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Genomic Origin and Organization of the Allopolyploid *Primula egaliksensis* Investigated by *in situ* Hybridization

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• *Background and Aims* Earlier studies have suggested that the tetraploid *Primula egaliksensis* ($2n = 40$) originated from hybridization between the diploids *P. mistassinica* ($2n = 18$) and *P. nutans* ($2n = 22$), which were hypothesized to be the maternal and paternal parent, respectively. The present paper is aimed at verifying the hybrid nature of *P. egaliksensis* using cytogenetic tools, and to investigate the extent to which the parental genomes have undergone genomic reorganization.
• *Methods* Genomic *in situ* hybridization (GISH) and fluorescent *in situ* hybridization (FISH) with ribosomal DNA (rDNA) probes, together with sequencing of the internal transcribed spacer (ITS) region of the rDNA, were used to identify the origin of *P. egaliksensis* and to explore its genomic organization, particularly at rDNA loci.
• *Key Results* GISH showed that *P. egaliksensis* inherited all chromosomes from *P. mistassinica* and *P. nutans* and did not reveal major intergenomic rearrangements between the parental genomes (e.g. interchromosomal translocations). However, karyological comparisons and FISH experiments suggested small-scale rearrangements, particularly at rDNA sites. *Primula egaliksensis* lacked the ITS-bearing heterochromatic knobs characteristic of the maternal parent *P. mistassinica* and maintained only the rDNA loci of *P. nutans*. These results corroborated sequence data indicating that most ITS sequences of *P. egaliksensis* were of the paternal repeat type.
• *Conclusions* The lack of major rearrangements may be a consequence of the considerable genetic divergence between the putative parents, while the rapid elimination of the ITS repeats from the maternal progenitor may be explained by the subterminal location of ITS loci or a potential role of nucleolar dominance in chromosome stabilization. These small-scale rearrangements may be indicative of genome diploidization, but further investigations are needed to confirm this assumption.

Key words: Diploidization, FISH, genome evolution, GISH, hybridization, ITS, polyploidy, *Primula egaliksensis*, 45S rDNA.

INTRODUCTION

Polyploidization has played a major role in plant speciation (Soltis and Soltis, 1993; Bretagnolle *et al.*, 1998; Otto and Whitton, 2000; Levin, 2002; Soltis *et al.*, 2003). At least 70 % of all angiosperms are thought to have polyploid origins (reviewed by Soltis, 2005), including species of small genome size and chromosome number such as *Arabidopsis thaliana* (Henry *et al.*, 2006). Two types of polyploids are commonly recognized according to the degree of homology among co-existing genomes. Autopolyploids, which arise within a single species, contain more than two sets of homologous chromosomes in their nuclear genome, while allopolyploids contain more than two sets of homeologous chromosomes that diverged from each other prior to interspecific hybridization (Ramsey and Schemske, 1998).

Polyploids generally undergo rapid genome restructuring following their formation (Wendel, 2000; Adams and Wendel, 2005; Chen and Ni, 2006) as demonstrated, for instance, by the studies on synthetic and natural allopolyploids of *Nicotiana* (Skalická *et al.*, 2003, 2005; Kovarík *et al.*, 2004; Lim *et al.*, 2004*a*), wheat (Levy and Feldman, 2004) and *Arabidopsis* (Pontes *et al.*, 2004). Genomic changes range from intergenomic chromosome translocations to interlocus recombinations (e.g. unequal crossing-over and gene conversion), and can be accompanied by epigenetic modifications (e.g. cytosine methylation) at specific loci leading to altered gene expression patterns (Wendel, 2000; Liu and Wendel, 2003; Wang *et al.*, 2004; Chen and Ni, 2006). Altogether these changes may constitute a response to the cellular stress imposed by the novel hybrid chromosome composition and/or doubling of the gene dosage (i.e. genomic shock; McClintock, 1984; Madlung and Comai, 2004) and result in the gradual diploidization of polyploid taxa (Leitch and Bennett, 2004; Ma and Gustafson, 2005). Yet there are also examples of polyploids that have undergone only few changes in overall genome structure since their formation, e.g. in *Gossypium* (Liu *et al.*, 2001) and *Spartina* (Baumel *et al.*, 2002; Ainouche *et al.*, 2004).

The varied response of plant genomes to polyploidization is reflected in the nature and arrangement of ribosomal DNA (rDNA) repeats of polyploids. Ribosomal genes occur in hundreds or thousands of copies that are tandemly repeated over one or more loci (Long and Dawid, 1980). Some hybrids show a strict conservation in number, but not necessarily in size, of both parental rDNA loci (e.g. in *Nicotiana*; Matyásek *et al.*, 2003; Kovarík *et al.*, 2004), while others display gain (e.g. in *Gossypium*; Hanson *et al.*, 1996) or loss (e.g. in *Arabidopsis*; Pontes *et al.*, 2004) of rDNA loci. At the sequence level, similar

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contrasting scenarios of rDNA loci evolution were observed as sequences from putative allopolyploids were found to be either additive for differing parental nucleotides (e.g. Campbell *et al.*, 1997), or homogenized towards one parental repeat type (e.g. Wendel *et al.*, 1995; Kovarík *et al.*, 2004), due to processes of intra- and interlocus recombination (referred to as concerted evolution; Elder and Turner, 1995) or loss of rDNA loci.

The genus *Primula* (totalling approx. 430 species; Richards, 2002) constitutes an ideal model system to investigate the effects of hybridization and polyploidization on plant evolution. On the one hand, primulas have been hybridized for gardening purposes since the 16th century and several sections are known for their high levels of interspecific hybridization in the wild (e.g. sects. *Auricula* and *Primula*; Richards, 2002). On the other hand, the ploidy level varies from diploid to 14-ploid, with some sections being entirely polyploid (e.g. sects. *Auricula* and *Parryi*; Richards, 2002). Interestingly, morphological, caryological, distributional and phylogenetic data suggest that most polyploids belonging to sect. *Aleuritia* are of allopolyploid origin (Bruun, 1932; Vogelmann, 1956; Hultgård, 1990, 1993; Kelso, 1991, 1992; A. Guggisberg, G. Mansion and E. Conti, unpubl. res.).

The tetraploid *Primula egaliksensis* ($2n = 40$) is widely distributed across North America and is taxonomically ascribed to sect. *Armerina* owing to its entire petiolate leaves, narrow elongated capsules and the absence of farina (Kelso, 1991; Richards, 2002). Karyological and morphological evidence suggest, however, that it may be an intersectional allopolyploid involving one species (out of 27) of sect. *Aleuritia* and one species (out of 14) of sect. *Armerina* (Kelso, 1991, 1992). This hypothesis relies primarily on the additivity of chromosome numbers, the intermediacy of gland types, pollen sizes, colpi numbers and exine reticulation patterns (Kelso, 1991, 1992). Within sect. *Aleuritia*, four diploid species occur in North America (*P. alcalina*, *P. anvilensis*, *P. mistassinica* and *P. specuicola*), but chloroplast DNA- (cpDNA) based phylogenies suggest that *P. egaliksensis* is more closely related to *P. mistassinica* ($2n = 18$) than to any other species of *Primula* (Fig. 1; Mast *et al.*, 2001, 2006; Guggisberg *et al.*, 2006; A. Guggisberg, G. Mansion and E. Conti, unpubl. res.). Within sect. *Armerina*, *P. nutans* ($2n = 22$) is the only diploid that occurs in North America and phylogenetic evidence based on rDNA sequences indicates a common origin for *P. nutans* and *P. egaliksensis* (Fig. 1; Guggisberg *et al.*, 2007). Hence *P. mistassinica* and *P. nutans* are the most likely progenitors of *P. egaliksensis*, the former probably acting as the maternal parent. Distributional data further suggest that *P. egaliksensis* originated in North America, because it is rarely found outside of this area and the current ranges of its putative parents only overlap in north-western Canada. Finally, the diversity of cpDNA haplotypes recovered by sequencing of multiple accessions of *P. egaliksensis* advocates for a recurrent origin of this taxon (Guggisberg *et al.*, 2006).

The numerous studies on synthetic and natural polyploids have shown that polyploid genomes are dynamic in their response to polyploidization, underlining the need for additional case studies looking at genomic, genetic and epigenetic changes following chromosome doubling. Cytogenetic investigations are powerful in verifying the origin of polyploid taxa and may provide preliminary insights into the extent of genomic rearrangements. The present paper aims to (1) confirm the hypothesized allopolyploid origin of *P. egaliksensis*, (2) investigate the genomic organization of this tetraploid by genomic *in situ* hybridization (GISH), and (3) understand the evolutionary history of the 45S rDNA loci – consisting of the 18S, 5·8S and 26S ribosomal genes and the internal transcribed spacers (ITS) – using fluorescent *in situ* hybridization (FISH) and ITS sequencing.

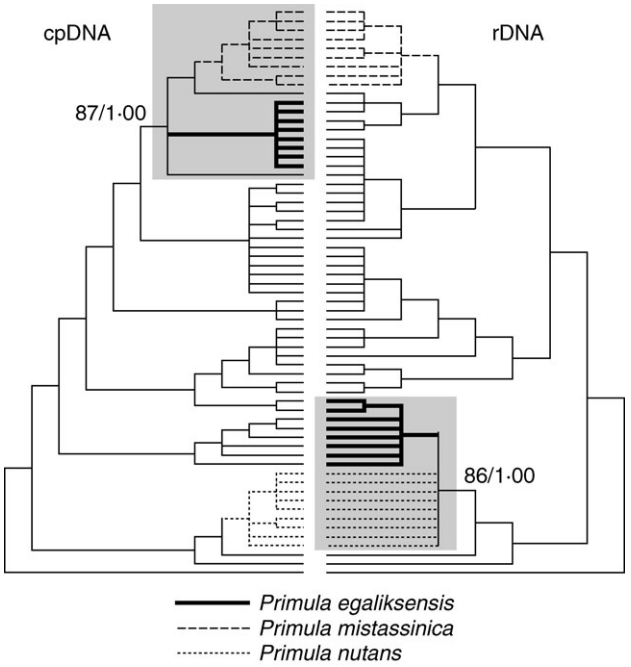

FIG. 1. Phylogenetic placement of *Primula egaliksensis* in relation to its putative parents, i.e. *P. mistassinica* and *P. nutans*, in the cpDNA- and rDNA-based phylogenies inferred by Bayesian analysis. Clades of interest are highlighted in grey and followed by bootstrap support values/ posterior probabilities. Redrawn from A. Guggisberg, G. Mansion and E. Conti, unpubl. res.

MATERIALS AND METHODS

Plant material

The plant material used in this study is listed in Table 1. Different accessions were used for DNA extractions and chromosome spreads because none of the sampled populations contained both the leaf and seedling tissues necessary for the two sets of analyses.

DNA extraction, PCR amplification, cloning and sequencing

Total genomic DNA (gDNA) was extracted using the DNeasy Plant Mini Kit (Qiagen, Switzerland). The two ITS and the 5·8S ribosomal gene of the rDNA cistron (hereafter called the ITS region, covering 638 bp) were amplified

TABLE 1. *Plant material of* Primula *used in this study. Voucher specimens were deposited at the herbarium of Z, Zürich, Switzerland*

Taxon	Tissue type	Provenance	Herbarium voucher
P. egaliksensis	Leaf	Alaska/USA	Guggisberg & Mansion 100703-1b[1]
	Leaf	Newfoundland/Canada	Guggisberg & Mansion 240604-6b[2]
	Root tip	Alaska/USA	Guggisberg & Mansion 060703-1
	Root tip	Alaska/USA	Guggisberg & Mansion 150703-1
P. mistassinica	Leaf	Wisconsin/USA	Anderson 210503-3
	Root tip	Alberta/Canada	Eveleigh 300603-3
P. nutans	Leaf	Alaska/USA	Guggisberg & Mansion 240603-2
	Root tip	Yukon Territory/Canada	Guggisberg & Mansion 250603-2
	Root tip	Yukon Territory/Canada	Bennett, Line & Hett-Seccombe 030604-1

[1]Abbreviated '*P. egaliksensis* 1' in Table 2.
[2]Abbreviated '*P. egaliksensis* 2' in Table 2.

with ITS.LEU and ITS4 primers (Baum *et al.*, 1998) using 2 mM $MgCl_2$, 200 μM dNTPs and 0·2 μM of each primer in a standard PCR buffer using an annealing temperature of 52 °C (with a ramp speed of 1 °C s^{-1}). PCR products destined for sequencing were purified (GFX PCR DNA and Gel Band Purification Kit, Biosciences Amersham, Switzerland) and cloned into pCR®II-TOPO® (TOPO TA Cloning® kit, Invitrogen, Switzerland) before sequencing (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, ABI Prism 3100 automated sequencer, Applied Biosystems, USA). Because preferential amplification of one sequence variant may lead to its overrepresentation in the final reaction mixture (cf. PCR drift and PCR selection; Wagner *et al.*, 1994), three PCR reactions of each sample were pooled for use in ligations (Mason-Gamer, 2004).

Sequencher 4·2 (Gene Codes Corp., USA) was used to check the quality of the electropherograms and compile the contiguous sequences for each PCR product. The starting and ending points of each sequence were determined by comparison with the partial rDNA sequence of *Rhododendron kanehirai* (GenBank AF172290) and deposited in GenBank (accession no. DQ993783-DQ993802, EU095369- EU095392). Cloned ITS sequences of *P. egaliksensis* were finally aligned with 18 sequences of *P. mistassinica* (GenBank DQ993741-DQ993749) and *P. nutans* (GenBank DQ993763-DQ993771) using Se-Al v2·0a9 (available at http://tree.bio.ed.ac.uk/) in order to determine the direction of concerted evolution at sites distinguishing the putative parents. The alignment was searched for variable positions using PAUP* 4·0·10b (Swofford, 1999).

Chromosome preparation and in situ *hybridization*

Seeds were imbibed in water, stratified for 6 weeks at 4 °C, and germinated on Murashige and Skoog basal medium in a constantly illuminated growth chamber at 20 °C. Two-week-old seedlings were treated with auxin (2·5 μM naphthalene acetic acid in medium) for 1 week to induce the formation of root meristems. Mitotic cells were stopped at the metaphase stage upon exposure to colchicine (0·05 % in liquid medium) for 2 h. Root tip samples of approx. 1 cm length were excised and fixed in ice-cold ethanol : acetic acid (3 : 1, v/v) and kept at −20 °C until use. Spread nuclei were prepared essentially as described by Lysak *et al.* (2006), but with 50 % acetic acid.

The preparations were stained with 1 mg mL^{-1} 4,6-diamino-2-phenylindole (DAPI) in Vectashield antifade (Vector Laboratories, Canada) and screened for the quality of metaphase, prophase and interphase nuclei under epifluorescence microscopy (Axioplan, Zeiss, Germany and DM6000, Leica, Germany). The best preparations were rinsed in PBS (10 mM sodium phosphate, pH 7·0, 143 mM NaCl), dehydrated in ethanol series (70 %, 90 %, 100 %, 2 min each), and air dried before FISH or GISH analyses.

For FISH and GISH experiments, slide and probe preparation, hybridization and detection were carried out as described by Lysak *et al.* (2006) with the following minor modifications: the RNase treatment was done for 30 min instead of 60 min; chromosomes were treated with 25 mg mL^{-1} pepsin in 0·01 M HCl for 2 min at 37 °C before hybridization; the probes were pre-denatured for 15 min at 75 °C and cooled on ice for 5 min before hybridization onto the chromosomes; post-hybridization washes were done at 45 °C for 10 min each, followed by rinses at room temperature; for GISH, the chromosomes were denatured in 70 % formamide in 2× SSC for 2 min at 80 °C, fixed in 70 % ethanol at −20 °C for 5 min and dehydrated in 90 % and 100 % ethanol at room temperature. The hybridization mixture consisted of 50–100 ng of labelled probes, 50 % deionised formamide, 10 % dextran sulphate, 2× SSC, and 50 μg sheared salmon sperm DNA. Pre-denaturation of the genomic probe mix was found to be essential to eliminate cross-hybridization at heterochromatic regions, possibly due to quick re-annealing of conserved repeat sequences in the probe mixture before target chromosomes were reached.

The 45S rDNA probe was prepared from the plasmid pTa71 (a gift from P. Fransz, University of Amsterdam) containing the 18S–25S ribosomal genes isolated from *Triticum aestivum* (Barker *et al.*, 1988), and labelled with digoxigenin (Nick Translation Mix, Roche, Switzerland). The ITS probes of *P. egaliksensis* (ITS-PE), *P. mistassinica* (ITS-PM) and *P. nutans* (ITS-PN) were

prepared from PCR products as described above, and labelled with biotin (Nick Translation Mix, Roche, Switzerland). ITS probes from *P. mistassinica* (ITS-PM) and *P. nutans* (ITS-PN) were used on their respective genomes, but also on nuclei of *P. egaliksensis*. For GISH, gDNA of *P. mistassinica* (gDNA-PM) and *P. nutans* (gDNA-PN) was extracted as described above, and sheared to approx. 1000 bp by sonication for 10 s (UW2200, Bandelin Electronic, Germany) before labelling with either digoxigenin- or biotin-labelled nucleotides (Nick Translation Mix, Roche, Switzerland). Digoxigenin-labelled probes were detected using a Mouse-anti-Digoxigenin (Roche, Switzerland) and a Goat-anti-Mouse antibody coupled to Alexa 488 (Molecular Probes, Invitrogen, Switzerland) as primary and secondary antibody, respectively (Lysak *et al.*, 2006). Biotin-labelled probes were detected with Avidin and Goat-anti-Avidin coupled to Texas Red as described by Lysak *et al.* (2006). All images were processed using Adobe Photoshop® (Adobe Systems, Switzerland) and treated for colour contrast and brightness.

RESULTS

GISH demonstrates the hybrid origin of P. egaliksensis

The investigation of root tip prophase and metaphase cells of *P. mistassinica*, *P. nutans* and *P. egaliksensis* confirmed previous reports of chromosome numbers. A diploid chromosome number was counted in both *P. mistassinica* ($2n = 18$; Fig. 2A) and *P. nutans* ($2n = 22$; Fig. 2B), and a tetraploid chromosome number was counted in *P. egaliksensis* ($2n = 40$; Fig. 2C). In addition, most chromosomes of *P. mistassinica* were characterized by heterochromatic knobs (i.e. highly condensed chromatin) corresponding to brightly stained chromosomal regions (cf. arrowhead and inset in Fig. 2A) that were distinct from pericentromeric regions. Noticeably, these genomic structures were absent in both *P. nutans* and *P. egaliksensis*.

In order to assess whether *P. egaliksensis* inherited the genome complements from both *P. mistassinica* and *P. nutans*, GISH was carried out. Control experiments using labelled gDNA of *P. nutans* to hybridize *P. mistassinica* nuclei, and vice versa, showed very little or no cross-hybridization with our experimental conditions (see Materials and Methods; data not shown). Hence, the genomes of the putative parents were differentially labelled and hybridized to *P. egaliksensis* spread nuclei of root tips. The results unambiguously demonstrate the hybrid origin of *P. egaliksensis*, for interphase nuclei as well as metaphase chromosomes of *P. egaliksensis* clearly revealed the presence of both *P. nutans* (red) and *P. mistassinica* (green) genomes (Fig. 2D–J).

Parental chromosomes of P. egaliksensis *remain globally preserved*

GISH on *P. egaliksensis* metaphase plates enabled the counting of 18 chromosomes hybridized with gDNA of *P. mistassinica* and 22 chromosomes hybridized with gDNA of *P. nutans*, as expected if all parental chromosomes were inherited by the allopolyploid (Fig. 2E–J). As evidenced by the relative homogenous hybridization pattern on each chromosome, no chromosomes of *P. egaliksensis* presented hybridization signals from both parents, suggesting that no major intergenomic rearrangements occurred between the parental genomes (e.g. interchromosomal translocations).

ITS sequences of P. egaliksensis *are mainly homogenized towards the* P. nutans *repeat type*

Sequencing of 44 ITS clones from two accessions of *P. egaliksensis* revealed 22 different ITS sequences. These clones varied at 21 of the 27 positions distinguishing the putative progenitors of *P. egaliksensis*, i.e. *P. mistassinica* and *P. nutans* (Table 2). Thirty-six clones (82 %; numbers 1–13, 15, 17–18, 20–21 of *P. egaliksensis* 1 and numbers 1–15, 17–19 of *P. egaliksensis* 2) were completely homogenized towards the ITS repeat type found in *P. nutans*. Of the remaining eight clones, six (numbers 14, 16, 19, 22 of *P. egaliksensis* 1 and 16, 20 of *P. egaliksensis* 2) were mainly, but not completely, homogenized towards the ITS repeat type found in *P. nutans*, while two (21–22 of *P. egaliksensis* 2) were mainly homogenized towards the ITS repeat type found in *P. mistassinica*. Hence only 5 % of the clones presented a nucleotide composition characteristic of *P. mistassinica* ITS sequences. Finally, two clones (numbers 22 and 20 of *P. egaliksensis* 1 and 2, respectively) showed a deletion of 34 bp in the ITS1 (e.g. positions 21 and 44 of the ITS alignment; Table 2). From this analysis, we can conclude that most, but not all, ITS sequences of *P. egaliksensis* are homogenized towards the paternal repeat type (*P. nutans*), suggesting that *P. egaliksensis* has progressively lost the maternally inherited ITS sequences. The incomplete homogenization may indicate that this is still a dynamic process.

rDNA loci of P. egaliksensis *have undergone drastic restructuring*

Because our sequencing results suggested that the rDNA of *P. egaliksensis* have undergone concerted evolution, perhaps involving intra- and interlocus recombination, we investigated the number and position of rDNA loci using FISH. Hybridization with the ITS-PM probe revealed at least 14 signals in *P. mistassinica* (Fig. 2K) and most of them co-localized with the heterochromatic knobs (indicated by arrowheads in Fig. 2K). However, only two ITS signals (indicated by arrows in Fig. 2K) belonged to the unique pair of 45S rDNA loci detected by FISH (Fig. 2L). Conversely, in *P. nutans*, the ITS-PN probe co-localized with the two 45S rDNA loci (Fig. 2M, N). A similar situation was found in the hybrid *P. egaliksensis*, where the ITS-PE probe co-localized with the two 45S rDNA loci (Fig. 2O, P). Likewise, the parental ITS probes (ITS-PN and ITS-PM) co-localized on two loci of *P. egaliksensis* chromosomes (data not shown). The latter experiment indicates that the parental ITS probes are not

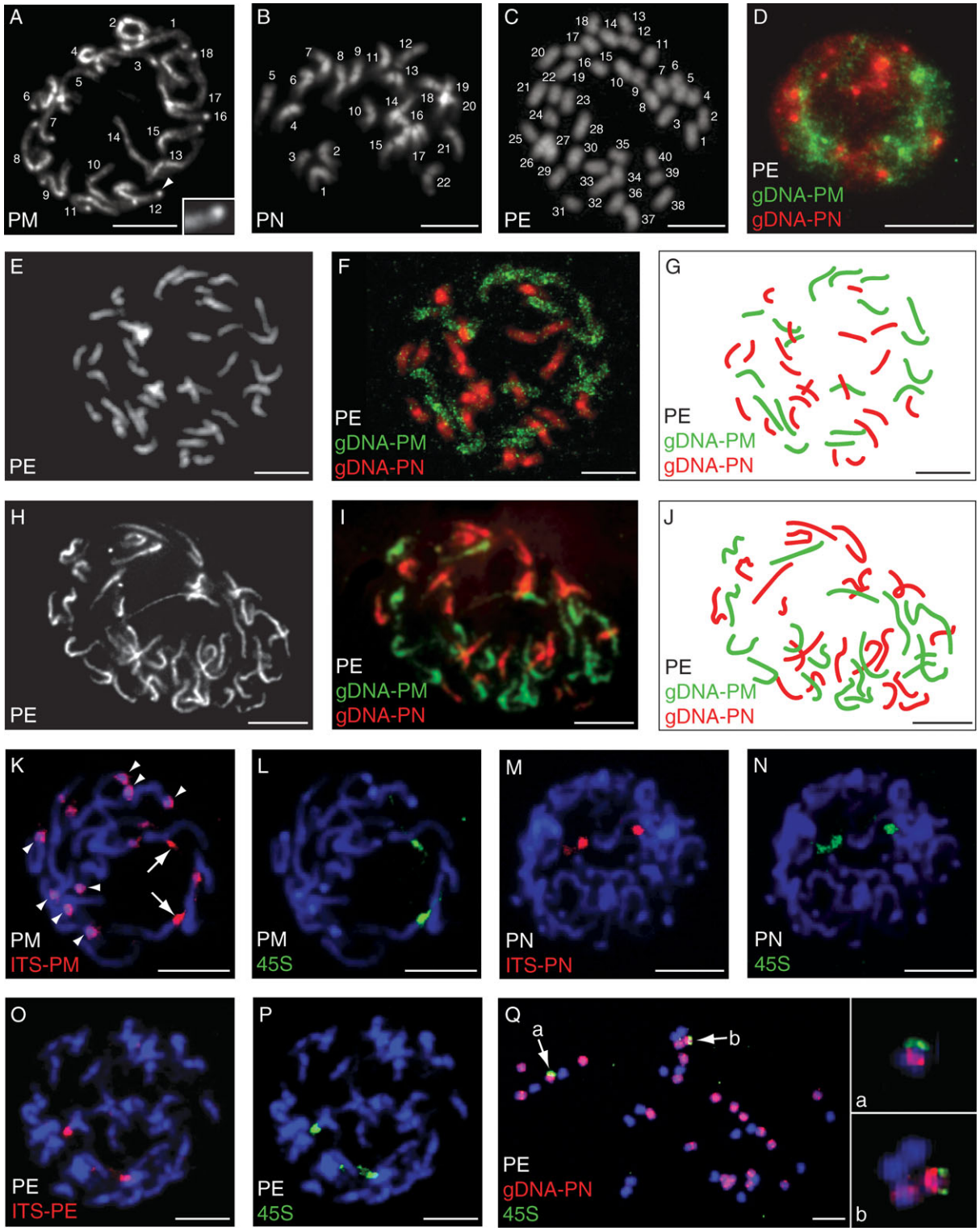

FIG. 2. (A–C) DAPI-stained root tip prophases/metaphases of (A) *Primula mistassinica* showing $2n = 18$ chromosomes, (B) *P. nutans* showing $2n = 22$ chromosomes, and (C) *P. egaliksensis* showing $2n = 40$ chromosomes; the arrowhead in (A) points to the heterochromatic knob shown in the inset. (D) GISH to interphase nuclei of *P. egaliksensis* using gDNA from *P. mistassinica* (green) and *P. nutans* (red). (E–J) Root tip prophases/metaphases of *P. egaliksensis* stained with DAPI (E, H), and resulting GISH signal (F, G, I, J) following *in situ* hybridization of gDNA from *P. mistassinica* (green) and *P. nutans* (red). (K–P) FISH localization of ITS (red) and 45S rDNA (green) loci on root tip prophases of (K, L) *P. mistassinica*, (M, N) *P. nutans*, and (O, P) *P. egaliksensis* counterstained with DAPI; in (K), arrows point to cross-hybridization of ITS and 45S rDNA probes, and arrowheads highlight heterochromatic knobs. (Q) Root-tip metaphase of *P. egaliksensis* counterstained with DAPI after GISH with gDNA of *P. nutans* (red) and FISH with 45S rDNA probes (green); arrows (a, b) point to the two 45S rDNA-bearing chromosomes shown in the insets. Scale bar = 10 μm. PE, *P. egaliksensis*; PM, *P. mistassinica*; PN, *P. nutans*.

TABLE 2. *Clonal variation within* Primula egaliksensis *at the 27 nucleotide positions of the ITS region distinguishing* P. mistassinica *and* P. nutans. *Site numbering follows ITS alignment. Sites identical to the first sequence are represented by dots and gaps are represented by dashes. Site 378 belonging to the 5-8S gene and separating the ITS1 from ITS2 is highlighted in bold.*

Taxon	1 1	2 1	4 4	5 2	5 7	6 8	8 5	9 0	9 7	1 0 5	1 0 6	1 0 9	1 1 9	1 2 6	1 3 0	1 4 2	1 9 3	1 9 7	2 0 2	**2 3 8**	4 4 1	4 9 8	5 5 4	5 7 4	5 9 5	5 8 4	5 9 5	6 1 7
P. nutans	C	C	T	T	G	A	A	T	C	T	G	T	T	T	C	T	A	G	T	**C**	A	C	C	C	T	G	C
P. egaliksensis 1 c1-13/15/17-18/20-21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**.**	.	.	.	.	.	.	.
c14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**.**	G	T	.	.	C	A	.
c16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**T**	.	.	.	.	.	.	.
c19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**.**	.	.	.	.	C	.	.
c22	.	.	–	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	**.**	.	T	.	.	.	.	.
P. egaliksensis 2 c1-15/17-19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**.**	.	.	.	.	.	.	.
c16	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	**.**	.	.	.	.	.	.	.
c20	.	–	–	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	**.**	.	T	.	.	.	.	.
c21	T	T	C	G	.	C	G	C	T	C	.	C	C	C	T	A	G	C	.	**.**	.	.	.	.	.	.	.
c22	T	T	C	G	.	C	G	C	T	C	.	C	C	C	T	A	G	C	G	**.**	G	T	.	.	.	C	A
P. mistassinica	T	T	C	G	T	C	G	C	T	C	A	C	C	C	T	A	G	C	G	**T**	G	T	T	T	C	A	T

different enough (max. 5 % divergence) to be distinguished with our experimental conditions (approx. 80 % washing stringency) and suggest that *P. egaliksensis* lost the numerous ITS-bearing heterochromatic knobs observed in *P. mistassinica* (see Discussion). Clearly, *P. egaliksensis* has lost one pair of rDNA loci from one parent. To determine the origin of the retained loci, we combined GISH and 45S-FISH and found that the rDNA probe hybridized with chromosomes from *P. nutans* (Fig. 2Q).

DISCUSSION

Previous studies have suggested that *P. egaliksensis* is an allotetraploid originating from hybridization of the diploids *P. nutans* and *P. mistassinica* (Kelso, 1991, 1992; A. Guggisberg, G. Mansion and E. Conti, unpubl. res.). The present paper unambiguously confirms this hypothesis and provides insights into the evolution of the parental genomes.

Genomic origin and organization of P. egaliksensis

The chromosome number of *P. egaliksensis* was previously found to be the sum of the chromosomes found in *P. mistassinica* and *P. nutans* (Kelso, 1991), but the origin of the chromosomes has never been investigated. Using GISH, we can now state that *P. egaliksensis* possesses 40 chromosomes corresponding to the entire genome complements of *P. mistassinica* and *P. nutans*, thereby confirming its allotetraploid nature.

The hybridization experiments did not detect major intergenomic rearrangements between the parental genomes (e.g. interchromosomal translocations) in *P. egaliksensis*, suggesting that the chromosomes have retained their parental integrity. Yet karyological comparisons advocate for intragenomic rearrangements, because the tetraploid lacks the heterochromatic knobs characteristic of its maternal progenitor, *P. mistassinica*. Heterochromatic knobs are heavily stained heterochromatic features that contain a low density of expressed genes interspersed with numerous retrotransposons and tandemly repeated elements (reviewed by Bennetzen, 2000). The observation that all *P. egaliksensis* chromosomes inherited from *P. mistassinica* do not present these knobs may suggest active mechanisms of chromosome rearrangement near heterochromatic knobs, as shown in maize (Buckler *et al.*, 1999). Alternatively, this result may argue for *P. mistassinica* being polymorphic for heterochromatic knobs, as it is found in maize (Brown, 1949), indicating that the original parent of *P. egaliksensis* may come from a different population of *P. mistassinica* (with no knobs) than the one sampled here (with knobs).

The most compelling evidence of genome restructuring in the allotetraploid *P. egaliksensis* comes from the analysis of rDNA loci. While the sequencing of ITS clones highlighted a vast homogenization towards the paternal parent *P. nutans*, FISH and GISH analyses demonstrated that the 45S rDNA loci from the maternal parent *P. mistassinica* were lost, in conjunction with the ITS-bearing heterochromatic knobs. The subterminal location of ITS loci in *P. mistassinica* and *P. nutans* may explain the rapid

elimination of ITS copies from the maternal parent in the allopolyploid *P. egaliksensis*. Indeed, studies on cotton, tobacco and peonies indicated that loci located near the telomeres are more prone to recombination (leading to possible homogenization) than sites situated near the centromeres (Wendel *et al.*, 1995; Cronn *et al.*, 1996; Hanson *et al.*, 1996; Zhang and Sang, 1999; Fulnecek *et al.*, 2002). Alternatively, the loss of rDNA genes may be related to nucleolar dominance, a common epigenetic phenomenon in interspecific hybrids whereby only rDNA genes inherited from one parent are transcribed (Reeder, 1985; Pikaard, 2000). Recent investigations on *Brassica* × *Orychophragmus* hybrids have suggested that nucleolar dominance may play a role in chromosome stabilization by inducing genome-specific rearrangements (Li and Ge, 2007). However, remnant nucleotidic signatures of *P. mistassinica* in *P. egaliksensis* ITS clones imply that the loss of maternal ITS sequences occurred later than in the first hybrid generation, since recombination apparently occurred between *P. nutans* and *P. mistassinica* ITS repeats.

Primula egaliksensis *vs. other allopolyploid models*

According to the nucleo-cytoplasmic interaction (NCI) hypothesis proposed by Gill (1991), newly formed polyploids must undergo rapid structural chromosomal changes to lift the 'sterility resulting from the adverse interaction between the male nuclear genome and both the nuclear and cytoplasmic genomes of the female'. Molecular and cytogenetic investigations on synthetic allotetraploid lines of tobacco support this hypothesis, because first-generation polyploids are the genomic sum of their parents, but interchromosomal translocations can be detected after three generations, along with changes in number and composition of rDNA loci (Skalická *et al.*, 2003, 2005; Lim *et al.*, 2006, 2007).

Primula egaliksensis is an intersectional hybrid (Kelso, 1991, 1992; Richards, 2002), and thus the lack of major intergenomic rearrangements between the parental genomes may be a consequence of the considerable genetic divergence between the putative parents. In synthetic allopolyploids, frequencies of intergenomic recombination were shown to be positively correlated with degrees of divergence between the putative progenitors (Song *et al.*, 1995), but studies on hybrids of distantly related mouse strains also indicated that substantial chromosomal divergence suppresses recombination (Shao *et al.*, 2001). Hence, there might be a positive correlation between the genetic divergence of the parents and the frequency of interchromosomal translocations in the hybrids until this genetic divergence reaches a level where homeologous recombination becomes extremely rare or even impossible.

The NCI hypothesis further predicts that the paternal genome should evolve more rapidly than the maternal one because it functions within an alien maternal genomic environment (Gill, 1991). This assumption has been supported by recent studies on synthetic tobacco (Skalická *et al.*, 2003, 2005), but comparable assays on natural *Nicotiana* allopolyploids showed that genetic changes may also be targeted at the maternal genome donor (Lim *et al.*, 2000; Kovarík *et al.*, 2004; Clarkson *et al.*, 2005). The present data on the natural allotetraploid *P. egaliksensis* argue for genome restructuring primarily affecting the genome of the maternal progenitor (*P. mistassinica*). Yet paternally targeted rearrangements at the gene level cannot be excluded since our investigations were restricted to whole-chromosome structure and rDNA loci.

Primula egaliksensis is supposed to have originated during the Pleistocene glaciations (i.e. between 1·8 million to 10 000 years ago) as a result of repeated contact between its putative parents following glacial advancement and retreat (Kelso, 1991, 1992; Richards, 2002; Guggisberg *et al.*, 2006; A. Guggisberg, G. Mansion and E. Conti, unpubl. res.). The efficiency of *in situ* hybridization of the parental genomes onto *P. egaliksensis* chromosomes suggests a relatively high degree of conservation of the progenitor sequences in the hybrid and corroborates the hypothesis of a 'geologically young' hybrid with a maximum age of approx. 1 million years. Indeed, recent studies on natural allopolyploids of *Nicotiana* showed that the effectiveness of GISH is considerably reduced after 1 million years of genome evolution and fails after 5 million years of divergence (Clarkson *et al.*, 2005; Lim *et al.*, 2007).

The concomitant loss of ITS-bearing heterochromatic knobs and 45S rDNA sites of *P. mistassinica* in *P. egaliksensis* may be indicative of ongoing genome diploidization (Leitch and Bennett, 2004; Ma and Gustafson, 2005), attesting to the dynamic nature of polyploid taxa (Soltis and Soltis, 1993; Wendel, 2000; Soltis *et al.*, 2003). Future investigations on the *P. egaliksensis* species complex will be aimed at (1) dating the origin of the allopolyploid; (2) assessing the copy number of 45S rDNA repeats in order to ascertain that the rDNA loci of *P. egaliksensis* were lost and not fused; and (3) identifying the ITS repeats borne on heterochromatic knobs of *P. mistassinica*, as they may not be linked to functional 45S rDNA loci (Maggini *et al.*, 1991; Stupar *et al.*, 2002; Lim *et al.*, 2004*b*).

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