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Altitudinal Patterns in Adaptive Evolution of Genome Size and Inter-Genome Hybridization Between Three *Elymus* Species From the Qinghai–Tibetan Plateau

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Genome size variation and hybridization occur frequently within or between plant species under diverse environmental conditions, which enrich species diversification and drive the evolutionary process. *Elymus* L. is the largest genus in Triticeae with five recognized basic genomes (St, H, P, W, and Y). However, the data on population cytogenetics of *Elymus* species are sparse, especially whether genome hybridization and chromosomal structure can be affected by altitude are still unknown. In order to explore the relationship between genome sizes, we studied interspecific hybridization and altitude of *Elymus* species at population genetic and cytological levels. Twenty-seven populations at nine different altitudes (2,800–4,300 m) of three *Elymus* species, namely, hexaploid *E. nutans* (StHY, 2n = 6x = 42), tetraploid *E. burchan-buddae* (StY, 2n = 4x = 28), and *E. sibiricus* (StH, 2n = 4x = 28), were sampled from the Qinghai–Tibetan Plateau (QTP) to estimate whether intraspecific variation could affect the genomic relationships by genomic *in situ* hybridization (GISH), and quantify the genome size of *Elymus* among different altitude ecological groups by flow cytometry. The genome size of *E. nutans, E. burchan-buddae,* and *E. sibiricus* varied from 12.38 to 22.33, 8.81 to 18.93, and 11.46 to 20.96 pg/2C with the averages of 19.59, 12.39, and 16.85 pg/2C, respectively. The curve regression analysis revealed a strong correlation between altitude and nuclear DNA content in three *Elymus* species. In addition, the chromosomes of the St and Y genomes demonstrated higher polymorphism than that of the H genome. Larger genome size variations occurred in the mid-altitude populations (3,900–4,300 m) compared with other-altitude populations, suggesting a notable altitudinal pattern in genome size variation, which shaped genome evolution by altitude. This result supports our former hypothesis that genetic richness center at medium altitude is useful and valuable for species adaptation to highland environmental conditions, germplasm utilization, and conservation.

Keywords: *Elymus*, altitude, genome size, flow cytometry, GISH

INTRODUCTION

Strong correlations between variation in genomic attributes and species diversity across the plant tree of life suggest that polyploidy or other mechanisms of genome size change confer selective advantages due to the introduction of genomic novelty (Barrett et al., 2019). Genome size and nuclear DNA content are both important biodiversity characteristics, which provide a practical and predictive element in biology (Höfer and Meister, 2010; Sliwinska, 2018). Determination of genome size in species is of great significance not only for molecular and cell genetics, but also for plant genomics and evolution study. Numerous correlations between genome size and physiologically or ecologically relevant phenotypes have been observed, including nucleus size (Baetcke et al., 1967), plant cell size (Pegington and Rees, 1970), seed size (Beaulieu et al., 2007), body size (Gregory et al., 2000), and growth rate (Cavalier, 1978). Adaptive models of genome size evolution suggest that positive selection drives genome size toward an optimum due to selection on these or other traits and that stabilizing selection prevents expansions and contractions away from the optimum (Gregory and Hebert, 1999; Müller et al., 2019). Correlations between intraspecific variation in genome size and other phenotypes or environmental factors have been observed (Long et al., 2013; Kang et al., 2015; Hoang et al., 2019), suggesting the possibility that some of the observed variations may be adaptive. Much of the discussion about genome size variation has focused on variation among species, and intraspecific variation has often been downplayed as the result of experimental artifacts (Hoang et al., 2019) or argued to be too small to have much evolutionary relevance (Hannes et al., 2021). However, there is substantial controversy about the genetic process that controls the size of plant genomes (Pellicer et al., 2014), but most researchers believed that the main mechanism that drives the rapid amplification of plant genomes is polyploidy or transposable elements (TEs), especially transposons (Pellicer et al., 2014). Recent research has suggested that genome size varies many orders of magnitude across species, due to changes in both ploidy and haploid DNA content, and we still know relatively little about the makeup of many eukaryote genomes, the impact of genome size on phenotype, or the processes that govern variation in repetitive DNA and genome size among taxa (Wendel et al., 2016; Li et al., 2020).

Genome size can be an important feature that predicts the response of different species to climate and environmental changes. Meanwhile, genome size has a significant influence on some epigenetic traits and plant growth and development (Müller et al., 2019). During the last decades, flow cytometry became the preferred method for genome size measurement in plants. Flow cytometry is a powerful technology, and cells and particles can be analyzed in a cell nuclei suspension because of its precision, high speed, objectivity, and relative simplicity (Yumni et al., 2021). Species relationships can be elucidated by correlating the chromosome numbers with genome sizes (Huang et al., 2013). The application of flow cytometry has been well-documented for the determination of the accurate nuclear DNA contents, adding hints on the ploidy status and the evolutionary pattern in the varying genome sizes (Saha et al., 2017). Besides the easiness of sample preparation and high throughput, the capability to estimate genome size, nuclear replication state, and ploidy and endopolyploidy levels is an advanced feature of this method compared with other approaches such as Feulgen densitometry or genome sequencing. Flow cytometry has been extensively used in plant genetics, plant physiology, and other fields (Ochatt, 2008). Genomic *in situ* hybridization (GISH) has been considered to be an effective and illustrative method for the rapid and reliable identification of genomic homology in allopolyploid and related species, alien chromatin in interspecific and intergeneric species, and identification of genetic constitution on chromosome level in polyploid species (Qie et al., 2007; Badaeva et al., 2021; Chaves et al., 2021).

The genus *Elymus* comprises ∼150 species in the Triticeae tribe of the grass family (Poaceae) and is widely distributed throughout the Tropics. Asia is an important center of origin and diversity of the perennial species in the Triticeae (Sun, 2002), where more than half, ∼80, of the known *Elymus* species have originated (Dewey, 1984). Cytogenetic analyses have identified that all *Elymus* species include the St genome in combination with one or more of four other genomes (H, Y, P, and W). The St, H, P, and W genomes are known to be denoted by *Pseudoroegneria* (Nevski) Á, Löve *Hordeum L.*, *Agropyron Gaertn.*, and *Australopyrum* (Tzvelev) Á. Löve, respectively (Jensen, 1990; Torabinejad and Mueller, 1993). However, the origin of genome Y has always been controversial. The *Elymus* genus, tetraploid *E. burchan-buddae* (StY), *E. sibiricus* (StH), and hexaploid *E. nutans* (StHY) have similar morphologies, different genome constitutions, and overlapping habitats (Sun and Salomon, 2009), which display a continuous distribution at different altitudes on the Qinghai–Tibetan Plateau (QTP) of western China. An altitudinal gradient, as an important index of spatial niche measurement, greatly affects the distribution range and pattern of organisms. Intense stresses (low temperature, low oxygen supply, and high UV radiation) caused by high-altitude environments may result in noticeable genetic adaptations in native species (Beall, 2014). Recently, several studies have reported differences in the genome size and evolutionary processes driving the adaption of alpine plants along altitudinal gradients, and an adaptation of genome size to an unknown ecological parameter connected to altitude (Hämälä and Savolainen, 2019; Bohutínská et al., 2021). Savas et al. (2019) also determined that a geographical origin (localization, altitude) had a statistically significant effect on genome size. Our previous study showed significant correlations between altitude and morphological traits, and tetraploid *Elymus* species had a higher sensitivity to altitude than hexaploid *E. nutans* (Chen et al., 2015). The variation in intraspecies genome size is especially obvious when the geographical distance is relatively long or the climatic conditions are quite different. As an important ecological factor, altitude has an obvious influence on ecological factors such as temperature and moisture and thus affects genetic variation and population differentiation (Buehler et al., 2012). For example, a positive correlation was detected between genome size and growing altitude in wild populations of *Corchorus olitorius* (Benor et al., 2011). A variation in genome

size along altitudinal gradients has also been reported in maize (Díez et al., 2013), showing that altitude affects genomic variation and population differentiation possibly due to the dramatic influence on ecological factors including temperature and moisture. However, potential geographical patterns of genome size variation with the increase in altitudinal gradients are still not explicit. The limited number of reports on the genome size and chromosome counts is controversial for the rarely studied species of *Elymus*. We aim to explore how *Elymus* has adapted and evolved to the highly heterogeneous environments in QTP. Thus, we hypothesize that genome size is reduced in populations at high elevations. The objectives of this study were: (1) to estimate the nuclear DNA content in allohexaploid hexaploid *E. nutans* (StHY, 2n = 6x = 42), allotetraploid *burchan-buddae* (StY, 2n = 4x = 28), and *E. sibiricus* (StH, 2n = 4x = 28), (2) to investigate the genomic constitution and genomic homology between the St, H, and Y genomes, and (3) to examine the pattern between genome size and altitude to preliminary explore altitude change affecting the adaptability, fitness, competitiveness, and colonizing ability in *Elymus* species.

MATERIALS AND METHODS

Plant Materials

Seeds from three *Elymus* species, namely, *E. nutans*, *E. sibiricus*, and *E. burchan-buddae*, were sampled at nine different locations across the QTP at varying altitudes from 3,000 to 4,300 m within a small geographical scope of 60 km between 34°28′26.2"N and 34°40′43.7" N of latitude and from 100°22.1′42.6"E to 100°40′33.9" E of longitude (**Figure 1**, **Supplementary Table 1**). Three *Elymus* species were sampled at the same field at each altitude level, and 10 individuals were sampled for each population with individuals at least 10 m apart. The seeds were stored in the Animal Forage Laboratory of Henan Agricultural University. Morphological traits of each individual, including plant height, flag leaf length and width, spike weight and length, glume length and width, lemma length and width, palea length and width, floret number, and awn length, were determined (Chen et al., 2015). According to the required standards of Dolezel (2003), barley *(Hordeum vulgare L.)* was chosen as an internal reference standard, which was provided by Xiong Da-Bing at the National Engineering Research Center for Wheat. Plants of *Elymus* and barley were grown from seeds in an optical incubator at 25°Cwith a photoperiod of 12 h and 15°C in the dark per day, and their fresh tender leaves were collected within 2 to 3 weeks after planting.

Preparation of Nuclei Suspension

The 50-mg fresh leaves were sampled from each individual of *E. nutans*, *E. burchan-buddae*, and *E. sibiricus*, then washed with distilled water, dried with filter paper, and placed in a plastic Petri dish containing 250 μl of precooled extraction buffer. After vertically fast shredding with a sharp single-edge blade (maintaining equal weight to protect the organization from adhesions), 250 μl of extraction buffer was added, and the tissues were filtered through a 30 μm filter head (Celltrics, Partec, Germany) into a 1.5-ml centrifuge tube. Following filtration, the samples were centrifuged at 11,000 rpm at 4 °C for 15–20 s. The supernatant was discarded, and the nuclei were resuspended in 500 μl of a solution containing propidium iodide (PI) staining solution (0.02 mg/ml RNase solution+0.02 mg/mL PI solution). The nuclei suspension of *Hordeum vulgare* L. was obtained using the same method and mixed with the nuclei suspension of three *Elymus* species individually by the same volume. The samples were incubated at a low temperature in the dark for ∼30–60 min.

Genome Size Evaluation by Flow Cytometry Measurement

The nuclear DNA content was determined using a flow cytometer (CyFlow® Cube8, Partec, Germany). The excitation wavelength was set at 488 nm at 270 V, the excitation FL2 fluorescence channel was collected, and the propidium iodide (PI) emission fluorescence intensity was detected. A minimum of 10,000 nuclei per sample were analyzed. Three measurements were obtained for each sample, while 10 replications were determined for each population per species. The nuclear DNA content was calculated as follows: sample nuclear DNA content (pg/2C) = [(mean value of the sample peak)/(mean value of the internal standard)] × known nuclear DNA. The mean genome sizes were presented as 1C value in picograms (pg) or megabase pairs (1 pg = 978 Mbp) (Dolezel, 2003).

Preparation of Root tip and Mitotic Chromosome

Root tips were pretreated in ice-cold water at 0–4°C for 20–24 h and placed in 2 mM of 8-hydroxyquinoline (Sigma) at room temperature (25°C) under dark treatment for 2–4h. After being washed with distilled water for 15 min, they were dried with filter paper, fixed in ethanol–glacial acetic acid (3:1, v/v) for 24 h at room temperature, and then stored at 4°C in a refrigerator for 2–4 days. Again, they were washed two times with distilled water for 5 min and transferred to a EP tube filled with 4% cellulose–pectinase [2.7% Cellulase Onozuka R10 (Phytotech, USA) and 1.3% pectolyase (Sigma, Germany), pH 4.8] at 37°C for 1–2 h. After the unnecessary enzymolysis liquid was removed carefully using a pipette, they were washed with distilled water for 10 min, transferred onto slides with distilled water, and covered with the cover glass. Each root tip was squashed in a drop of 45% acetic acid. The slides with well-spread chromosomes were kept in a freezer at −80°C for more than 30 min, and the slides were air-dried for GISH.

Probe Preparation

Pseudoroegneria strigosa and *Hordeum bogdanii* Wilensky were grown about 3 weeks in pots for probe preparation. The total genomic DNAs of *P. strigosa* and *H. bogdanii* were extracted by the CTAB method (Murray and Thompson, 1980). Before labeling, the total genomic DNA was randomly fragmented into a size of 100–500 bp by DNase I (Sigma, Germany). The treated genomic DNAs of *P. strigosa* and *H. bogdanii* were labeled with biotin-11-dUTP (Thermo, USA) and digoxigenin-11-dUTP (Roche, Switzerland), respectively, by a nick translation method and used as probes in GISH analyses.

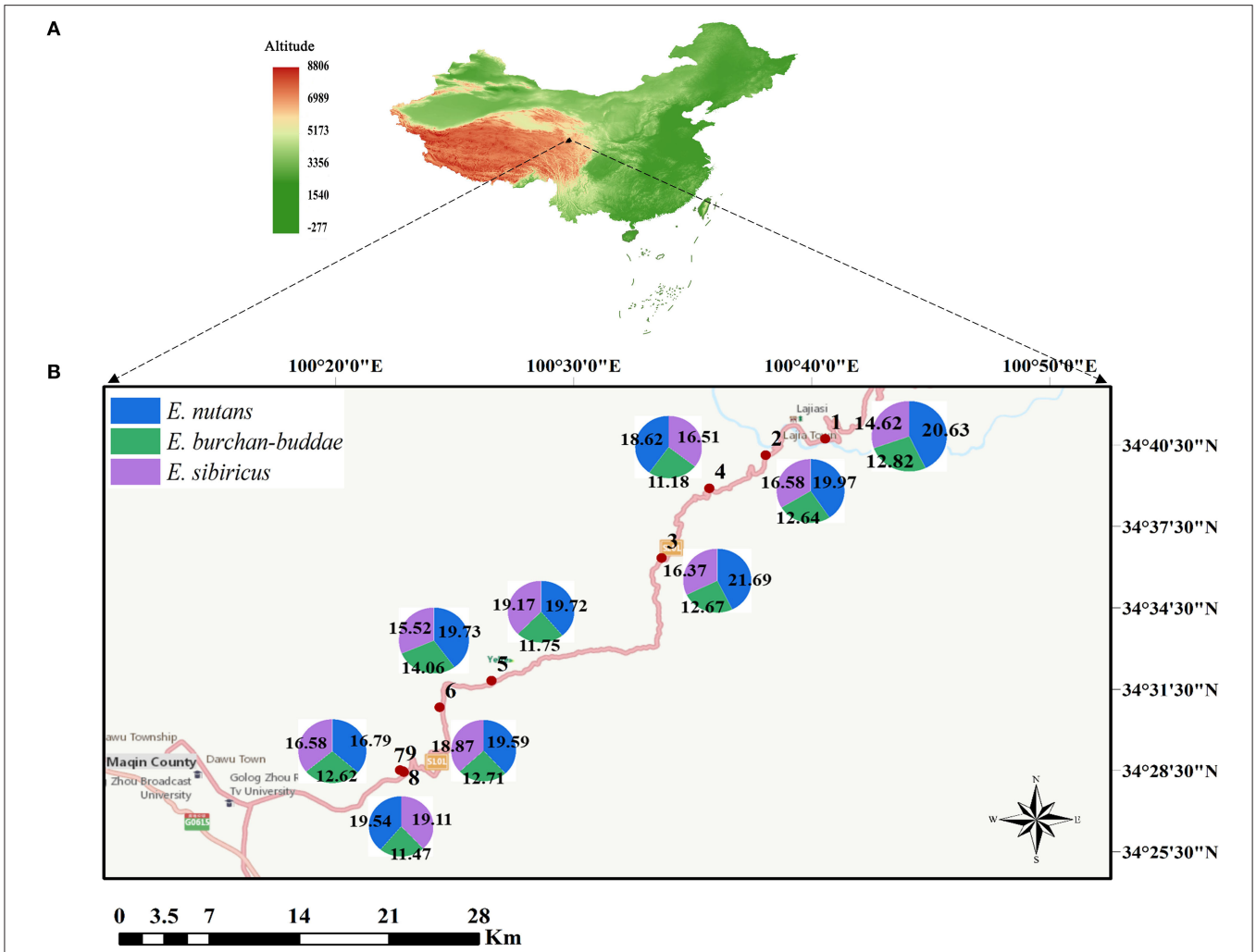

FIGURE 1 | Geographical distribution of the nine sampling sites and genome size of three *Elymus* species (*E. nutans*, *E. burchan-buddae*, and *E. sibiricus*, the detailed altitudes are given in **Supplementary Table 1**). **(A)** Sampling location at the Qinghai–Tibetan Plateau center of China. **(B)** The geographical location of the population sampled. The crook lines indicate various sizes of national highways; the pizza charts show the genome size of three *Elymus* species; and the numbers in the charts represent the population number.

Genomic *in situ* Hybridization

The slides were re-fixed in ethanol–glacial acetic acid (3:1, v/v) for 30 min, washed two times for 10 min in ethanol, and dried at 37°C for over 24 h. Chromosomes were denatured in 70% formamide in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 80–85°C for 90 s, then dehydrated through an ice-cold ethanol series (50, 70, 100%) for 5 min each, and air-dried. The probe hybridization mixture [containing 100% deionized formamide (by vol.), biotin-labeled DNA or dig-labeled DNA, 50% dextran sulfate (by vol.), 20 × SSC, and ddH_2O] was denatured at 90°C for 10 min.

Fluorescence Microscopy Detection

Following hybridization, the slides were removed and then placed in 2 × SSC at room temperature for 5 min and 42°C for 5 min, 1 × TNT [0.1M Tris, 0.15M NaCl, 0.05% Tween-20 (by vol.)]. The slides were placed in a mixture of 1×TNB buffer [containing 1M Tris (pH 7.5), 3M NaCl, blocking reagent], ddH_2O, streptavidin–Texas red (Invitrogen, USA), or anti-DIG-fluorescein (FITC) (BOSTER, USA), hybridized in a humidified box at 37°C for 1 h, then washed three times with 1×TNT at room temperature for 5 min, and air-dried. After that, a drop of 4′,6-diamidino-2-phenylindole (DAPI) was placed on each slide. Fluorescence was viewed with a double rotary laser confocal imaging real-time analyzer (PerkinElmer, UltraVIEW VoX, USA).

Data Analysis

Statistical Analysis

The CyViewTM Cube software was used for flow cytometry/FCS analysis, and histograms with a coefficient of variation (CV) above 5% were rejected. Statistical analyses were performed

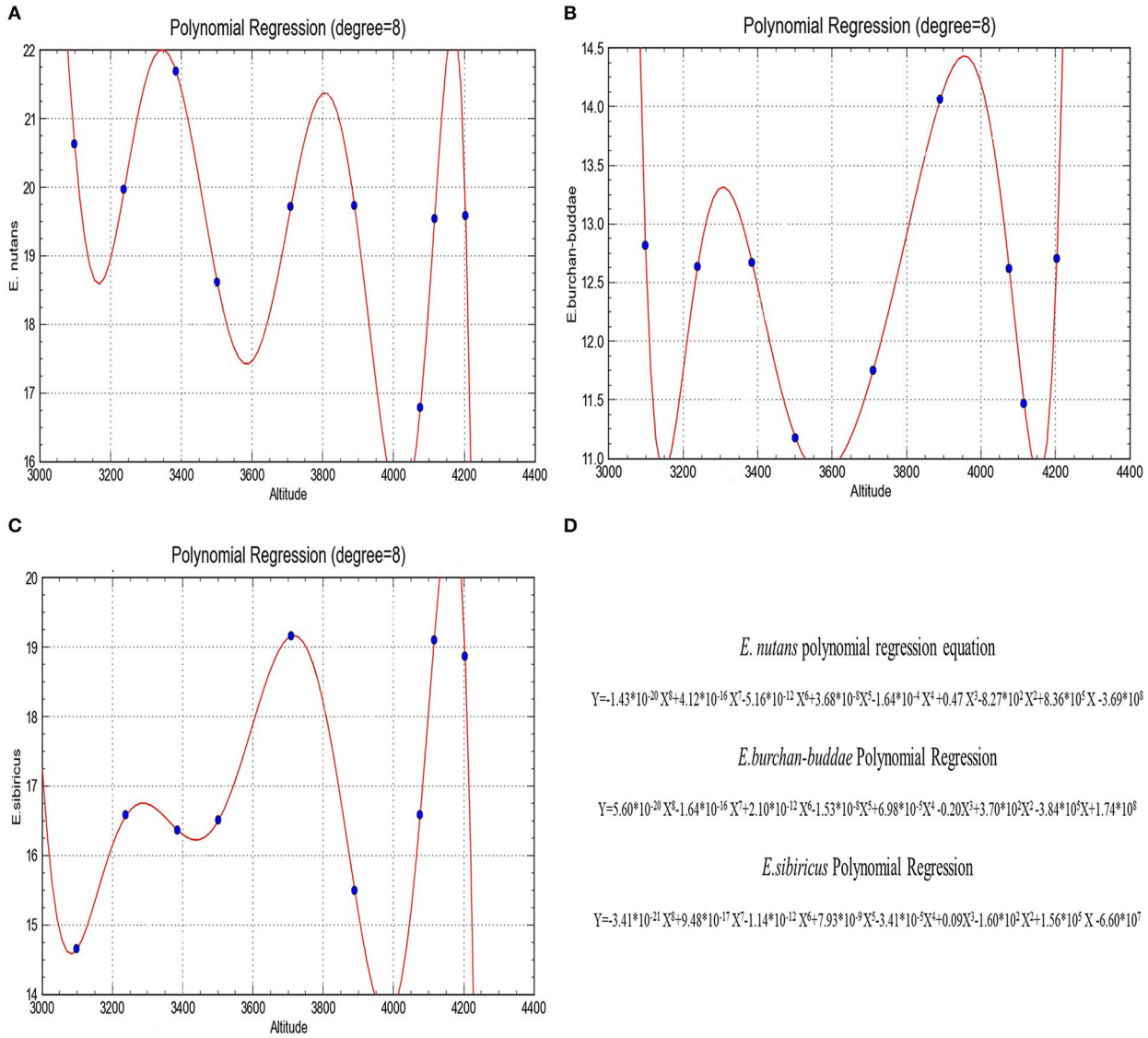

FIGURE 2 | Curve regression analyses of three *Elymus* species: **(A)** *E. nutans*, **(B)** *E. burchan-buddae*, and **(C)** *E. sibiricus*; **(D)**, polynomial regression analyses of three *Elymus* species.

using a one-way ANOVA (SPSS 20.0.) with least significant difference (LSD) and Duncan's multiple comparisons to evaluate significance within and among populations of three *Elymus* species.

Curve Fitting Analysis

In order to explore the specific varying pattern between altitude and nuclear DNA content of each population, curve regression analysis was used. The regression analysis was performed by Curve Expert Professional 2.3.0 to estimate the relationship between nuclear DNA content and altitude. The curve regression of three *Elymus* species is shown in (**Figure 2**), where the independent variables X and Y represent the altitude and the nuclear DNA content, respectively, and the *r*-value represents the correlation between two quantities. The curve regression analysis between altitude and nuclear DNA content estimated by flow cytometry of PI-stained samples showed a strong correlation with r values of 0.963, 0.987, and 0.998 of *E. nutans, E. burchan-buddae*, and *E. sibiricus*, respectively, which accounted for an obvious biological significance.

RESULTS

Altitudinal Variation in Nuclear DNA Content of Three Elymus Species

The mean 2C nuclear DNA of *E. nutans*, *E. burchan-buddae*, and *E. sibiricus* was in the range of 12.38–22.33, 8.81–18.93, and 11.46–20.96 pg/2C with averages of 19.59, 12.39, and 16.85

TABLE 1 | Nuclear DNA content (pg/2C) of three *Elymus* species in nine altitudes (between 3,098 and 4,203 m).

Species	Altitude	Minimum	Maximum	Average ± SE	CV (%)	Species Average
E. nutans	3,098	18.18	21.94	$20.63^{ab} \pm 0.53$	7.24	19.59
	3,238	16.14	21.89	$19.97^{ab} \pm 0.73$	10.36	
	3,385	21.17	22.33	$21.69^{a} \pm 0.15$	1.96	
	3,501	16.80	20.43	$18.62^{b} \pm 0.46$	6.99	
	3,709	17.81	21.52	$19.72^{ab} \pm 0.53$	7.65	
	3,889	17.59	21.66	$19.73^{ab} \pm 0.52$	7.42	
	4,075	12.38	20.21	$16.79^{c} \pm 0.83$	13.91	
	4,115	16.90	21.79	$19.54^{b} \pm 0.56$	8.04	
	4,203	17.38	21.09	$19.59^{b} \pm 0.71$	5.12	
E. burchan-buddae	3,098	11.13	16.53	$12.82^{ab} \pm 0.60$	13.34	12.39
	3,238	10.73	13.89	$12.64^{ab} \pm 0.41$	9.19	
	3,385	11.10	14.11	$12.67^{ab} \pm 0.38$	8.39	
	3,501	8.81	12.63	$11.18^{b} \pm 0.58$	14.70	
	3,709	10.71	13.36	$11.75^{b} \pm 0.39$	9.40	
	3,889	11.45	18.93	$14.06^{a} \pm 1.15$	20.00	
	4,075	10.36	15.71	$12.62^{ab} \pm 0.66$	14.88	
	4,115	9.10	15.69	$11.47^{b} \pm 0.77$	19.10	
	4,203	10.73	14.08	$12.71^{ab} \pm 0.49$	10.98	
E. Sibiricus	3,098	11.76	17.51	$14.65^{c} \pm 0.83$	15.94	16.85
	3,238	13.61	19.11	$16.58^{bc} \pm 0.58$	9.81	
	3,385	12.74	19.31	$16.37^{c} \pm 0.88$	13.13	
	3,501	11.46	19.62	$16.51^{bc} \pm 0.92$	15.76	
	3,709	17.50	20.96	$19.17^{a} \pm 0.42$	5.77	
	3,889	12.79	18.72	$15.52^{c} \pm 0.73$	13.33	
	4,075	14.86	18.53	$16.58^{bc} \pm 0.48$	8.18	
	4,115	16.70	20.74	$19.11^{a} \pm 0.45$	6.69	
	4,203	17.56	20.17	$18.87^{ab} \pm 1.30$	9.78	

Average, mean of nuclear DNA content (pg/2C) of 10 individuals in each population; Minimum, the minimum of nuclear DNA content (pg/2C) of 10 individuals in each population; Maximum, the maximum of nuclear DNA content (pg/2C) of 10 individuals in each population; the different letters indicate the significant differences ($P < 0.05$); SE, standard error; CV, coefficient of variation; Species Average, mean of nuclear DNA content (pg/2C) of all individuals of each species.

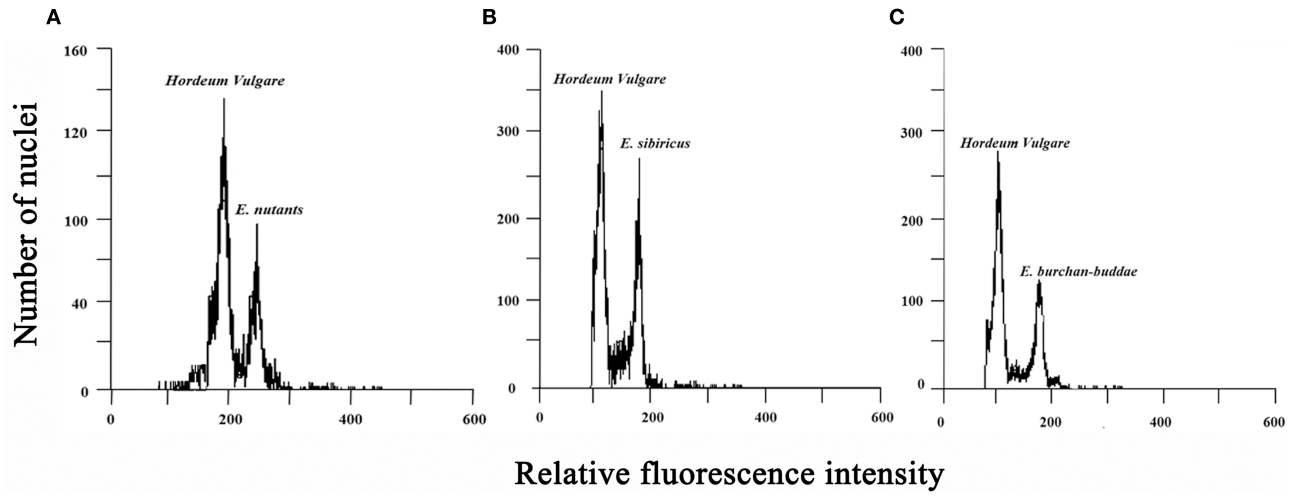

FIGURE 3 | Frequency distribution of the genome size of three *Elymus* species: **(A)**, *E. nutans*; **(B)**, *E. sibiricus*; and **(C)**, *E. burchan-buddae*. Internal standard: *Hordeum vulgare* L.

pg/2C, respectively (**Table 1**). The largest nuclear DNA content in *E. nutans* was observed for the population at an altitude of 3,385 m (22.33pg/2C), while the smallest was at an altitude of 4,075 m (12.38 pg/2C). For *E. burchan-buddae*, the population at an altitude of 3,889 m (18.93 pg/2C) and an altitude of 3,501 m (8.81 pg/2C) showed the largest and the smallest DNA content, respectively (**Table 1**). The largest and the smallest nuclear DNA content of *E. sibiricus* appeared at altitudes of 3,709 m (20.96 pg/2C) and 3,501 m (11.46pg/2C), respectively (**Table 1**).

The coefficient of variation ranges of *E. nutans*, *E. burchan-buddae*, and *E. sibiricus* were 1.96–13.91, 8.39–20.00, and 5.77–15.94%, respectively (**Table 1**). The largest CV values of *E. nutans*, *E. burchan-buddae*, and *E. sibiricus* were 13.91, 20.00, and 15.94% at 4,075, 3,889, and 3,098 m, respectively (**Table 1**). Hexaploid *E. nutans* showed a larger variation at 3,000–3,400 m; a high variation of tetraploid *E. sibiricus* occurred at 3,400–3,900 and 3,900–4,300 m; and *E. burchan-buddae* showed a high variation at 3,400–3,900 and 3,000–3,400 m. For hexaploid *E. nutans*, the nuclear DNA content was high in populations at 3,000–3,400 m, while the nuclear DNA content of tetraploid *E. burchan-buddae* and *E. sibiricus* was high in populations at 3,400–3,900 and 3,900–4,300 m, respectively (**Table 1**). There was a negative correlation between genome size variation and altitudinal gradient in *E. nutans*, while no correlation was found in *E. sibiricus* and *E. burchan-buddae*, which expounded significantly complex evolutionary of genome size of *Elymus* along an altitudinal gradient (**Figure 3**; **Supplementary Table 2**). The plant height ($r = 0.394$, $p < 0.05$) and palea length ($r = -0.720$, $p < 0.05$) were correlated with genome size in *E. nutans* (**Supplementary Table 2**).

Hybridization Between Elymus Species and Genome Donor Species

Genomic relationships of 27 populations (10 individuals for each population) among three *Elymus* species, namely, hexaploid *E. nutans,* tetraploid *E. burchan-buddae,* and *E. sibiricus*, at nine different altitudes were estimated by GISH with a total genomic DNA from *P. strigosa* labeled with biotin-11-dUTP and detected with a streptavidin Texas-Red conjugated antibody and a total genomic DNA from *H. bogdanii* labeled and detected with an anti-DIG-fluorescein (FITC). GISH clearly classified all chromosomes into three subgenomes, namely, H, St, and Y. Sequential GISH permitted the identification of individual chromosomes within each subgenome (**Figure 4**).

After DAPI staining, 28 chromosomes of *E. burchan-buddae* could be counted and showed blue fluorescence signals on metaphase (**Figure 4A**). When a total DNA probe from *P. strigosa* was hybridized into the chromosome of *E. burchan-buddae*, the strong hybridization of red fluorescence signals was observed on 20–24 chromosomes (**Figure 4B**). The same *in situ* hybridization pattern was obtained when using a total DNA probe from *H. bogdanii* on chromosomes of *E. burchan-buddae*; 8–12 chromosomes showed green fluorescence signals (**Figure 4C**). We found that the St genome had partial homology to the Y genome, and the H genome possibly had some relationship with the St or Y genome. Compared with the two groups of hybridization, the genomes St and H could be distinguished.

For *E. sibiricus*, 28 chromosomes showed blue fluorescence signals by DAPI (**Figure 4D**). When total DNA probes from *P. strigosa* and *H. bogdanii* were applied to chromosomes of *E. sibiricus*, some labeling could be detected as revealed by dispersed and spotted fluorescent signals. The major hybridization signals were the same, and 18–22 chromosomes showed red and green fluorescence signals (**Figures 4E,F**, respectively). We found that the St genome had partial homology to the H genome. The results verified the St genome in *E. sibiricus* from *P. strigosa* and the H genome in *E. sibiricus* from *H. bogdanii*.

Blue fluorescence signals were observed on 42 chromosomes of *E. nutans* after DAPI staining (**Figure 4G**). *In situ* hybridization with total DNA from *P. strigosa* and *H. bogdanii* revealed strong signals on chromosomes with 26–32 red and 20–26 green fluorescence (**Figures 4H,I**, respectively). The St genome had partial homology to the H genome, and the H genome could be distinguished. The relationship between the St genome and the Y genome was closer than that between the Y genome and the H genome compared with the two groups of hybridization.

DISCUSSION

In our study, we first applied FCM to evaluate intraspecific and interspecific genome size variations among nine populations of hexaploid *E. nutans* (StHY), tetraploid *E. sibiricus* (StH), and *E. burchan-buddae* (StY), which are morphologically very similar and have partly overlapping distribution areas (a continuous distribution on the Qinghai–Tibetan plateau at a certain altitude range), different ploidy and genome combinations, and genome presenting obvious regional differentiation (Ohsawa and Ide, 2008). It is no wonder that confusion sometimes arises during sampling, propagating, and investigating germplasm collections. However, based on FCM and genome sequencing, all three wild *Elymus* species can be recognized easily. In addition, *Hordeum vulgare* L. was considered an ideal internal standard due to its very few secondary compounds and approximate genome size to *Elymus* as well as strong genetic stability (Tuna et al., 2006; Chang et al., 2022). The most plausible reason for interspecific and intraspecific genome size variation was suggested to be one or the other kind of repetitive sequences along with complex genetic mechanisms, karyotypic, environmental, and phylogenetic factors (Du et al., 2017). Therefore, our study provided accurate and reliable data for assessing genome size variation.

Intraspecific and Interspecific Variation Among Elymus Species

Our study revealed highly statistical intraspecific variations of genome size along altitudinal gradients in three *Elymus* species despite their same geographical distribution. A significant intraspecific variation in maize (Lai et al., 2005) and *Brachionus asplanchnoidis* (Stelzer et al., 2019) was also reported. However, the mechanism resulting in genome size variation evolution is

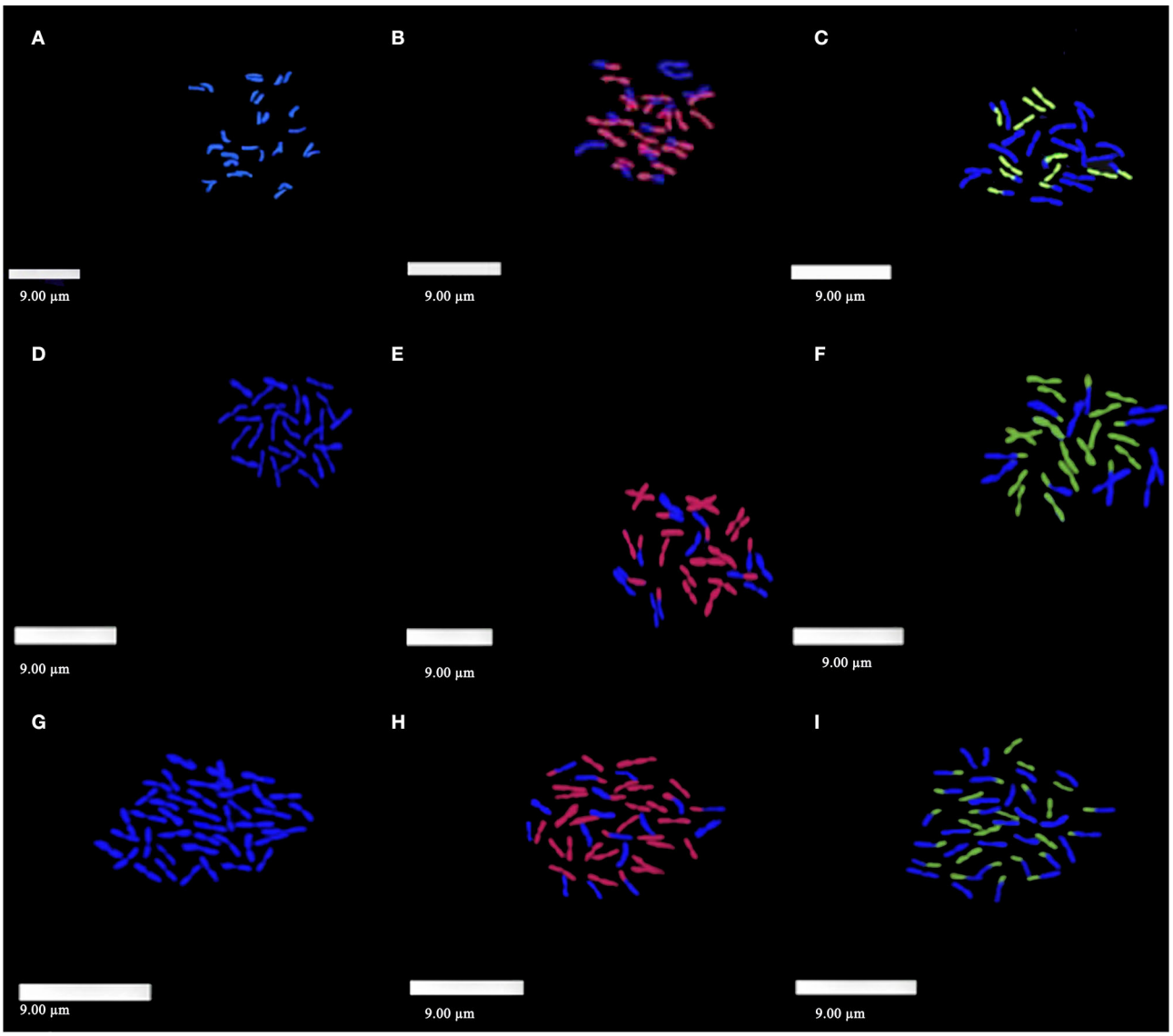

FIGURE 4 | Genomic *in situ* hybridization on somatic metaphase cells from root tips of *E. nutans*, *E. burchan-buddae*, and *E. sibiricus*, with *Pseudoroegneria strigosa* and *H. bogdanii* as probes. **(A–C)** *E. burchan-buddae*. **(A)** Twenty-eight chromosomes counterstained by DAPI on metaphase; **(B)** 24 chromosomes showed red fluorescence when probing with St-genome DNA of *P. strigosa*; **(C)** 12 chromosomes showed green fluorescence when probing with H-genome DNA of *H. bogdanii*. **(D–F)** *E. sibiricus*. **(D)** Twenty-eight chromosomes counterstained by DAPI on metaphase; **(E)** 22 chromosomes showed red fluorescence when probing with St-genome DNA of *P. strigosa*; **(F)** 22 chromosomes showed green fluorescence when probing with H-genome DNA of *H. bogdanii*. **(G–I)** *E. nutans*. **(G)** Forty-two chromosomes counterstained by DAPI on metaphase; **(H)** 32 chromosomes showed red fluorescence when probing with St-genome DNA of *P. strigosa*; **(I)** 26 chromosomes showed green fluorescence when probing with H-genome DNA of *H. bogdanii*.

complicated. One probable explanation is that populations of plants in high-altitude mountainous regions are always inclined to be geographically isolated and altitude can shape genome size to adapt to diverse and infertile environmental conditions. In addition, the distinct intraspecific differences observed in genome size in this study could be attributed to its non-strict self-pollinating in *Elymus*, resulting in a larger variation in genome size than self-pollinating species and unstable genome size (Eilam et al., 2007). Consequently, it is convincing that genome size diverges widely in populations, indicating highly genomic variability. No (Oney-Birol and Tabur, 2018) strong association (positive by Šmarda et al., 2008; negative by Charles and Jeremy, 2008) between genome size and plant height was previously reported. Different sampling strategies and plant species in these studies would lead to various associations; however, we found a positive association between genome size and plant height and between genome size and ploidy level. Our finding will be helpful in selecting germplasm with high forage productivity at the cellular level.

Our comprehensive study discriminated a significantly large genome size variation among the three wheatgrasses; therefore, they can be differentiated efficiently by genome size and

unambiguously regardless of their similar morphological features and overlapping distributing location. Our results showed a larger genome size in hexaploid *Elymus* species compared with that in tetraploid *Elymus* species. Larger genomes are considered to be more complicated with more frequency repeat sequences, larger intergenic spacer, and more introns, which might accelerate the evolutionary process of plant species. Moreover, greater genome size changes in hexaploid species demonstrated a wider genomic differentiation and indicated a faster evolution in the hexaploid species (Abbott and Lowe, 2004; Nakano et al., 2021) than in the tetraploid species. Large differences in evolutionary pressures, especially the additional H genome in hexaploid *E. nutans*, accelerated genomic variation, accommodating adequate adaptability and accounting for extensive altitudinal distribution in these areas with high altitude (Zhao et al., 2019). Besides, genome combination differentiation between the genomes Y and H in *E. sibiricus* and *E. burchan-buddae* can be the potential reason for an interspecific genome size variation, which corresponds to the previous research (Sun and Salomon, 2009). The origin of Y genome has always been a very interesting scientific question in wheatgrass phylogenetic studies (Sun and Salomon, 2009; Sun and Komatsuda, 2010; Tan et al., 2022). Our data showed that Y genome size is smaller than the H genome size, which provides a hint for discovering the potential progenitor and donor of the Y genome. Furthermore, interspecific genomic changes that occurred in the formation of polyploids resulted from hybridization, sequence rearrangements, and homologous recombination (Han et al., 2003; Urfusová et al., 2021).

Varying Pattern Between Genome Size and Altitude

Genome size variation of plant species has a momentous adaptive and evolutionary significance and is always related to environmental characteristics (Mráz et al., 2009; Meyerson et al., 2016), including latitude, longitude, and altitude generally. Our study claimed a wide variation of genome size among wheatgrass populations along altitudinal gradients and revealed a complex and strong association between altitude and genome size. In our previous work, higher genetic diversity was observed in populations at 3,400–3,900 m (medium altitudes) than those at 3,000–3,400 and 3,900–4,300 m (low and high altitudes, respectively) for the *Elymus* species (Yan et al., 2009). Interestingly, in this study, larger genome size variations occurred at the 3,400–3,900 m regions compared with the *Elymus* populations at other altitudes. The results yielded notable genomic evolution information within and among *Elymus* populations, especially at 3,400–3,900 m, revealing abundant genome size diversity. And population genetic relationships seemed to be closer with a similar altitude, and the curve regression analysis showed a strong association between genetic relationships of populations and their genome size at a particular altitude. Our study result also is consistent with that of Ohsawa and Ide (2008). Primarily, species abundance typically peaks at mid-altitude compared with the high- and low-altitude areas coupled with a severe ecological environment including low oxygen content, cold temperature, infertile soil, and water depletion, leading to a deteriorating habitat suitability for plants from the center to the edge (Herrera and Bazaga, 2008; Ohsawa and Ide, 2008). In addition, genetic variation across the altitudinal ranges is often centrally distributed and highly diverse at the mid-altitude regions in *Elymus* species as well as other widespread species (Brown, 1984). In contrast, marginal altitudes including high (3,900–4,300 m) and low (3,000–3,400 m) altitudes limited the opportunities for hybridization or gene introgression, which may narrow genomic variation. A larger temperature variation at the 3,900–4,300 m altitude compared with the other altitudes is a possible reason for generating more plentiful genomic variation among and within species (Koskey et al., 2018). Under the circumstances, a stronger plasticity and higher genomic variation at mid-altitudes (3,900–4,300 m) within *Elymus* populations under varying environmental conditions are well-annotated. These findings of the genomic variation patterns in *Elymus* across altitudinal gradients are favorable to polyploidy evolution and species formation, contributing to explaining the powerful adaptability of *Elymus* to the highland environment. From the standpoint of germplasm conservation and utilization, the mid-altitude was the conducive locality for collecting and developing QTP wheatgrass germplasm resources.

The Genomic Relationships Between the St, H, and Y Genomes in Elymus Species

Genome is generally defined as a full set of chromosomes in a haploid cell of a genome or all the genes in haploid cells as a genome. We found 1C nuclear DNA contents (genome sizes) of *E. nutans* (StHY), *E. burchan-buddae* (StY), and *E. sibiricus* (StH) were 9.82, 6.195, and 8.425 pg/1C, respectively. The nuclear DNA content of genome St (4.8 pg) is larger than that of genomes H (3.625 pg) and Y (1.395 pg), whereas the nuclear DNA content of genome Y is smaller than that of genome H. When hybridizing the probe *P. strigosa* with *E. burchan-buddae* and *E. sibiricus*, we found the relationship between the St genome and the Y genome was closer than that between the St genome and the H genome. Hybridization of *H. bogdanii* with *E. burchan-buddae* and *E. nutans* showed that the H genome had partial homology to the Y genome, and they are from different donors. Our results supported the hypothesis that the Y genome evolved in a diploid species and has a different origin from the St genome (Yan et al., 2011). RPB2 genes and transcription elongation factor G (EF-G) sequence analysis also indicates the Y genome has a different origin from the St genome (Sun and Salomon, 2009; Yan et al., 2011).

Our GISH results on *Elymus* showed the close homology between *Elymus* and the other two species including *P. strigosa* and *H. bogdanii*. Simultaneously, GISH data showed that the St, H, and Y genomes were closely related, and genomic homology was found among them. Lu and Bothmer (1990) suggested that genomes St, H, and Y have lower homology. A certain degree of homology of the St and Y genomes was reported in the intergeneric hybridizations among interspecific hybrids of three diploid species of *Pseudoroegneria* and four tetraploid species of

Roegneria (StY) and with three species of *Elymus* (StH, StYW) (Zhang et al., 2009). Zhang and Yan (2000) reported the St genome and Y genome in *Roegneria grandis* had high homology. We found the relationship between the St genome and the Y genome was closer than that between the St genome and the H genome, and the Y genome and the H genome.

CONCLUSION

An apparent genome size variation within the population of native *Elymus* species was observed along the altitude on the QTP, accounting for their strong adaptation to a changeable and harsh highland environment. Ploidy levels are regarded as the main biological character for genome variation, and genome size can significantly positively influence morphological traits such as plant height. More importantly, we found a higher chromosome variation in the population at 3,900–4,300 m. We speculated that more complicated genomic changes were associated with escalating altitudes in the Tibetan Plateau, and the mid-altitude (3,900–4,300 m) area fosters more genomic variation. We believe that this altitudinal range is ideal for the investigation, collection, and utilization of superior plant germplasm resources. These genomic and chromosomal changes promote the genetic variability and enable the newly formed allopolyploids to adapt to more changeable and harsher environments during the evolution of polyploid species, thus facilitating their rapid and successful establishment in nature.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

ZC and YoG conceived the project, designed the experiments, and wrote the paper. MH, JZ, ZG, and YuG helped perform the experiments. XY and GS revised the manuscript. All authors discussed the results, commented on the manuscript, and read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.923967/full#supplementary-material

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