Multiple horizontal transfers of nuclear ribosomal genes between phylogenetically distinct grass lineages

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The movement of nuclear DNA from one vascular plant species to another in the absence of fertilization is thought to be rare. Here, nonnative rRNA gene [ribosomal DNA (rDNA)] copies were identified in a set of 16 diploid barley (*Hordeum*) species; their origin was traceable via their internal transcribed spacer (ITS) sequence to five distinct Panicoideae genera, a lineage that split from the Pooideae about 60 Mya. Phylogenetic, cytogenetic, and genomic analyses implied that the nonnative sequences were acquired between 1 and 5 Mya after a series of multiple events, with the result that some current *Hordeum* sp. individuals harbor up to five different panicoid rDNA units in addition to the native *Hordeum* rDNA copies. There was no evidence that any of the nonnative rDNA units were transcribed; some showed indications of having been silenced via pseudogenization. A single copy of a *Panicum* sp. rDNA unit present in *H. bogdanii* had been interrupted by a native transposable element and was surrounded by about 70 kbp of mostly noncoding sequence of panicoid origin. The data suggest that horizontal gene transfer between vascular plants is not a rare event, that it is not necessarily restricted to one or a few genes only, and that it can be selectively neutral.

Hordeum | Triticeae | Panicoideae | transposable elements | horizontal gene transfer

The exchange and recombination of genetic material are major driving forces of evolution: in eukaryotes, the process operates via sexual fertilization, whereas in prokaryotes, horizontal gene transfer (HGT) is commonplace. The extent to which HGT has contributed to the evolution of multicellular eukaryotes is debatable (1–3), largely because of the supposed low frequency of HGT events. Plant to plant exchanges of nonnuclear DNA are relatively common (4–6), but the exchange of nuclear DNA has been recorded, at best, sporadically. Most of the established horizontal transfer events involving a plant genome result from interactions between a plant and a pathogen or parasite (7–11). Plant to plant transfers outside of the fertilization process are thought to be rare (12–17), presumably because they require a vector to move the DNA from one plant to the other.

Eukaryotic genomes harbor thousands of copies of ribosomal DNA (rDNA) arranged in tandem arrays. Thanks to the sequence diversity of their spacer sequences (ITS), this class of repetitive DNA has been highly informative concerning phylogenetic relationships. In an attempt to use ITS variation to identify the progenitors of hexaploid couch grass (*Elymus repens*, Triticeae, Pooideae), a nonnative ITS type was uncovered that was considered to have originated from a species of *Panicum* (18), a panicoid genus that separated from the pooids some 60 Mya (19). *Elymus* spp. are not known to intercross with *Panicum* spp. (20–22), presenting a puzzle of how the exotic rDNA was acquired. Because *E. repens* is an allopolyploid harboring genomes derived from both *Pseudoroegneria* and *Hordeum* (18, 23), it was of interest to determine whether the transfer pre- or postdated the allopolyploidization event. Using an assay that selectively amplified the panicoid ITS (18), it was possible to show the presence of panicoid rDNA in both progenitor species, although the sequence data favored the notion that *E. repens* had inherited its panicoid rDNA unit from *Hordeum bogdanii* (18).

Here, the aim was to characterize the occurrence and nature of panicoid-derived rDNA in Triticeae genomes, with a focus on *Hordeum* and *Pseudoroegneria* species. The sequence polymorphism characteristic of the ITS offered an opportunity to place a specific HGT in relation to the evolutionary history of the host. A detailed analysis of the structure of one HGT was carried out by sequencing a BAC clone of *H. bogdanii*, which harbored a panicoid rDNA unit. In conjunction with cytogenetic analyses, this information allowed for both the nature of the nonnative genetic material and its location in the barley genome to be identified.

Results and Discussion

The initial PCR-based screen of a set of Triticeae species (a full list is in Table S1) revealed that nonnative panicoid rDNA was present only in species belonging to the two genera *Hordeum* and *Pseudoroegneria* (Table S2). It was found in all 16 diploid *Hordeum* species belonging to section *Stenostachys*, all of which harbor the I genome, but not found in any of the remaining *Hordeum* taxa (Table S2). Two of the five *Pseudoroegneria* species (*Pseudoroegneria strigosa* and *Pseudoroegneria spicata*) also tested positive. Sequence variation within the panicoid ITS was established by cloning and sequencing the relevant stretch of DNA (amplicon 2) (Fig. S1) based on a template of either *Hordeum*

Significance

A screen of *Hordeum* (barley) spp. genomes identified several instances of the presence of ribosomal DNA of panicoid origin. The Pooideae and Panicoideae lineages separated from one another around 60 Mya and are sexually incompatible. During the past 1–5 My, at least nine independent transfers of panicoid DNA into *Hordeum* seem to have occurred, confirming that the transfer of exotic DNA is not an isolated event, at least among the grasses. The supposed rarity of this event in plant genomes more likely reflects technical limitations in its detection rather than it being a genuine biological phenomenon.

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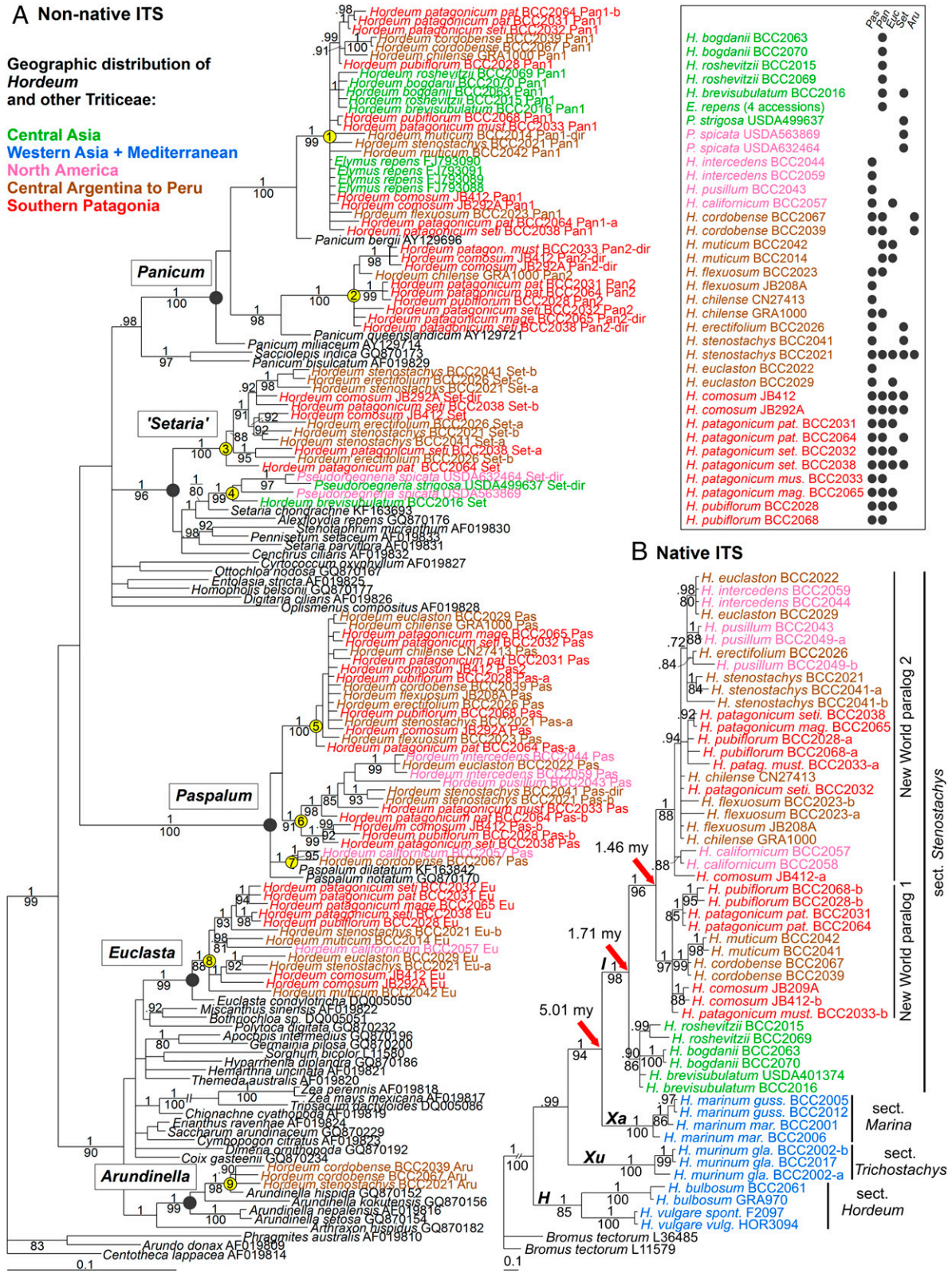

Fig. 1. Phylogenetic analysis of (*A*) nonnative (panicoid) and (*B*) native (pooid) rDNA ITS sequences. (*A*) The numbers inserted above and below the branches refer to the Bayesian posterior probabilities and the bootstrap values for maximum parsimony, respectively; –a and –b are variants of the same rDNA subtype, and –dir indicates a direct sequence of the respective rDNA type. Yellow circles including the numbers 1–9 indicate subclades referred to in the text and Table S4. *Inset* reports a summary of the distribution of panicoid rDNA (corresponding to five genera) across *Hordeum*, *Pseudoroegneria*, and *Elymus* species/accessions. (*B*) The New World species contain two rDNA loci (24). The dating of the nodes follows (25). H, Xu, Xa, and I are genome designators (26).

or *Pseudoroegneria* spp. DNA. The unexpected outcome was that the source of the rDNA was traceable to five distinct Panicoideae genera, namely *Arundinella*, *Euclasta*, *Paspalum*, *Panicum*, and *Setaria* (Fig. 1*A*). A PCR assay was then designed to amplify each of the five panicoid lineages separately (Table S3), and the screen was repeated to pick up any hitherto undetected nonnative ITS types. Between one and five panicoid ITS types were found in the various *Hordeum* and *Pseudoroegneria* individuals (Table S2). Although a single nonnative ITS derived from *Setaria* spp. was present in the *Pseudoroegneria* genomes, the *Hordeum* genomes harbored up to five distinct nonnative rDNA. Most of the Central Asian and North American taxa harbored a single nonnative ITS type, but the South American ones have accumulated multiple copies within individual plants (Fig. 1*A* and Table S2). The panicoid ITS sequences occurred in derived positions of the phylogenetic tree (Fig. 1*A*), suggesting a relatively recent acquisition. Some clustered with sequences of extant panicoid species (e.g., *Paspalum dilatatum* and *Arundinella hispida*), whereas others seemed to be derived from either nonsampled or extinct species belonging to one of the five panicoid genera. Some sequence variation was noted within each of the five lineages, resulting in distinct clusters of highly similar sequence shared by several *Hordeum* and *Pseudoroegneria* species; these patterns were most parsimoniously interpreted as being the outcome of a number of independent transfer events, in some cases involving more than one per panicoid genus (1–9 in Fig. 1*A*; a detailed evaluation is in Table S4).

In an attempt to reveal the evolutionary origin of the transfers, the distribution of the nonnative ITS sequences was compared with a phylogeny of genus *Hordeum* based on native ITS sequences (Fig. 1*B*), incorporating preexisting information regarding its speciation and phylogeography (25, 27, 28) (Table S4). According to this analysis, the likely most ancient transfer event was *Panicum 1*, because this rDNA type was found in all of the Central Asian and most of the South American taxa (Fig. 1*A*). This event, therefore, likely occurred in their common ancestor, which predated the diversification of section *Stenostachys*, thereby establishing the timing of the transfer to 1.7–5.0 Mya and locating it to Central Asia. Of the other events, only *Setaria 4* did not involve New World (American) species. *Setaria 4* was present in the Central Asian species *Hordeum brevisubulatum* and two *Pseudoroegneria* species (Table S4). The acquisition of this nonnative rDNA by *Pseudoroegneria* spp. could have occurred independently of its acquisition by *Hordeum*, but it is more likely that the transfer was disseminated by wide hybridization given the number of established *Elymus* spp. allopolyploids, which have arisen from various *Hordeum* × *Pseudoroegneria* hybrids. The New World *Hordeum* species seem to have experienced a number of transfers, some of which (*Paspalum 5* and *6* and *Euclasta 8*) were represented in a number of species, whereas others (*Panicum 2* and *Arundinella 9*) were restricted to just a few species (Fig. 1*A*, *Inset*). The most parsimonious scenario for the former group posits that a small number of events transferred the panicoid rDNA into a New World *Hordeum* species before the major speciation period (about 1.46 Mya) and that these transfers were then carried vertically into the various new species. A more detailed evaluation of individual transfer events and their interpretation in relation to host phylogeny and speciation are provided in Table S4.

A characterization of the structural features of the panicoid rDNA was undertaken by constructing a BAC library of *H. bogdanii*, a Central Asian species harboring only the *Panicum 1* ITS type. Sequencing of the single positive clone (BAC 46L9) recovered from the library revealed a retrotransposon-rich ∼70-kbp region of *Panicum*-derived DNA, which included parts of an rDNA unit. The nonnative sequence was interrupted by two *Hordeum*-specific *Copia*-like transposable elements (TEs), which implied that two transposition events must have followed the integration of the panicoid rDNA segment into the *Hordeum* genome (Fig. 2). One of the TEs has disrupted the 18S rRNA gene within the *Panicum* rDNA, thereby effectively disabling the unit. On the basis of sequence divergence in the 5′ and 3′ long-terminal repeats (LTRs) of TEs, the timing of the transposition events was estimated to be 0.25–0.29 Mya, which is substantially later than the date of the panicoid rDNA acquisition. The retention of such a large block of horizontally transferred nuclear DNA shows that even presumably selectively neutral material can be maintained over a significant (in evolutionary terms) time period.

The FISH technique, using BAC 46L9 as the probe, was used to locate the insertion site of the *Panicum*-derived rDNA segment in *H. bogdanii*. The hybridization mix also included a labeled 45S rDNA probe comprising the 18S, 5.8S, and 26S rDNA genes and the ITS to locate the native rDNA sites of *Hordeum*. Both the *Panicum* and the native rDNA mapped to the same chromosome, but whereas the former corresponded to an interstitial site on the long arm, the latter located to a cluster on the short arm (Fig. 3*A*). When the experiment was repeated using BAC 46L9 and *Panicum bergii* genomic DNA as probes, the two hybridization sites fully overlapped with one another (Fig. 3*B*), showing that *H. bogdanii* harbored only a single segment derived from *Panicum*. A similar experiment was conducted on *Hordeum pubiflorum*, which harbored rDNA derived from both *Panicum* and *Paspalum*. When its mitotic chromosomes were hybridized with labeled genomic DNA of *P. bergii* and *Paspalum dilatatum*, the only *Panicum* rDNA site detected mapped to the identical site as in *H. bogdanii* (Fig. 3*C*), whereas the *Paspalum* rDNA mapped to a different site, although on the same chromosome (Fig. 3*D*). This outcome supports the phylogeny-based hypotheses concerning independent acquisitions from different Panicoideae sources as well as establishes the presence of the most ancient material at identical sites in two distantly related *Hordeum* species.

To determine whether any of the panicoid rDNA segments had retained their functionality, RNA was isolated from the leaf of seven *Hordeum* and one *Pseudoroegneria* species, reverse-transcribed, and then, subjected to RT-PCR using Panicoideae-specific PCR primer pairs (Table S3). The experiment failed to show even one example of the transcription of panicoid rDNA. The mechanism underlying the silencing of these genes was explored by searching for base substitutions in the five most conserved motifs within both the 5.8S gene and the ITS2 region and also, alterations that may have compromised the ability of 5.8S RNA transcripts to form the necessary secondary structures (Dataset S1 and Table S5). Most of the nonnative rRNA present in both *Hordeum* and *Pseudoroegneria* spp. showed evidence of sequence alterations, which could have disrupted functionality. Thus, although it remains a possibility that some of the nonnative rDNA have retained functionality, the abundance of their transcript cannot be high enough in a background of a large excess of native rDNA transcript to offer any selective advantage to the host.

HGT may represent the most plausible explanation for the presence of the nonnative rDNA in these Triticeae species, although it is acknowledged that the evidence for it is only circumstantial. The alternative possibility requires a successful hybridization between Pooideae and Panicoideae grasses, which is hindered by crossing barriers (20–22). Even if the barriers to such wide crosses were overcome (which is possible when plants—particularly polyploid species—are raised under controlled conditions), the introduction via this route of segments derived from as many as five distinct Panicoideae genera into a diploid barley seems unlikely. Only few proven natural triple hybrids exist (ref. 29 and references therein), and to our knowledge, no natural intergeneric or interspecific hybrid with more than three parents has ever been reported. In contrast, both aphids and endophytic fungi represent potential vectors for HGT. A number of aphid species feed on a range of grass genera, whereas it has recently been shown that endophytic fungal symbionts of the genus *Epichloë* are capable of horizontally acquiring genes from their grass host (30). The proliferation of fungal endosymbionts

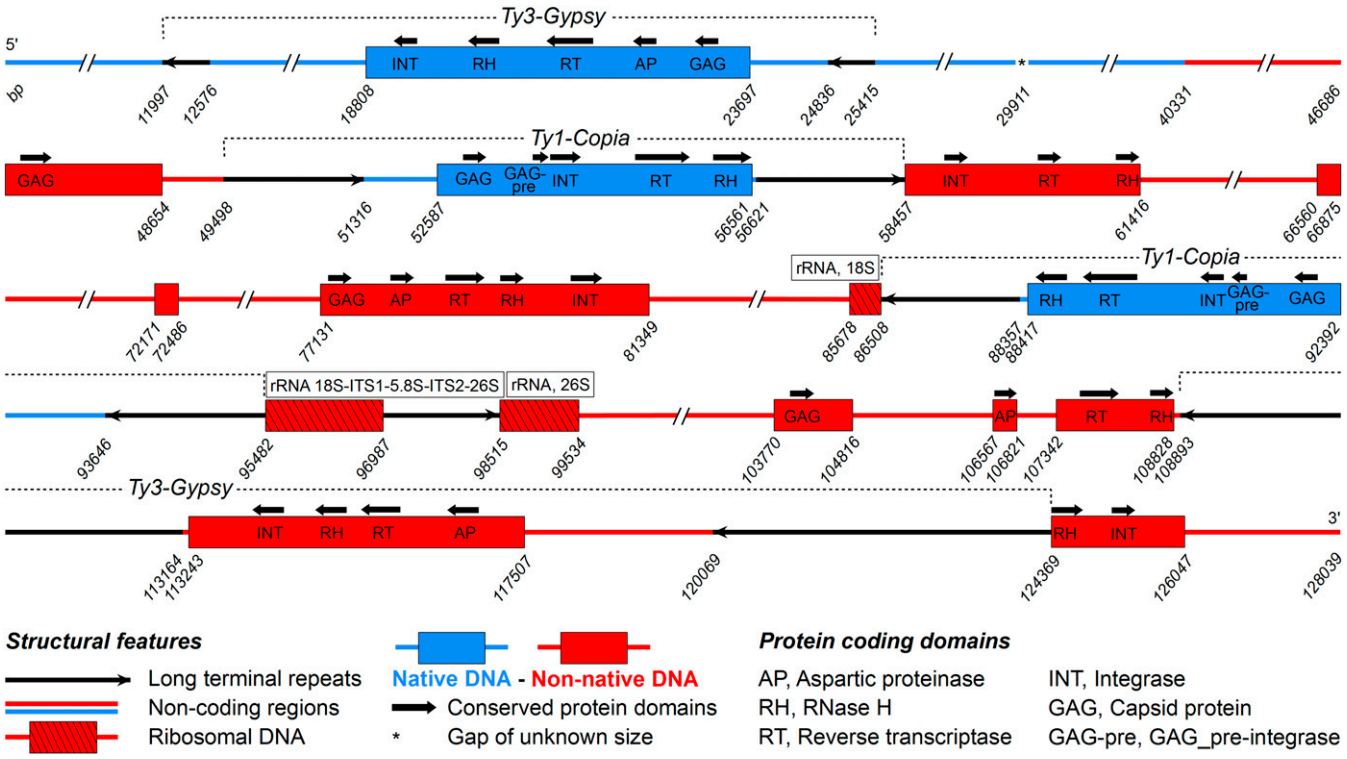

Fig. 2. A physical map of BAC clone 46L9 harboring a genomic fragment of panicoid origin.

in seeds ensures a prolonged physical contact with their host, which is necessary for HGT to occur.

The exchange of genetic material requires that the partners coincide with one another in time and space, which is no longer the case for all plant taxa involved here. It is known that the distribution of species is strongly influenced by climate, which has changed greatly over the past 5 My (31), resulting in historical opportunities for species to have overlapped that are no longer possible today (32). The inferred timing of the transfer events implies interactions between Central Asian and New World Pooideae species with subtropical or tropical Panicoideae. After established in a *Hordeum* host, the various panicoid DNA sequences could have been transferred from progenitor to a derived species and/or spread via hybridization between the mostly interfertile species of section *Stenostachys*.

The real frequency with which HGT occurs is highly uncertain (2, 3), which is also the case for the consequences (if any) of the presence of exotic DNA (14, 33). To date, the detection of HGT has been haphazard, and a systematic search for it in a large plant genome, such as that of *Hordeum*, is scarcely feasible. The evidence presented here is based on an approach that was specifically designed to target rDNA genes of Panicoideae after an initial chance discovery (18), and it is doubtful whether a single copy of exotic rDNA could be detected using a whole-genome approach given the large number of native copies present. These data suggest that HGT may not be an exceptional event in the grasses and by implication, similarly so for other angiosperm families. Although the capture of panicoid rDNA may have contributed to the rapid diversification experienced by section *Stenostachys*, the most species-rich *Hordeum* group (25), it seems more likely that the transfers were selectively neutral and hence, random.

Materials and Methods

Plant Materials. Only diploid taxa ($2n = 14$) were considered here, with the exception of one autotetraploid accession of *Hordeum bulbosum* (GRA970; $2n = 28$) (Table S1). All currently recognized diploid species belonging to the genus *Hordeum* were sampled (20 species along with nine subspecies); each was represented by two accessions, with the exception of *Hordeum erectifolium*, for which only a single population was available. The screen also included 7 *Pseudoroegneria* spp. accessions (spread across five species) and 18 accessions (15 species) belonging to other Triticeae genera (Table S1). The materials were sourced from the Leibniz Institute of Plant Genetics and Crop Plant Research GenBank, the US Department of Agriculture (USDA) National Small Grains Collection, the Plant Genetic Resources Canada (PGR Canada) and a private collection of F.R.B.

rDNA Amplification. The total rDNA ITS was PCR-amplified from each accession using the Poaceae-specific primer pair ITS-Poa-f/ITS4 (34) (Fig. S1, step 1) in three independent reactions to reduce PCR bias. The resulting amplicons (amplicon 1) produced from a *Hordeum* sp. template were sequenced from both ends, and the acquired sequences were used to construct a native rDNA-based phylogeny (Fig. 1*B*). Where sequence reads were disturbed because of heterogeneity in the amplicon, the amplicon was first cloned using a TOPO TA Cloning Kit (Invitrogen); then, eight clones per amplicon were sequenced based on the M13 forward primer.

The presence of panicoid rDNA in the sample species genomes was detected in a PCR assay based on a Panicoideae-specific primer pair (18). Subsequently, a number of specific primer combinations were designed to selectively amplify particular ITS variants (Table S3), and these primers were applied to the full set of accessions. The steps involved in the screening procedure are described in detail in *SI Materials and Methods*.

Phylogenetic Analyses. The nonnative rDNA present in the *Hordeum* and *Pseudoroegneria* genomes was placed in a phylogenetic context within the Panicoideae by carrying out a Bayesian inference and maximum parsimony analysis. Details regarding the necessary sequence alignments and the parameters chosen for the phylogenetic analysis are provided in *SI Materials and Methods*.

Construction and Analysis of an *H. bogdanii* BAC library. A low-coverage genomic BAC library was constructed from *H. bogdanii* accession number BCC2063 (Table S1) based on high-molecular weight DNA prepared from flow-sorted nuclei. The procedure was modified from that in ref. 35. A 30-g sample of fresh leaf tissue was fixed in formaldehyde and homogenized. Nuclei were concentrated from the resulting suspension by passing it through an FACSAria device (Becton Dickinson), and they were embedded in agarose plugs (each

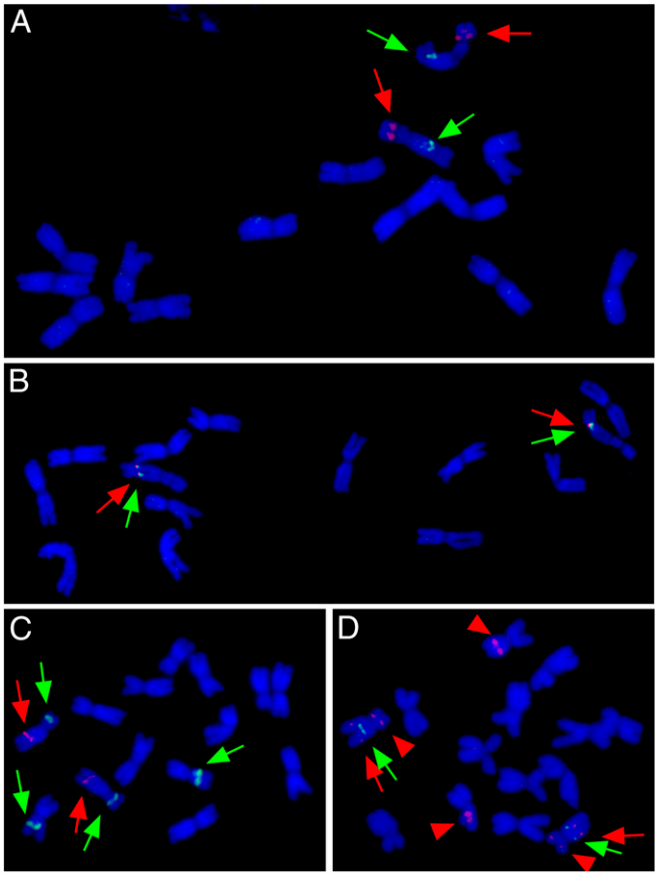

Fig. 3. Molecular cytogenetic analysis of (*A* and *B*) *H. bogdanii* and (*C* and *D*) *H. pubiflorum*. (*A*) The BAC clone 46L9 signal (green arrows) localizes to a site on the long arm of the same chromosome that carries the 45S-rDNA cluster (red arrows). (*B*) The BAC clone 46L9 signal (green arrows) colocalizes with the signal obtained from the *P. bergii* genomic DNA probe (red arrows) to a site on a single chromosome pair. (*C*) The signal obtained from the *P. bergii* genomic DNA probe (red arrows) localizes to a site on the long arm of the same chromosome that carries one of two native rDNA clusters present (green arrows). (*D*) The signal obtained from the *Pa. dilatatum* genomic DNA probe (green arrows) highlights a site proximal to one hybridizing to *P. bergii* genomic DNA (red arrows). The red arrowheads show the native rDNA clusters.

plug harbored around 10^5 nuclei), which were then treated with *Hin*dIII. The partially digested DNA was separated by pulsed-field gel electrophoresis (two size selection steps), and fragments in the range 100–300 kbp were isolated from the gel and ligated into a dephosphorylated pCC1BAC plasmid (Epicentre). The recombinant plasmid was transformed into *Escherichia coli* strain MegaX DH10B T1 (Life Technologies/Invitrogen) via electroporation. In total, 18,432 clones were picked and ordered in a set of 384-well microtiter plates.

Screening of the BAC Library and Sequencing of Selected BAC Clones. The BAC library was spotted onto 22.2 × 22.2-cm nylon filters (4 × 4 pattern; 18,432 clones on each filter in duplicate). To identify BAC clones possessing *Panicum* rDNA, a hybridization probe specific for each of the *Panicum*-like ITS1 and ITS2 sequences present in *H. bogdanii* was amplified, labeled with α^{32}P using a Prime-It II Random Primer Labeling Kit (Agilent) according to the manufacturer's protocol, and hybridized to the filters. DNA from 21 positive BAC clones was isolated using an Invitek Plant Mini DNA Kit (www.thistlescientific.co.uk) and prepared for sequencing using a TruSeq DNA PCR-Free Kit (Illumina). The BACs were sequenced using a MiSeq instrument and the MiSeq Reagent Kit v3 to achieve pair-end reads of length of 300 nt, giving an at least 100× coverage for each BAC.

Assembly and Analysis of the BAC Clone Sequences. The BAC sequence reads were initially filtered to remove bacterial sequences using ERNE v.1.2 software (36). Adaptors were trimmed using the TRIMMOMATIC v.0.30 tool (37) with the following parameters: ILLUMINACLIP 2:30:10, LEADING:20, TRAILING:20, SLIDINGWINDOW:4:20, and MINLEN:85. The BAC assembly was performed using Ray v. 2.3.1 software (38), applying a range of K-mer values, and the best assembly was chosen based on a set of criteria (*N*50, BAC coverage, and *N* content). The sequences were subjected to a BLAST search to select which BAC clones contained a panicoid rDNA unit. The BAC sequences were scanned for the presence of the *Panicum*-like ITS sequence and the native ITS sequence of *H. bogdanii* (GenBank accession nos. FJ793096 and KU513503). The sequence of BAC 46L9 (two scaffolds comprising 128,039 bp) was analyzed using BLAST2GO v. 3.1.3 (39). BLASTN and BLASTX analyses were run to identify the highest similarity sequences and assess the function of the sequence, respectively. A conserved domain search was performed on the National Center for Biotechnology Information web server. The online version of *LTR_FINDER* (40) was used to identify retrotransposon LTRs. Subregions of the rDNA sequence were identified by reference to the sequence of *Alloteropsis semialata* rDNA [GenBank accession no. KT281159 (41)].

Inferring the Timing of TE Insertion. The insertion times of the two *Hordeum*-like *Copia* retrotransposons nested within the panicoid DNA segment in *H. bogdanii* were estimated following the work in ref. 42. Paired LTR sequences were aligned, and the number of base substitutions was counted. The age of the insertion was estimated using the expression $N/(2 \times L \times K)$, where *N* represented the number of substitutions between the two LTRs and *L* was the length of the LTR. A value of 6.5×10^{-9} was assumed for *K* (mutation rate per synonymous site per year) (43).

Cytogenetic Analyses. FISH was used to localize native and nonnative rDNA sequences on mitotic chromosomes of selected *Hordeum* species as described earlier (18). DNA of BAC 46L9 was isolated and labeled with digoxigenin using a DIG-Nick Translation Kit (Roche Applied Science) according to the manufacturer's protocol. The rDNA clone pTa71 (44), which contains the 18S-5.8S-26S cluster of rDNA genes and the ITS region, was labeled with biotin using a Biotin-Nick Translation Kit (Roche Applied Science) according to the manufacturer's protocol. A second set of experiments used as probes genomic DNA of *P. bergii* (labeled with biotin) and BAC 46L9 (labeled with digoxigenin). In two additional experiments, genomic DNA of *P. bergii* (labeled with biotin) and pTa71 (labeled with digoxigenin) were hybridized to *H. pubiflorum* (BCC2028) chromosomes, and genomic DNA of *Pa. dilatatum* (labeled with digoxigenin) and genomic DNA of *P. bergii* (labeled with biotin) were hybridized to *H. pubiflorum* chromosomes. The latter preparations were reprobed with biotin-labeled pTa71. In each case, the site of probe hybridization was detected using a combination of anti–digoxigenin-FITC conjugate (Roche Applied Science) and streptavidin-Cy3 conjugate (Life Technologies/Invitrogen). The chromosomes were counterstained with 1.5 μg/mL DAPI in Vectashield Antifade Solution (Vector Laboratories).

Assay for the Transcription of Nonnative rDNA. Real-time PCR was used to screen selected *Hordeum* accessions and *Ps. spicata* using primer pairs targeting the various panicoid rDNA types (Table S3). RNA was extracted from the leaf of *H. bogdanii* BCC2063, *Hordeum roshevitzii* BCC2015, *Hordeum chilense* GRA1000, *Hordeum cordobense* BCC2039, *Hordeum patagonicum* ssp. *setifolium* BCC2038, *Hordeum patagonicum* ssp. *magellanicum* BCC2065, *H. pubiflorum* BCC2028, and *Ps. spicata* USDA563869 using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. Contaminating genomic DNA was removed by treatment with a TURBO DNA-Free Kit (Life Technologies/Invitrogen), and cDNA was then synthesized using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche) based on random hexamer priming. The PCRs were identical to the DNA-based ones described above, except that cDNA rather than genomic DNA was used as the template. The concentration of the cDNA was varied from 0.125 to 12.5 ng/μL. Two control PCRs were carried out: one was primed with ITS-Poa-f/ITS4 to ensure that the cDNA preparation was satisfactory, and the other was supplied with DNase-treated RNA as the template to ensure that the DNase treatment had been effective. The PCR products were visualized by EtBr staining after electrophoretic separation through a 2.5% (wt/vol) agarose gel.

Nucleotide Substitutions in the Nonnative rDNA Sequences. The functionality of the nonnative rDNA sequences across all accessions and each of the ITS types was assessed by querying the sequence of the highly conserved motifs of the 5.8S gene and the ITS2 (45); in addition, the ability of 5.8S RNA transcripts to form constrained secondary structures based on a prediction model was assessed. The analyses were performed as described in ref. 46, and only the latter was affected using the ViennaRNA Package 2.0 (47).

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