Draft genomes assembly and annotation of *Carex parvula* and *Carex kokanica* reveals stress-specific genes

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*Kobresia* plants are important forage resources on the Qinghai-Tibet Plateau and are essential in maintaining the ecological balance of grasslands. Therefore, it is beneficial to obtain *Kobresia* genome resources and study the adaptive characteristics of *Kobresia* plants on the Qinghai-Tibetan Plateau. Previously, we have assembled the genome of *Carex littledalei* (*Kobresia littledalei*), which is a diploid with 29 chromosomes. In this study, we assembled genomes of *Carex parvula* (*Kobresia pygmaea*) and *Carex kokanica* (*Kobresia royleana*) via using Illumina and PacBio sequencing data, which were about 783.49 Mb and 673.40 Mb in size, respectively. And 45,002 or 36,709 protein-coding genes were further annotated in the genome of *C. parvula* or *C. kokanica*. Phylogenetic analysis indicated that *Kobresia* in Cyperaceae separated from Poaceae about 101.5 million years ago after separated from *Ananas comosus* in Bromeliaceae about 117.2 million years ago. *C. littledalei* and *C. parvula* separated about 5.0 million years ago, after separated from *C. kokanica* about 6.2 million years ago. In this study, transcriptome data of *C. parvula* at three different altitudes were also measured and analyzed. *Kobresia* plants genomes assembly and transcriptome analysis will assist research into mechanisms of plant adaptation to environments with high altitude and cold weather.

*Kobresia* plants (Cyperaceae) are the most important component of alpine grasslands on the Qinghai-Tibet Plateau\(^1\). *Kobresia* plants are important forage resources in alpine areas and are essential in maintaining the ecological balance of grasslands for their tolerance of cold, radiation, drought and strong wind\(^2\). The sedge *C. parvula* is the dominant species of high-altitude pastures in Tibet, and it is the most important source of forage in animal husbandry. It grows in alpine steppe, alpine meadow and swamp meadow on river beach, hillside, valley and terrace at an altitude of 3700–5300 m. *C. parvula* reaches only 2–3 cm in height, making it highly grazing tolerant. *C. kokanica* grows in moist grassland, swamp meadow and meadow grassland on hillsides, gullies, lakeside, alluvial fan and flood plain at an altitude of 3100–5200 m. *C. kokanica* reaches 10–60 cm in height and the morphological difference of this species is largely differed with altitudes.

Reference genomes of various individuals can provide new insights into genomic structure and evolution, genetic diversity, and phylogeny\(^3\). We have previously assembled the genome of *Carex littledalei*, which is a diploid with 29 chromosomes\(^4\). Basic chromosome numbers and ploidy are varied in different *Kobresia* species and difficult to verify, which take great challenge to the assembly of *Kobresia* genomes. Combined application of chromosome counts, new microsatellite markers and flow cytometry confirmed tetraploidy in *C. parvula* (2n = 4x = 64)\(^5\). Chromosome numbers of *Kobresia esenbeckii* (2n = 66), *Kobresia duthiei* (2n = 84), *Kobresia curvata* (2n = 50), *Kobresia schoenoides* (2n = 32) were reported and it is concluded that chromosomal evolution in the unispicate *Kobresia* species may have been caused by both polyploidy and aneuploidy\(^6\).

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This study assembled and annotated genomes of *C. parvula* and *C. kokanica* based on long reads from the PacBio Sequel sequencing platform and short reads from the Illumina Hi-seq 2500 sequencing platform. The final genome assembly of *C. parvula* is approximately 783.49 Mb with a contig N50 of 468.079 kb. We predicted 45,002 protein-coding genes from the generated assembly of *C. parvula*, and 94.7% (42,630 genes) of all protein-coding genes were annotated. At the same time, genome assembly of *C. kokanica* is approximately 673.40 Mb with a contig N50 of 1.179 Mb. We predicted 36,709 protein-coding genes from the generated assembly of *C. kokanica*, and 96.6% (35,477 genes) of all protein-coding genes were annotated. The results of comparative genomic analysis have tentatively clarified the origin of the two species and revealed their genomic characteristics, which provide new insights for studies exploring genome evolution and reveal stress-specific genes of them.

**Data description**

**Sample sequencing and genome size estimation.** *C. parvula* (E: 91°59.7597′ N: 31°35.9755′) and *C. kokanica* (E: 91°10.5554′ N: 30°29.8873′) in anthesis stage were collected in July 2018 from Dangquka Village, Dangquka Town, Damxung County in the Tibet Autonomous Region of China. *C. parvula* and *C. kokanica* located at altitudes of up to 4600 m and 4275 m, respectively (Fig. 1). High-quality genomic DNA of *C. parvula* and *C. kokanica* was extracted from leaf tissue separately. Illumina’s Genomic DNA Sample Preparation kit was used for preparation of sequencing library. DNA library with insert sizes of 350 bp was sequenced on an Illumina HiSeq 2500 platform. 53.07 Gb and 55.31 Gb of short cleaned reads were generated for k-mer analysis and base correction, and their coverage were 137.59 × and 128.48 × of *C. parvula* and *C. kokanica* respectively (Supplemental Table S1, coverage were calculated based on genome size of haploid). The genome size was calculated based on k-mer (k = 17) statistics using the modified Lander–Waterman algorithm. As a result, the genome size of *C. parvula* was estimated to be 385.7 Mb (genome size of haploid) with 1.74% heterozygosity and a repeat sequence ratio of 47.97%, and *C. kokanica* was estimated to be 430.51 Mb (genome size of haploid) with 2.00% heterozygosity and a repeat sequence ratio of 47.84% (Supplemental Table S2, Supplemental Fig. S1). Meanwhile, we inferred that *C. parvula* is tetraploid and *C. kokanica* is triploid from k-mer analysis (Supplemental Fig. S1).

**Assembly of *C. parvula* and *C. kokanica* genome.** First, pre-assembly reads were obtained by self-correcting of PacBio long reads and assembled into contigs by FALCON (falcon-kit = 0.7) through the “Overlap-Layout-Consensus” algorithm. Contigs were corrected using PacBio long reads with quiver (smrtlink_6). Then, one round of polishing were applied to the assembled contigs using Pilon-1.18 with the Illumina short reads. To improve the assembly of *C. parvula*, contigs were linked into scaffold using 93.27G 10 × data using FragScaffold (PBSuite_15.8.24). The preliminary genome assembly of *C. parvula* includes 5261 contigs with N50 = 468,079 bp and longest scaffold = 7,682,949 bp. The genome is approximately 783.49 M in length and the GC content of the genome is 35.41% (Table 1). The preliminary genome assembly of *C. kokanica* includes 1504 contigs with N50 = 1,179,729 bp and longest scaffold = 4,432,967 bp. The genome is approximately 673.40 M in length and...
Table 1. Statistics of assembled Carex parvula and Carex kokanica assembly and annotation.

| Genome assembly | Carex parvula (Kobresia pygmaea) | Carex kokanica (Kobresia royleana) |
|-----------------|----------------------------------|-----------------------------------|
| Number of scaffolds | 5261 | 1504 |
| Total length of scaffolds (bp) | 783,486,840 | 673,403,748 |
| N50 of scaffolds (bp) | 468,079 | 1,179,279 |
| Longest scaffold (bp) | 7,682,949 | 4,432,967 |
| GC content (%) | 35.41 | 34.68 |

Repeat annotation

| Total (bp) | 411,079,196(52.47%) | 373,538,444(55.47%) |
| TRF (bp) | 38,080,191(4.86%) | 37,108,299(5.31%) |
| Transposable element (bp) | 403,711,071(51.53%) | 367,847,237(54.63%) |

Gene annotation

| Number of genes | 45,002 | 36,709 |
| Total coding sequence length (bp) | 154,945,486 | 124,438,004 |
| Mean gene length (bp) | 3443.08 | 3389.85 |
| Mean number of exons per gene | 5 | 5.73 |
| Mean exon length (bp) | 221.49 | 212.93 |
| Average CDS length (bp) | 1107.86 | 1220.16 |

the GC content of the genome is 34.68% (Table 1). Compared with the estimated genome size, 1.56 (C. kokanica)–2.03 (C. parvula) times of genome sequences were assembled.

Repeat annotation of Carex parvula and Carex kokanica genome. We used LTR Finder\(^{10}\), RepeatScout (http://www.repeatmasker.org/) and RepeatModeler to identify ab initio repeat sequence library. Then RepeatMasker was used to predict repeat sequences of the genome through similarity searching of repetitive elements released by Repbase\(^{11}\) and our ab initio identified repeat sequence library. We also predicted repetitive elements by RepeatProteinMask and tandem repetitive sequences by TRF\(^{12}\). For C. parvula, a total of ~411.08 M repetitive elements including ~37.11 M tandem repetitive sequences and 367.85 M TE, which was 55.47% of the genome (Supplemental Table S3). Among them, DNA transposons accounted for 18.60% of the genome. Retrotransposon including long terminal repeat (LTR), long interspersed nuclear elements (LINE) and short interspersed nuclear elements (SINE) accounted for 23.84%, 6.77% and 0.03% of the genome, respectively (Supplemental Table S4).

Prediction and functional annotation of protein-coding genes. Ab initio, homology-based and RNA-seq-based pipelines were integrated for subsequent prediction of protein-coding genes of C. parvula genome and C. kokanica genome. SNAP\(^{13}\), Augustus\(^{14}\), Genie\(^{2}\), GenieHMM\(^{16}\) and GENSCAN\(^{17}\) were used for ab initio gene prediction. For homology annotation, we used proteomes of Zea mays, Setaria italica, Brachypodium distachyon, Ananas comosus, Oryza sativa and Arabidopsis thaliana to predict protein-coding genes by GeneWise. Simultaneously, we used the RNA-seq data of 9 samples from different habitats and altitudes for C. parvula (HC26, HC27, HC28 (3381, alpine meadow), HC01, HC02, HC05 (4400, alpine meadow), HC04, HC59, HC60 (4250, Alpine swamp)) and 4 samples from different tissues (leaf, root, spike and stem) for C. kokanica to generate annotation results based on transcripts. After integrating results of the three sources of evidence by EVM, 58,702 (Supplemental Table S5) and 48,549 (Supplemental Table S6) primitive gene models were predicted for C. parvula and C. kokanica separately. We then filtered and polished these gene models through evidence number and expression level, and 45,002 genes in C. parvula and 36,709 genes in C. kokanica supported by at least two lines of evidence or with FPKM > 1 were retained. The average length of genes are 3443.08 bp and 3389.85 bp, while the average length of CDSs are 1107.86 bp and 1220.16 bp in C. parvula and C. kokanica, separately. There are 5 exons in each gene on average with length of 221.49 bp per exon in C. parvula, while there are 5.73 exons in each gene on average with length of 212.93 bp per exon in C. kokanica (Supplemental Table S7).

Functional annotation was obtained by mapping predicted protein sequences to KEGG\(^{18}\), SwissProt\(^{19}\) and Non-redundant protein NCBI databases\(^{20}\). Simultaneously, to inferring the functional annotation of protein coding genes by domain, the protein sequences were searched against member databases of Interpro using InterProScan\(^{11,21}\). Meanwhile, Gene Ontology (GO) terms were obtained by Blast2GO. Finally, a total of 44,796 (96.01%) out of 45,002 genes have integrated functional annotation in C. parvula, and a total of 36,579 (97.40%) out of 36,709 genes have integrated functional annotation in C. kokanica (Supplemental Table S8). In C. parvula, 30,282 (67.3%) genes have blast hit in all four databases, and 26,267 (71.5%) genes of C. kokanica have blast hit in all four databases (Supplemental Fig. S2). Gene sets were assessed with BUSCO Version 3.0.2\(^{22}\), in which 85.9% and 88.2% complete gene models of 1440 embryophyta core genes (odb9) were identified in C. parvula and C. kokanica.
We also compared the colinear gene pairs between C. parvula/C. kokanica and C. littledalei. There are 8926 and 4010 genes have 1:1 ortholog to C. littledalei in C. parvula and C. kokanica respectively. And There are 4437 and 10,061 genes have 1:2 ortholog to C. littledalei in C. parvula and C. kokanica respectively (Supplemental Table S10). We also illustrated the synteny between contigs of C. parvula, C. kokanica and C. littledalei Chromosome 2 (Supplemental Fig. S3). The comparison between C. kokanica and C. littledalei clearly revealed the 2:1 collinear relationship. However, the comparison between C. parvula and C. littledalei didn’t show remarkable 2:1 collinearity, which is likely due to the comparatively shorter contigs.

Comparative genome analysis and divergence time estimation. The assembly of C. kokanica, C. parvula and C. littledalei genomes provides an opportunity to resolve the relationships among Kobresia plants. Using 3 eudicots (Arabidopsis thaliana, Crucihimalaya himalaica and Rhodiola crenulata) and 12 monocots (Brachypodium Distachyon, Oryza sativa, Zea mays, Sorghum bicolor, Phyllostachys heterocycle, Elaeis guineensis, Musa acuminate, Setaria italic, Carex kokanica (Ckok), Carex parvula (Cpar), Crucihimalaya himalaica (Chim), Rhodiola crenulata (Rcre)).

Comparative analyses of Carex parvula and Carex kokanica with other plants. (A) The distribution of genes (single-copy genes, multiple-copy genes, unique genes, other and uncluster genes number) in 15 different species. Carex littledalei (Clit), Arabidopsis thaliana (Ata), Brachypodium Distachyon (Bdis), Oryza sativa (Osat), Zea mays (Zmay), Sorghum. Bicolor (Bic), Phyllostachys heterocycle (Phet), Elaeis guineensis (Egui), Musa acuminate (Macu), Ananas comosus (Acom), Setaria italic (Sita), Carex kokanica (Ckok), Carex parvula (Cpar), Crucihimalaya himalaica (Chim), Rhodiola crenulate (Rcre). (B) Common and unique gene families between Acom, Osat, Macu, Clit, Egui, Cpar and Ckok. (C) Gene family expansions and contractions and estimation of divergence time in Carex parvula, Carex kokanica and 13 other species. (D) Enriched KEGG terms of the expanded genes of C. parvula and C. kokanica (K. royleana).
Global transcriptome analysis of *Carex parvula* at different altitudes. Transcriptome analysis of *Kobresia* plants at different altitudes can provide a crucial systems-level insight into the molecular mechanisms underlying the mechanisms of plant adaptation to environments with high altitude and cold weather. Three representing materials, at altitudes of 4400 m (Alpine grassland), 4250 m (Alpine meadow) and 3381 m (Alpine swamp), were chosen and respectively named ALT1, ALT2 and ALT3 with three biological duplicates. Differentially expressed genes (log2 (relative expression level) ≥ 1 and ≤ –1, padj ≤ 0.05, DEGs) between ALT2 vs ALT1, ALT2 vs ALT3 and ALT3 vs ALT1 were identified by RNA-Seq (Supplemental Tables S11/S12/S13). Principal component analysis (PCA) of the DEGs revealed that the separation of gene expression profiles among these three groups were quite different from each other, which proved the representativeness of these material (Fig. 3A). 3767 upregulated and 5090 downregulated genes were observed in ALT2 compared with ALT1, 3612 upregulated and 3560 downregulated genes were observed in ALT2 against ALT3, and 4404 upregulated and 3726 downregulated genes were observed in ALT1 vs ALT3 (Fig. 3B). Venn diagram showed that only 561 genes were shared, but a large number of specific differentially expressed genes were identified among these three groups were quite different from each other, which proved the representativeness of these materials. However, there was no obvious enrichment result in the constricted genome families of these two genomes. But it is worth noticing that although the results of GO enrichment analysis were relatively similar, the results of KEGG pathway enrichment of these two genome were significantly different (Fig. 2D).

Transcriptome differences in photosynthesis and stress response. Gene Ontology (GO) enrichment analyses were performed with the DEGs between ALT1, ALT2 and ALT3 (Fig. 4). By comparison, genes in photosynthesis (GO:0015979), response to heat (GO:0009048) and response to water stimulus (GO:0009415) were significantly enriched. 9 of the 12 heat stress transcription factors (HSF) had lower expression level in ALT2 vs ALT1, ALT2 vs ALT3 and ALT3 vs ALT1 were identified by RNA-Seq (Supplemental Tables S11/S12/S13). Principal component analysis (PCA) of the DEGs revealed that the separation of gene expression profiles among these three groups were quite different from each other, which proved the representativeness of these material (Fig. 3A). 3767 upregulated and 5090 downregulated genes were observed in ALT2 compared with ALT1, 3612 upregulated and 3560 downregulated genes were observed in ALT2 against ALT3, and 4404 upregulated and 2482 downregulated genes were observed in ALT1 compared with ALT3 (Fig. 3B). Venn diagram showed that only 561 genes were shared, but a large number of specific differentially expressed genes were identified among these three comparable groups (Fig. 3C). The clustering analysis results of the relative expression levels of the upregulated genes in the three groups are also shown in Fig. 3D, which also proved the representative of these materials.

Conclusion

In this study, we presented the scaffold-level genomes of *Carex parvula* and *Carex kokanica* and described their genetic attributes. Comparative genomic analysis revealed their evolutionary status and the preference of genes positive selection in the genus *Kobresia* under the plateau environment stress. The difference analysis of transcriptome data of *Carex parvula* at different altitudes and habitats further revealed mechanisms of plant adaptation to environments with high altitude and cold weather. This study provides a valuable reference for further study on genomic evolution, preservation and utilization of excellent traits of *Kobresia* plants.
Figure 3. Overall differentially expressed genes (DEGs) of Carex parvula from different altitudes and environments. (A) Principal component analysis (PCA) of differentially expressed genes (log2 (relative expression level) ≥ 1 and ≤ −1, DEGs) in 3 groups. (B) The histogram of the statistical results for the DEGs in 3 groups. The orange for down-expressed genes and the blue for up-expressed genes. (C) Venn diagram of the overlap between the DEGs between ALT2 vs ALT1, ALT2 vs ALT3 and ALT3 vs ALT1. D, The clustering analysis results of the relative expression levels of the different groups.

Figure 4. DEGs in photosynthesis and stress response. GO enrichment analysis clustering results (red for the biological process; green for the molecular function) of the up-expressed and down-expressed genes. The clustering analysis results of genes, which belonged to the photosynthesis, response to heat and water stimulus, relative expression levels of these three different groups based on the results of RNA-seq analysis.
Methods

Sample collection, DNA extraction and sequencing. C. parvula (E: 91°59.7597΄ N:31°35.9755΄) and C. kokanica (E: 91°10.5554΄ N:30°29.8873΄) were collected in July 2018 from public land of Dangquka Village, Dangquka Town, Damxung County in the Tibet Autonomous Region of China. Guangpeng Qu undertook the formal identification of the plant material. All collected specimens were submitted to the herbaria of Tibet Academy of Agriculture and Animal Husbandry Science, Lhasa, Tibet, China. The deposition number of C. parvula is 1807002, and the deposition number of C. parvula was 1807003.

For Illumina sequencing, Genomic DNA was extracted from the leaf tissues of all samples using the CTAB method. DNA from each sample was randomly fragmented by nebulization to an average size of 350 bp and processed by the Illumina DNA sample preparation protocol. The DNA library was prepared according to the instructions of the manufacturer and sequencing was performed on the Illumina HiSeq 2500 platform (paired-end 2 × 150 bp).

For PacBio sequencing, a total of 10 μg of sheared DNA was used for a 20-kb insert size library construct and the DNA library were sequenced on the PacBio RS II platform.

For 10× sequencing, DNA sample preparation, indexing, and barcoding were done using the GemCode Instrument from 10× Genomics. About 0.7 ng input DNA with 50 kb length was used for GEM reaction procedure during PCR, and 16-bp barcodes were introduced into droplets. Then, the droplets were fractured following the purifying of the intermediate DNA library. Next, we sheared DNA into 500 bp for constructing libraries, which were finally sequenced on the Illumina HiSeqX Ten.

Genome assembly. To estimate the genome size of C. parvula and C. kokanica, we used reads from paired-end libraries to determine the distribution of K-mer values. According to the Lander–Waterman theory, genome size can be determined by the total number of K-mers divided by the peak value of the K-mer distribution. Given the number of peaks in the K-mer distribution of C. parvula and C. kokanica, it was indicated that C. parvula is tetraploid and C. kokanica is triploid. The estimated ploidy of C. parvula is consistent with the report of Seeber et al.4 With the major peak at the expected K-mer depth and the formula genome size = total K-mer/expected K-mer depth, the size of the haploid genome of C. parvula and C. kokanica was estimated to be 396.40 M and 447.68 M.

For genome assembly, pre-assembly reads were obtained by self-correcting of PacBio long reads and assembled into contigs by FALCON (falcon-kit = 0.7) through the “Overlap-Layout-Consensus” algorithm. Contigs were corrected using PacBio long reads with quiver (smrtlink_6). Then, using Illumina short reads to improve the precision in Pilon-1.18. To improve the assembly of C. parvula, contigs were linked into scaffold using 10× data using FragScaff (PBSuite_15.8.24).

Genome annotation. Transposable elements in the assembly were identified both at DNA and protein levels. We used RepeatModeler, RepeatScout and LTRFinder10 to de novo identify and classify repeated sequences in each genome. RepeatMasker was applied for DNA-level identification using Repbase11 and the de novo transposable element library. At the protein level, RepeatProteinMask was used to searches against the transposable element protein database. Overlapping transposable elements belonging to the same type of repeats were merged.

Ab initio, homology-based and RNA-seq-based pipelines were integrated for subsequent prediction of protein-coding genes of C. parvula and C. kokanica genome. SNAP13, Augustus14, Geneid15, GlimmerHMM16 and GENSCAN17 were used for ab initio gene prediction. For homology annotation, we used proteomes of Zea mays, Setaria italica, Brachypodium distachyon, Ananas comosus, Oryza sativa and Arabidopsis thaliana to predict protein-coding genes by GeneWise. Simultaneously, we used the RNA-seq data from 10 samples from different habitats and altitudes for C. parvula (HC26, HC27, HC28 (3381, alpine meadow), HC01, HC02, HC05 (4400, alpine meadow), HC04, HC59, HC60 (4250, Alpine swamp) and 4 sample from different tissues (leaf, root, spike and stem) for C. kokanica to generate annotation results based on transcripts.

Functional annotation was obtained by mapping predicted protein sequences to KEGG18, SwissProt and Non-redundant protein NCBI databases for plants. Simultaneously, to inferring the functional annotation of protein coding genes by domain, the protein sequences were searched against member databases of InterPro23 using InterProScan24. Meanwhile, Gene Ontology (GO) terms were obtained by Blast2GO.

Comparative genomics and divergence time estimation. Orthologous groups of selected plant species were constructed using OrthoMCL26. Protein sequences of each single-copy orthologous group were aligned with MAFFT (v.7.313)45 with parameters ‘--maxiterate 1000 --localpair’. Alignments of resulting orthologous groups with family-wide p < 0.05 were defined as rapidly evolving gene families whereas, the Viterbi p < 0.05) was used to identify branches with gene families significantly expanded or contracted compared with their last common ancestor.

Sample collection, RNA extraction and sequencing. Three representing materials, altitude of 4400 m (ALT1, Alpine grassland, Dangxiong, Tibet), 4250 m (ALT2, Alpine meadow, Dangxiong, Tibet) and 3381 m (ALT3, Alpine swamp, Qinghai) were collected from public land. We collected three samples in each site. Fresh leaves were stored in liquid nitrogen and transported to Novogene (Tianjin, China) for subsequent procedures.
Total RNA of leaves of each sample was extracted with NEB Next Ultra™ RNA Library Prep Kit, sequenced by Illumina HiSeq2500.

**Differentially expressed gene analysis.** RNA-seq reads were trimmed by Trimmomatic and then mapped to the *C. parvula* genome by HiSAT2. FPKM was calculated on the basis of unique mapping reads using the HTseq package. Different expressed genes (Foldchange > 2 and p value < 0.05) were identified using cuffdiff in cufflinks-1.3.0.

We confirm that all methods were performed in accordance with the relevant guidelines and regulations.

**Data availability**

We have uploaded our raw data and assemblies into NCBI with project accession ID: PRJNA734565 (*C. kokanica*) and PRJNA731336 (*C. parvula*).

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**Author contributions**

G.Q. conceived and designed the project. Y.B., Y.L., S.C., M.B., Y.L., D.T. and L.W. collected the samples. H.Z. and C.L. conducted data analysis. G.Z. helped with the data analysis and examined the results. C.L. wrote the original draft of the manuscript. M.C. performed the review and editing of the manuscript. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare no competing interests.

**Additional information**

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