/Hybrids	Genome	2n	Accesssion	Locality	GenBank No.
26	*Elymus glaucus* Buckley	**StH**	4x	PI 593652	Oregon United States	FJ695163* FJ695162*
27	*Elymus virginicus* L.	**StH**	4x	PI 490361 PI 882397	United States Sichuan, China	GQ855195* GQ855196*
28	*Elymus wawawaiensi*	**StH**	4x	PI 506284	Sichuan, China	
29	*Roegneria caucasica* K. Koch	**StY**	4x	H 3207	Xinjiang, Armenia	HM770785* HM770784*
30	*Roegneria ciliaris*(Trin.) Nevski	**StY**	4x	87-88 335 88-89-238	Sichuan, China	KU160610* KU160617*
31	*Roegneria dura* Keng	**StY**	4x	Y 2124	Neimenggu, China	KX578879*
32	*Roegneria grandis* Keng	**StY**	4x	ZY 3189 Y 3189	Xizang, China	KU160615* KU160618*
33	*Roegneria hondai* Kitagawa	**StY**	4x	Y 0362	Sichuan, China	KX578840* KX578841*
Number	Species/Hybrids	Genome	2n	Accesssion	Locality	GenBank NO. *DMC*1 *rps*16
34	*Roegneria longearistata* (Boiss.) Drob.	**StY**	4x	Y 2259	Inner Mongolia, China	KX578848
35	*Roegneria shandongensis* (B. Salomon) J. L. Yang & C.Yen	**StY**	4x	ZY 3150	Shanxi, China	KX578862*
36	*Roegneria ugamica* (Drob.) Nevski	**StY**	4x	Y 1698	Sichuan, China	KX578877* KX578878*
37	*Pseudoroegneria libanotica* (Hackel) D. R. Dewey	**St**	2x	PI 228389 PI 228392	Iran	FJ695174*
38	*Pseudoroegneria spicata* (Pursh) A. Löve	**St**	2x	PI 547161 PI 632532	United States	FJ695175* KY636118*

Number	Species/Hybrids	Genome	2n	Accesssion	Locality	GenBank No.
39	*Pseudoroegneria stipifolia* (Czern. ex Nevski)	**St**	2x	PI 325181	Stavropol, Russian	FJ695176*
40	*Pseudoroegneria strigosa* (M. Bieb.) A. Löve	**St**	2x	PI 595164 PI 499637	Xinjiang, China	FJ695177*
41	*Pseudoroegneria tauri* (Boiss.) A. Löve	**St**	2x	PI 401329 PI380650	Iran	KU160613
42	*Agropyron cristatus* (L.) Gaertn	**P**	2x	H 4349 PI 598628	China Kazakhstan	AF277241* KY126307*
43	*Australopyrum retrofractum* (Vickery) A. Löve	**W**	2x	H 6723 PI 531553	China United States	AF277251* KY636080.1*
44	*Hordeum chilense* Roem. & Schult.	**H**	2x	PI 531781	Chile	FJ695173*
45	*Hordeum pubiflorum* Hook.f.	**H**	2x	BCC 2028		KY636108*
46	*Hordeum bogdanii* Wilensky	**H**	2x	PI 531761	China	FJ695172* MH331641*
Number	Species/Hybrids	Genome	2n	Accesssion	Locality	GenBank NO. *DMC*1 *rps*16
47	*Hordeum vulgare* L.	**I**	2x	H 3878	Italy	EF115541*
48	*Lophopyrum elongatum* (Host) A. Löve	$\mathbf{E^e}$	2x	PI 531719 PI531718	Israel	AF277246* MH331643*
49	*Thinopyrum bessarabicum* (Savul. & Rayss) A.	$\mathbf{E^b}$	2x	PI 531711 W6 21890	Russia	AF277254* KY636145*
50	*Psathyrostachys huashanica* Keng ex P.C Kuo	**Ns**	2x	PI 531823	Shanxi, China	GU165826*
51	*Aegilops speltoides* Tausch.	**B**	2x	H 6779		DQ247833*
52	*Aegilops tauschii* Coss.	**D**	2x	H 6668 AE429		AF277235* JQ754651*

Number	Species/Hybrids	Genome	2n	Accesssion	Locality	GenBank No.
53	*Dasypyrum villosum* (K. Koch) Nevski	**V**	2x	H 5552 W6 7264		AF277236* MH285850*
54	*Secale cereale* L.	**R**	2x			KC912691*
55	*Taeniatherum copmedusae* (L.) Nevski	**Ta**	2x	H 10254 PI220591		AF277249* MH285856*
56	*Triticum urartu* Tum.	**A**	2x	H 6664		DQ247826*
57	*Bromus sterilis* L.			OSA 420		AF277234*

Note: 1* Data from published sequences in the GenBank (http://www.ncbi.nlm.nih.gov)

Figure legends

FIGURE 1 Morphological characteristics of the natural hybrids and their parents. (a) – (c) Natural distribution of hybrids. (a) hybrid RH1 (arrowed). (b) hybrid RH2 (arrowed). (c) hybrid RH1 (arrowed). (d) – (g) Leaves of hybrids and parents. (d) *R. turczaninovi* i (arrowed). (e) *R. stricta*(arrowed). (f) hybrid RH1 (arrowed). G: hybrid RH2 (arrowed). (h) – (k) Stem segments of hybrids and parents. (h) *R. turczaninovi* i (arrowed). (i) *R. stricta* (arrowed). (j) hybrid RH1 (arrowed). K: hybrid RH2 (arrowed). (l) – (o) Basal leaves of hybrids and parents. (l) *R. turczaninovi* i (arrowed). (m) *R. stricta* (arrowed). (n) hybrid RH1 (arrowed). O: hybrid RH2 (arrowed).

FIGURE 2 Morphological analysis of hybrids RH1, hybrids RH2,*R. stricta* and *R. turczaninovii* . (a) Plant height. (b) Number of tillers. (c) Length of flag leaf. (d) Width of flag leaf. (e) Length of top second leaf. (f) Width of top second leaf. (g) Length of spike. (h) Number of florets. (i) Length of awn. Note: Different letter shows the significance of difference of ANOVA.

FIGURE 3 Pollen fertility and seed set of hybrids RH1, hybrids RH2, *R. stricta* and *R. turczaninovii*

FIGURE 4 Karyotype analysis of hybrids RH1, hybrids RH2,*R. stricta* and *R. turczaninovii.* (a) *R. turczaninovii* . (b) *R. stricta* . (c) RH1-13. (b) RH2-10.

FIGURE 5 Meiotic associations in PMCs of the parental species and hybrids. (a) and (b) *R. turczaninovii* with 14 II. (c)*R. stricta* with 14 II. (d) RH1-15 with 14 II (12 ring + 2 rod). (e) RH1-7 with 13 II (9 ring + 8 rod) + 2 I. (f) RH2-38 lagging chromosomes. (g) RH2-29 with 14 II (12 ring and 2 rod). (h) RH2-11 with 13 II (11 ring and 2 rod) + 2 I. (i) RH2-11 chromosome bridge.

FIGURE 6 Analysis of FISH and GISH in *R. stricta* and*R. turczaninovii* . (a) and (b) *R. stricta* . (c) – (f)*R. turczaninovii* . (a), (c) and (e) Used St_2–80 as probe (red), 14 chromosomes were labeled as **St** type (arrowed) and 14 chromosomes were labeled as non-St type. (b), (d) and (f) With total genomic DNA of *R. ciliaris* (**StY** genome) was labeled with Texas-red-5-dCTP (red) as probe, 28 chromosomes were labeled as red fluorescent signals.

FIGURE 7 Analysis of FISH and GISH in hybrids. (a) and (b) RH1-11. (c) and (d) RH2-12. E and F RH2-15. (a), (c) and (e) Using St_2–80 as probe (green), 14 chromosomes were labeled as **St** type (arrowed) and 14 chromosomes were labeled as non-St type. (b), (d) and (f) With total genomic DNA of *R. ciliaris* (**StY** genome) was labeled with fluorescein-12-dUTP (green) as probe, 28 chromosomes were labeled as green fluorescent signals.

FIGURE 8 Phylogenetic tree based on *DMC* 1 sequences of hybrids using ML. Numbers with bold above nodes are bootstrap values, and numbers below nodes are Bayesian posterior probability values.

FIGURE 9 Phylogenetic tree based on *rps* 16 sequences of hybrids using ML. Numbers with bold above nodes are bootstrap values, and numbers below nodes are Bayesian posterior probability values.

supplementary material

TABLE S1 The primers used in this study

Gene	Name of primers	Sequence of primer (5´- 3´)	Profiles
*DMC*1	T*DMC*1e10F	TGCCAATTGCTGAGAGATTTG	1 cycle: 4 min 94 ; 35 cycles: 1 min 94 , 1 min 52 , 2 r
	T*DMC*1e15R	AGCCACCTGTTGTAATCTGG	
*rps*16	*rps*16F	AAACGATGTGGTAGAAAGCAAC	1 cycle: 3 min 95 ; 35 cyclies: 40 s 94 , 40s 55 , 1 min
	*rps*16R	ACATCAATTGCAACGATTCGATA	

TABLE S2 Meiotic associations at metaphase I in PMCs of the parental species and hybrid

Species and hybrids	No. of cells observed	Chromosome pairing	Chromosome pairing	Chromosome pairing	Chromo
		I	Ring II	Rod II	Total II
R. stricta	463	0	13.55	0.44	13.99
R. turczaninovii	198	0	13.53	0.43	13.96
RH1-1	24	1.6	11.45	1.75	13.2
RH1-2	28	0.9	12.05	1.6	13.65
RH1-3	21	0.6	12.15	1.55	13.7
RH1-4	35	1.9	11.1	1.95	13.05
RH1-5	23	0.93	10.98	2.53	13.51
RH1-6	24	0.5	12.15	1.65	13.8
RH1-7	20	1.1	10.85	2.6	13.45
RH1-8	34	0.53	11.44	2.29	13.73
RH1-9	20	1.3	11.75	1.6	13.35
RH1-10	20	1	10.7	2.8	13.5
RH1-11	25	0.5	12.15	1.6	13.75
RH1-12	21	0.9	11.75	1.8	13.55
RH1-13	35	0.51	11.97	1.77	13.74
RH1-14	20	2	11.5	1.5	13
RH1-15	20	1.2	11.15	2.25	13.4
RH1-16	20	0.8	12	1.6	13.6
RH1-17	32	0.42	12.12	1.67	13.79
RH2-1	20	0.6	11.5	2.2	13.7
RH2-2	20	0.5	12.7	1.05	13.75
RH2-3	20	1.1	11.05	2.4	13.45
RH2-4	20	0.5	11.65	2.1	13.75
RH2-5	23	1.3	11.1	2.25	13.35
RH2-6	20	2.15	11.3	2.15	13.45
RH2-7	31	1.7	11.11	2.05	12.97
RH2-8	20	1.1	11.5	1.95	13.45
RH2-9	22	1.3	11.4	1.95	13.35
RH2-10	20	0.7	10.6	2.35	12.95
RH2-11	24	3.45	11.25	2.05	13.3
RH2-12	20	0.5	12.25	1.45	13.7

Species and hybrids	No. of cells observed	Chromosome pairing	Chromosome pairing	Chromosome pairing	Chromo
RH2-13	20	0.9	11.2	2.35	13.55
RH2-14	20	0.85	11.2	2.45	13.65
RH2-15	20	2.1	10.6	2.35	12.95
RH2-16	20	0.5	10.95	2.8	13.75
RH2-17	20	0.95	10.7	2.85	13.55
RH2-18	20	0.7	11.6	2.05	13.65
RH2-19	20	1.2	12.2	1.2	13.4
RH2-20	20	0.1	11.5	2.45	13.95
RH2-21	19	0.42	11.32	2.21	13.53
RH2-22	47	0.46	12.66	1.07	13.73
RH2-23	20	0.2	11.75	2.15	13.9
RH2-24	20	0.4	11.45	2.35	13.8
RH2-25	27	0.2	12	1.9	13.9
RH2-26	35	0.6	11.85	1.85	13.7
RH2-27	21	0.5	11.95	1.8	13.75
RH2-28	24	1	11.95	1.55	13.5
RH2-29	22	0.8	11.65	2.05	13.7
RH2-30	26	0.4	11.8	2	13.8
RH2-31	31	0	11.55	2.45	14
RH2-32	38	0.68	11.82	1.87	13.69
RH2-33	37	0.7	11.81	1,81	13.62
RH2-34	24	0.8	11.35	2.25	13.6
RH2-35	24	0.5	11.69	1.98	13.67
RH2-36	40	1.2	11.65	1.75	13.4
RH2-37	23	0.8	12.65	0.85	13.5
RH2-38	19	1.48	11.32	1.92	13.24
RH2-39	48	0.58	11.81	2.1	13.91
RH2-40	39	0.1	12.21	1.72	13.93