Chromosomal localization of genes conferring desirable agronomic traits from *Agropyron cristatum* chromosome 1P

Cui Li Pan, Qingfeng Li, Yuqing Lu, Jinfeng Zhang, Xinning Yang, Xiuquan Li, Lihui Li*, Weihua Liu*

1 National Key Facility for Crop Gene Resources and Genetic Improvement, Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing, China, 2 College of Agronomy, Northwest A&F University, Yangling, Shaanxi, China

*These authors contributed equally to this work.

Abstract

*Agropyron cristatum* (L.) Gaertn. (2n = 4x = 28, PPPP), a wild relative of common wheat, possesses many potentially valuable genes for wheat breeding. To transfer these genes into wheat, a series of wheat-*A. cristatum* derivatives have been obtained in our laboratory. In this study, a wheat-*A. cristatum* derivative II-3-1 was obtained, which was proven to contain a 1P (1A) disomic substitution and 2P disomic addition line with 40 wheat chromosomes and two pairs of *A. cristatum* chromosomes by genomic in situ hybridization (GISH) and molecular markers analysis. By further backcrossing with the wheat parent Fukuhokomugi (Fukuho) and self-fertilization, three different lines were separated from II-3-1, including wheat-*A. cristatum* 1P disomic addition line II-3-1a, 2P disomic addition line II-3-1b and 1P (1A) disomic substitution line II-3-1c. Because 2P addition line had been reported before, we aimed to investigate 1P disomic addition line II-3-1a and wheat-*A. cristatum* 1P (1A) disomic substitution line II-3-1c. Analysis of different genetic populations demonstrated that 1P chromosome harbored multiple agronomic traits, such as elevated spike length, increased tillering ability, reduced plant height and spikelet density. Besides, bristles on the glume ridges as an important morphological marker was located on 1P chromosome. Therefore, the novel 1P addition and substitution lines will be used as important genetic materials to widen the genetic resources of wheat.

Introduction

Wild relatives of wheat belonging to tertiary gene pools are valuable sources of new genetic variation for wheat improvement [1]. *Agropyron* genus, which mainly includes *A. cristatum* (L.) Gaertn., *A. desertorum* (Fisch.) Schult., *A. fragile* (Roth.) Candargy, *A. michanoi* Roshev. and *A. mongolicum* Keng, is an important genus related to wheat and has three ploidy levels in nature, i.e. diploid (2n = 2x = 14, PP), tetraploid (2n = 4x = 28, PPPP) and hexaploid (2n = 6x = 42, PPPPPP) [2]. *A. cristatum* is the most common species of *Agropyron* genus distributed on...
Desirable agronomic traits from chromosome 1P

Materials and methods

Plant materials

Common wheat cv. Fukuho and *Agropyron cristatum* cv. Z559 were used as recipient parent and control, respectively. II-3-1 was identified from the F3 progenies of Z559 and Fukuho. II-3-1a and II-3-1b were isolated from the BC2F2 derived from the hybridization between II-3-1 and Fukuho. II-3-1 was backcrossed with common wheat Fukuho firstly, followed by the self-pollination for three times. II-3-1c was selected from F4 generation. The genetic populations BC1F2 and BC2F2 were from then backcrossing and self-pollination of II-3-1a and Fukuho. All the materials were developed and provided by the Center of Crop Germplasm Resources Research in the Institute of Crop Science, Chinese Academy of Agricultural Sciences (CAAS), Beijing, China.

Cytological analyses of mitosis and meiosis

Chromosome preparations of root tips were made according to the previously described procedure [26]. For the pollen mother cells meiosis studies, the procedures were followed by Jauhar and Peterson [27].
Genome In Situ Hybridization (GISH)

The alien *A. cristatum* chromosomes were detected by GISH using *A. cristatum* cv. Z559 genome DNA as probe and common wheat cv. Fukuho genome DNA as blocker. The total genomic DNA of common wheat Fukuho and *A. cristatum* Z559 were isolated using a modified CTAB method [28]. The GISH procedure was described by Liu et al. [29].

Fluorescence In Situ Hybridization (FISH)

Probe pAs1 from *Aegilops tauschii* Coss. combined with probe pHvG39 from barley could distinguish A, B and D genome of common wheat [30]. *A. cristatum* repeats sequence pAcTRT1 and pAcpCR2 probes presenting different signals on different *A. cristatum* chromosomes could detect which *A. cristatum* chromosomes was included [24]. FISH procedure was described by Luan et al. [15]. All cytological images were observed under an OLYMPUS AX80 (Olympus Corporation, Tokyo, Japan) fluorescence microscope and captured with a CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

Molecular markers analysis

In order to identify alien chromosomes and determine homoeologous relationships between *A. cristatum* and wheat chromosomes in II-3-1, II-3-1a, II-3-1b, and II-3-1c, a total of 92 *A. cristatum* EST-STS markers from *A. cristatum* transcriptome sequences that corresponding to wheat homoeologous group 1 and 2 were used [25]. To detect presence/absence of wheat chromosomes in II-3-1 and II-3-1c, 33 wheat SSR markers located on wheat chromosome 1A, 1B and 1D were screened from the GrainGenes 2.0 website (https://wheat.pw.usda.gov/GG3/). All the information of primers was listed in S1 and S2 Tables. PCR amplification procedure and electrophoresis were described by Luan et al. [15].

Evaluation of agronomic traits

The addition line II-3-1a, substitution line II-3-1c, and their parents II-3-1 and Fukuho were planted at the experimental farm of Xinxiang of Henan province in China in two sowing seasons (2014–2015 and 2015–2016). All the materials were planted in the randomized block arrangement with three repeats with spacing 30 cm apart and rows 2.0 m long with 20 grains per row. Plant height, spike length, spikelets per spike, kernels per middle spikelet, kernels per main spike, fertile tiller numbers, thousand grain weight and bristles on the glume ridges were evaluated. Similarly, two populations developed from continuous backcrosses and selfcrosses of II-3-1a and the recurrent parent Fukuho. These populations were planted in the 2014–2015 and 2015–2016 growing season to evaluate the key agronomic traits. Each individual was identified with the P genome-specific STS markers, and each population was then divided into two groups according to the absence of *A. cristatum* 1P specific markers. All the traits were measured on each plant from the segregating populations and on 20 plants randomly selected from the parents II-3-1, II-3-1a, II-3-1c and Fukuho. The data was analyzed by the Statistical Analysis System version 9.2 (SAS Institute Inc., Cary, NC, USA) adopting Duncan’s multiple range tests analysis of variance at the P = 0.05 significance levels.

Results

Chromosome composition analysis of II-3-1

Chromosome counting and GISH analysis showed that there were 44 chromosomes in the somatic cells of II-3-1, including 40 wheat chromosomes (shown in blue) and 4 *A. cristatum* chromosomes (shown in red) (Fig 1a). Chromosomal configurations at the metaphase I of
pollen mother cells were counted in II-3-1, showing 2n = 22 II, with averages of 0.36 univalents, 2.18 rod bivalents, 19.55 ring bivalents, and 0.06 trivalents (Table 1). The presence of twenty wheat bivalents (shown in blue) and two A. cristatum bivalents (shown in red) suggested that the behaviors of chromosome pairing were regular (Fig 1b). All the results indicated that II-3-1 containing two pairs of A. cristatum chromosomes was cytologically stable.

**Fig 1.** GISH-FISH patterns of the root cells and pollen mother cells at meiotic metaphase I of II-3-1. (a) GISH analysis of II-3-1 showing red hybridization signals evenly distributed on P chromosomes with A. cristatum genomic DNA as the probe and Fukuho DNA as the blocker. Wheat chromosomes were counterstained with DAPI (blue). (b) Pollen mother cells of II-3-1 at meiotic metaphase I. (c) FISH detection of chromosome 1P and 2P using pAcTRT1 and pAcPCR2 as probes. (d) FISH detection using pAs1 (red) and pHvG38 (green) repetitive DNA as probes showing that 1A chromosomes are missing in II-3-1.

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FISH using pAcTRT1 and pAcpCR2 as probes demonstrated that the two pairs of chromosomes added to II-3-1 were 1P and 2P, respectively (Fig 1c). In order to detect which wheat chromosomes were absent in II-3-1, GISH-FISH and wheat SSR markers were used. GISH-FISH results demonstrated that a pair of *A. cristatum* chromosomes were replaced with a pair of wheat 1A chromosomes (Fig 1d). Thirty-three wheat SSR markers on wheat 1A, 1B and 1D were chose to further confirm detect which wheat chromosomes were absent in II-3-1. The results showed that 6 markers on chromosome 1B and 9 markers on chromosome 1D displayed specific bands in II-3-1 (Fig 2a and 2b), while 18 markers on chromosome 1A displayed no specific bands (Fig 2c). This result was consistent with the result of GISH-FISH identification, both of which indicated that the 1A chromosomes were substituted in II-3-1. In addition, three 1A-specific markers (barc287, cfe267, and cfe77) polymorphism between chromosomes 1P and 1A were screened (Fig 2d), suggesting that the added alien chromosomes and the absent wheat chromosomes belonged to the same homoeologous group. What’s more, fifty-six *A. cristatum* EST-STS markers aligned to wheat homoeologous group 1 and thirty-six aligned to wheat homoeologous group 2 can both amplified specific products in II-3-1 (Fig 2e and 2f). In conclusion, wheat-*A. cristatum* derivative II-3-1 contained a 1P (1A) disomic substitution and 2P disomic addition line.

Production and molecular cytogenetic identification of 1P chromosomal addition line

II-3-1a was obtained from the BC$_2$F$_2$ progenies of II-3-1 and Fukuho (Table 1). They formed 22 bivalents with an average pairing frequency of $2n = 44 = 0.61 + 2.29$ rod II + 19.41 ring II during meiotic metaphase I. GISH results of somatic cells showed that they contained 44 chromosomes including 42 wheat chromosomes and 2 of *A. cristatum* (Fig 3a and 3b). FISH identification with pAcTRT1 and pAcpCR2 as probes showed that the pair of chromosomes added were *A. cristatum* 1P (Fig 3c). Fifty-six *A. cristatum* EST-STS markers of group 1 also gave specific products to II-3-1a (Fig 2e). Those markers specific to 1P could not only be used to trace and identify chromosome 1P but also locate desirable genes.

Production and molecular cytogenetic identification of 1P chromosomal substitution line

II-3-1c was identified from the F$_4$ progenies of II-3-1 (Fig 4a). Chromosome configuration at meiotic MI of PMCs showed 21 bivalents with an average pairing frequency of $2n = 42 = 0.16$

| Materials | 2n | No. of cells | Univalent | Bivalent | Chromosome configuration | trivalent |
|-----------|----|--------------|-----------|----------|--------------------------|----------|
| II-3-1    | 44 | 120          | 0.36      | 2.18     | Rod: 19.55, Ring: 21.73   | 0.06     |
|           |    |              | (0–3)     | (1–4)    | (18–22)                 | (21–22)  |
| II-3-1a   | 44 | 120          | 0.60      | 2.29     | Rod: 19.41, Ring: 21.70  |          |
|           |    |              | (0–2)     | (0–3)    | (19–22)                 | (21–22)  |
| II-3-1c   | 42 | 120          | 0.16      | 1.23     | Rod: 19.60, Ring: 20.84  | 0.05     |
|           |    |              | (0–2)     | (1–3)    | (19–21)                 | (20–21)  |
| Fukuho    | 42 | 100          | 0.06      | 1.95     | Rod: 19.32, Ring: 20.97  |          |
|           |    |              | (0–2)     | (1–4)    | (17–21)                 | (20–21)  |

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I + 1.23 rod II + 19.60 II ring + 0.05 trivalents during meiotic metaphase I (Fig 4b) (Table 1).

FISH identification with pAcTRT1 and pAcpCR2 probes demonstrated that the two A. cristatum chromosomes in II-3-1c were a pair of A. cristatum 1P chromosomes (Fig 4c). All the fifty-six EST-STS markers specific to chromosome 1P could be amplified characteristic products in II-3-1c (Fig 2e). GISH-FISH identification indicated that the substituted wheat
chromosomes were a pair of chromosomes 1A (Fig 4d). The molecular cytogenetic identification suggested that II-3-1c was a stable wheat-\textit{A. cristatum} 1P (1A) substitution line.

The analysis of desirable genes for wheat improvement on chromosome 1P

The statistic analysis suggested that 1P addition line II-3-1a differed from Fukuho in plant height (Fig 5a), spike length (Fig 5b), spikelet density, effective tiller numbers and the bristles on the glume ridges (Fig 5c) in the sowing years 2014–2015 and 2015–2016 (Table 2). In order to define the source of these traits, 1P addition line II-3-1a was crossed with common wheat Fukuho to construct BC\textsubscript{1}F\textsubscript{2} and BC\textsubscript{2}F\textsubscript{2} populations. In BC\textsubscript{1}F\textsubscript{2} and BC\textsubscript{2}F\textsubscript{2} populations, the average height of plants with chromosome 1P were 89.55 cm and 75.85 cm, respectively, significantly lower than those without 1P which were 94.41 cm and 80.00 cm, respectively. The average spikelet density of plants carrying chromosome 1P were 15.58 and 16.58, respectively, significantly lower than those lack of chromosome 1P which were 17.20 and 18.37, respectively. The average spike length of plants with chromosome 1P were 11.12 cm and 10.75 cm, respectively, significantly longer than those without chromosome 1P which were 9.85 cm and 9.66 cm, respectively. The average tiller numbers of plants carrying chromosome 1P were

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**Fig 3.** GISH-FISH patterns of the root cells and pollen mother cells at meiotic metaphase I of II-3-1a. (a) GISH analysis of II-3-1a showing red hybridization signals evenly distributed on P chromosomes with \textit{A. cristatum} genomic DNA as the probe and Fukuho DNA as the blocker. Wheat chromosomes were counterstained with DAPI (blue). (b) Pollen mother cells of II-3-1a at meiotic metaphase I. (c) FISH detection of chromosome 1P and 2P using pAcTRT1 and pAcpCR2 as probes.

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23.18 and 18.00, respectively, more than those lack of chromosomes 1P which were 18.28 and 13.73, respectively. The bristles were present on the glume ridges in the plants with chromosome 1P and absent in the plants without 1P chromosomes, confirming that the gene controlling bristles on the glume ridges were located on chromosome 1P. The results above confirmed that the variation of plant height, spike length, spikelet density, tiller numbers and  

Fig 4. GISH-FISH patterns of the root cells and pollen mother cells at meiotic metaphase I of II-3-1c. (a) GISH analysis of II-3-1c showing red hybridization signals evenly distributed on P chromosomes with A. cristatum genomic DNA as the probe and Fukuho DNA as the blocker. Wheat chromosomes were counterstained with DAPI (blue). (b) Pollen mother cells of II-3-1c at meiotic metaphase I. (c) FISH detection of chromosome 1P and 2P use pAcTRT1 and pAcpCR2 as probes. (d) FISH using pAs1 (red) and pHvG38 (green) repetitive DNA as probes showing that 1A chromosomes are missing in II-3-1c.

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Table 2. Evaluation of agronomic traits of II-3-1a, II-3-1c, Fukuho and genetic populations derived from II-3-1a and Fukuho.

| Year | Materials | PH(cm) | SL(cm) | SNPS | SD | KNMS | GNPS | FTN | TGW(g) | WGB |
|------|-----------|--------|--------|------|----|------|------|-----|-------|-----|
| 2015 | Fukuho    | 94.32±4.91a | 10.18±1.01b | 18.55±1.21a | 17.37±2.04a | 4.55±0.52a | 64.27±7.04a | 18.91±5.32b | 34.96±2.98a | no  |
|      | II-3-1a   | 88.92±6.10b | 11.15±0.73a | 18.53±1.16a | 15.81±1.20b | 4.08±0.51a | 60.67±5.07ab | 24.58±4.94a | 32.72±5.19a | yes |
|      | II-3-1c   | 83.78±3.38c | 9.36±0.63c | 17.78±0.83a | 18.02±1.66a | 4.44±0.53a | 58.00±8.11b | 25.00±6.44a | 23.83±2.10b | yes |
|      | BC1F2    | 88.65±5.41b | 11.14±0.74a | 18.40±2.22a | 15.58±1.24b | 4.20±0.42a | 60.20±4.80ab | 24.10±3.41a | 33.16±4.40a | yes |
|      | BC1F2 +  | 94.45±4.50a | 10.34±0.58b | 18.73±1.19a | 17.20±1.57a | 4.45±0.52a | 64.09±5.17a | 18.27±3.44b | 34.64±4.07a | no  |
|      | BC1F2 -  | 94.45±4.50a | 10.34±0.58b | 18.73±1.19a | 17.20±1.57a | 4.45±0.52a | 64.09±5.17a | 18.27±3.44b | 34.64±4.07a | no  |
| 2016 | Fukuho    | 81.26±2.69a | 9.57±0.59b | 18.55±1.05a | 18.41±1.54a | 4.95±0.39a | 69.80±4.53ab | 13.75±3.46b | 36.77±3.60a | no  |
|      | II-3-1a   | 74.36±4.28b | 10.75±0.71a | 18.31±1.32a | 16.19±1.74b | 5.15±0.38a | 71.92±5.83a | 17.15±3.18a | 34.18±2.39a | yes |
|      | II-3-1c   | 69.74±2.21c | 8.49±0.40c | 17.07±0.83a | 18.97±1.30a | 5.07±0.28a | 65.14±4.07b | 17.21±2.33a | 26.38±1.15b | yes |
|      | BC2F2 +  | 75.85±2.82b | 10.75±0.49a | 18.82±1.08a | 16.58±1.03b | 4.91±0.30a | 71.91±10.27a | 18.00±2.28a | 34.95±3.32a | yes |
|      | BC2F2 -  | 81.33±2.79a | 9.66±0.36b | 18.73±0.70a | 18.37±0.80a | 4.93±0.26a | 69.73±5.09ab | 13.73±2.15b | 36.63±5.17a | no  |

Note: PH, Plant height; SL, Spike length; SNPS, Spikelet number per spike; SD, spikelets density; KNMS, Kernel number in the middle spikelet; GNPS, Grain number per spike; FTN, Fertile tiller number per plant; TGW, Thousand-grain weight; WGB, wheat glume bristles. '+' indicated the progenies contained A. cristatum 1P chromatin; '-' indicated the progenies contained no A. cristatum 1P chromatin. Significant differences in the mean are indicated at the P < 0.05 (lower-case letters) levels, based on Duncan’s multiple range tests.

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bristles on the glume ridges were affected by chromosome 1P. Therefore, wheat-\textit{A. crista\-tum}\ 1P addition line II-3-1a could be a potential breeding material to reduce plant height and spikelet density, and to improve spike length and tillering ability. Additionally, the presence of bristles on the glume ridges was a typical characteristic of chromosome 1P which could be used to identify and trace the plants carrying 1P chromosome in segregating progenies. The wheat-\textit{Agropyron cristatum} 1P addition line II-3-1a and 1P (1A) substitution line II-3-1c could provide novel genetic resources for producing translocation line for wheat improvement.

**Discussion**

**Potential value of production of wheat-\textit{A. crista\-tum} disomic 1P addition line and 1P (1A) substitution line**

Hybridization between wheat and its wild related species facilitates the utilization of exogenous desirable genes for wheat improvement. The disomic addition and substitution lines play bridge tools for the transfer of exogenous genes. To date, a large number of wild relatives of wheat have been used to establish disomic addition lines by hybridizing with wheat, such as \textit{Thinopyrum intermedium} [31–33], \textit{Secale cereale} L. [34], \textit{Dasypyrum villosum} [35], and \textit{Hordeum vulgare} [36–37]. Disomic addition lines could be used to locate genes on the alien chromosome and induce translocation line by \textit{Ph1b} gene, gametocidal chromosome or ionizing irradiation. However, alien chromosomes might be lost in the disomic addition line during self-fertilization because of its limited stability. Since alien chromosomes in the substitution lines showed the homoeologous compensation with the substituted wheat chromosomes, so wheat breeders tend to use substitution lines in breeding program. To produce disomic substitution lines, disomic addition lines were always backcrossed by the responding nullisomic of common wheat. For example, wheat-barely 2H addition line was backcrossed by 2D nullisomic of wheat to get 2H-2D substitution line [38]. The monosome substitution line produced by the cross of disomic substitution line with common wheat could generate the translocation line due to misdivision. So the translocation lines were more stable and more useful for investigating homoeologous compensation between the alien segments and the deleted wheat segments, and for breeding new variety. In this study, a wheat-\textit{A. crista\-tum} 1P addition line and a 1P (1A) substitution line was obtained respectively, which not only laid a good foundation for the research of genes on 1P chromosome but also provided materials for the production of translocation lines.

**Identification of \textit{A. crista\-tum} 1P chromosomes in addition and substitution lines with molecular markers**

Improvement of sequencing technologies and reduction of its cost laid good foundation for developing diverse functional molecular markers [20]. Molecular markers have potential to trace alien genes from wild species, identify homoeologous relationship between alien and wheat chromosomes and provide additional markers for comparative mapping. The transferability of wheat SSR markers was particularly important for genetic analysis, especially for the wheat-related species that had no genomic libraries [39]. Because EST sequences are always present in the expressed regions and conserved in gene transcripts, EST markers have been used to research homoeologous relationships among the species of \textit{Poaceae}. For examples, wheat EST-SSR markers were used to determine the chromosome 5Ns in wheat-\textit{Psathyrostachys huashanica} disomic addition line [40]. Four SSR markers showing length-polymorphisms between chromosomes 6B and 6G were obtained to monitor the 6B and 6G chromosomes in segregating generations involving the 6G (6B) substitution line [41]. In our study, three wheat
SSR markers (cfe267, cfe77, and barc287) that could distinguish wheat chromosome 1A and A. cristatum chromosome 1P were screened. These three polymorphic markers could not only be used to monitor the 1P chromosome in segregating populations but also demonstrate the homoeologous relationship between 1A and 1P.

A. cristatum P-genome-specific markers have also made great progress due to the improvement of sequencing technologies. Wu et al. [23] obtained three SCAR markers using A. cristatum repetitive sequence. Han et al. [24] separated two A. cristatum repetitive sequence which could recognize A. cristatum chromosomes using DOP-PCR, and P-genome-specific STS primers were developed from A. cristatum transcriptome sequences [25]. A. cristatum STS markers were used to determine wheat-A. cristatum 6P addition line and locate genes on different chromosome segments [8, 15–17, 42]. In this study, 56 EST-STS markers specific to chromosome 1P were screened from A. cristatum transcriptome sequences that aligned on wheat homoeologous group 1. Those results demonstrated that the alien chromosomes in II-3-1a and II-3-1c were homoeologous group 1.

Genes on chromosome 1P are potentially valuable in wheat breeding

Wild relatives of wheat are ideal gene pools for disease resistance, yield and quality. Many excellent alien genes have been transferred into wheat and played important roles in wheat improvement. In wheat production, the T1B.1R translocation line showing resistance against rust and powdery mildew, as well as good yielding capacity was efficiently used in wheat breeding programme [43–45]. A. cristatum, one important wild relatives of wheat, has various excellent genes. In this study, we found that multiple genes which controlling spike length, strengthen tillering ability, lower plant height and reduce spikelet density were located on chromosome 1P. The dwarfing genes Rht-B1, Rht-D1 and Rht8 had played great roles in deducing wheat plant height [46, 47]. Our results showed that the plants with chromosome 1P were significantly lower than those without chromosome 1P. It was predicted that chromosome 1P contained a novel dwarf genes which will provide new source for the dwarf gene family. Chromosome 1P also carried a gene (s) controlling the presence of bristles on the glume ridges which could be a typical characteristic to trace and identify 1P chromosomes. The wheat-A. cristatum 1P addition line and 1P (1A) substitution line will provide more useful genes and many great genetic diversity for wheat improvement.

Supporting information

S1 Table. Primer sequences of 56 1P-specific STS markers and 36 2P-specific STS markers. (DOC)

S2 Table. Primer sequences of wheat chromosome 1A, 1B, and 1D-specific SSR and EST markers. (DOC)

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Author Contributions

Conceptualization: LHL WHL.

Data curation: CLP QFL YQL JPZ XMY XQL.
Formal analysis: CLP QFL.

Funding acquisition: LHL YQL WHL.

Investigation: CLP.

Methodology: CLP QFL.

Project administration: LHL YQL WHL.

Resources: LHL WHL.

Software: CLP QFL.

Supervision: LHL WHL.

Validation: LHL WHL CLP.

Visualization: LHL WHL.

Writing – original draft: CLP QFL.

Writing – review & editing: CLP QFL YQL LHL WHL.

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