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Disentangling Reticulate Evolution in an Arctic–Alpine Polyploid Complex

ALESSIA GUGGISBERG*, GUILHEM MANSION, AND ELENA CONTI

Institut für Systematische Botanik, Universität Zürich, Zollikerstrasse 107, CH-8008 Zürich, Switzerland;
**Correspondence to be sent to: Department of Botany, University of British Columbia, 3529-6270 University Boulevard, Vancouver, British Columbia, Canada V6T 1Z4; E-mail: alessiag@interchange.ubc.ca.*

Abstract.—Although polyploidy plays a fundamental role in plant evolution, the elucidation of polyploid origins is fraught with methodological challenges. For example, allopolyploid species may confound phylogenetic reconstruction because commonly used methods are designed to trace divergent, rather than reticulate patterns. Recently developed techniques of phylogenetic network estimation allow for a more effective identification of incongruence among trees. However, incongruence can also be caused by incomplete lineage sorting, paralogy, concerted evolution, and recombination. Thus, initial hypotheses of hybridization need to be examined via additional sources of evidence, including the partitioning of infraspecific genetic polymorphisms, morphological characteristics, chromosome numbers, crossing experiments, and distributional patterns. *Primula* sect. *Aleuritia* subsect. *Aleuritia* (*Aleuritia*) represents an ideal case study to examine reticulation because specific hypotheses have been derived from morphology, karyology, interfertility, and distribution to explain the observed variation of ploidy levels, ranging from diploidy to 14-ploidy. Sequences from 5 chloroplast and 1 nuclear ribosomal DNA (nrDNA) markers were analyzed to generate the respective phylogenies and consensus networks. Furthermore, extensive cloning of the nrDNA marker allowed for the identification of shared nucleotides at polymorphic sites, investigation of infraspecific genetic polymorphisms via principal coordinate analyses PCoAs, and detection of recombination between putative progenitor sequences. The results suggest that most surveyed polyploids originated via hybridization and that 2 taxonomic species formed recurrently from different progenitors, findings that are congruent with the expectations of speciation via secondary contact. Overall, the study highlights the importance of using multiple experimental and analytical approaches to disentangle complex patterns of reticulation. [Concerted evolution; consensus network; hybridization; phylogenetic incongruence; *Primula*; recombination; ribosomal DNA polymorphism; secondary contact model.]

Polyploidization is one of the most important mechanisms of speciation in plants (Soltis D.E. and Soltis P.S. 1993; Bretagnolle et al. 1998; Otto and Whitton 2000; Levin 2002; Soltis et al. 2003). Indeed, up to 70% of all angiosperms may be of polyploid origin (Stebbins 1950; Grant 1981; Masterson 1994; Otto and Whitton 2000), including species of small genome size and chromosome number, for example, *Arabidopsis thaliana* (Vision et al. 2000; Henry et al. 2006). Two types of polyploids are commonly recognized according to the degree of homology among coexisting genomes. Autopolyploids, which arise within a single species, contain more than 2 sets of homologous chromosomes in their nuclear genome, whereas allopolyploids contain at least 2 sets of homologous chromosomes that diverged from each other prior to interspecific hybridization (Ramsey and Schemske 1998). Importantly, interspecific hybridization may also give origin to homoploid hybrids that maintain the same ploidy level as their parents (Arnold 1997, 2006; Rieseberg 1997; Rieseberg and Carney 1998). Recent evidence also suggests that recurrent polyploidization between the same species often generates polyploids of independent origins that may differ from each other either morphologically and/or genetically, adding to the complexity of reticulate histories (Soltis D.E. and Soltis P.S. 1993, 1999; Brochmann et al. 1998; Steen et al. 2000).

Species of hybrid origin, either at the homoploid or at the polyploid level, pose special problems in phylogenetic reconstruction, because they derive from the merging of different evolutionary lineages, whereas phylogenetic methods are designed to trace relationships among divergent branches of a genealogy (Rieseberg and Ellstrand 1993; McDade 1995; Linder and Rieseberg 2004; Vriesendorp and Bakker 2005; Arnold 2006). Morphologically, hybrids vary considerably because they may express derived, intermediate, or new character states when compared with the parents (Rieseberg and Ellstrand 1993; McDade 1995). Hybrids usually inherit the nuclear DNA from both parents and the organellar DNA of only one parent; thus, the incongruent placement of individual taxa in phylogenies generated from differently inherited markers can be instrumental in the identification of hypothetical hybrids and their putative parents (Rieseberg and Ellstrand 1993; Wendel and Doyle 1998; Linder and Rieseberg 2004). In angiosperms, comparisons between phylogenies derived from maternally and biparentally inherited chloroplast and nuclear DNA markers, respectively, have been used to reconstruct patterns of reticulation (e.g., Soltis and Kuzoff 1995; Sang et al. 1997).

Recently developed techniques of phylogenetic network estimation provide powerful new tools for the identification of potential hybrid origins because they allow for a more effective visualization of complex patterns of incongruence among trees, as compared with commonly used phylogenetic methods (Posada and Crandall 2001; Linder and Rieseberg 2004; Morrison 2005; Huson and Bryant 2006; Brysting et al. 2007). Importantly, tree incongruence can also be caused by paralogy, incomplete lineage sorting, concerted evolution, and recombination (e.g., Doyle 1992; Maddison 1997; Doyle and Davis 1998; Wendel and Doyle 1998; Álvarez and Wendel 2003; Bailey et al. 2003; Linder and Rieseberg 2004). Therefore, the inconsistent placement of taxa among trees can only provide an initial hypothesis of hybridization, which must be further examined by

integrating additional sources of evidence, including the partitioning of infraspecific genetic polymorphisms, chromosome number, distributional patterns, morphological characteristics, and interfertility experiments (Wendel and Doyle 1998; Arnold 2006).

Phylogenies generated from nuclear markers alone can allow for the identification of hybrids and their parents if both parental copies persist in the hybrid genome and if they were sufficiently diverged prior to hybridization (Doyle and Davis 1998). In this case, the detection, preferably by cloning, of intraindividual polymorphisms consisting of nucleotides that are each identical to the diverged nucleotides from other accessions (i.e., shared nucleotides) provides an indication of possible hybrid origins (Hughes et al. 2002; Smedmark et al. 2003; Devos et al. 2006; Guggisberg, Bretagnolle, et al. 2006; Noyes 2006). However, both intra- and interlocus recombination between repeats in multigene families can disrupt patterns of shared nucleotides, causing different copies to evolve in concert and potentially leading to complete homogenization of all repeat types toward one of the parental copies, with the concomitant loss of the other (Wendel et al. 1995; Kovarík et al. 2004, 2005). If homogenization has erased the hybrid signature in the nuclear marker under study, comparison with phylogenies derived from additional markers can still allow for the identification of reticulate patterns, when the taxa show incongruent placement in the different phylogenies (Wendel and Doyle 1998; Linder and Rieseberg 2004).

Despite its well-known drawbacks (Feliner and Rosselló 2007), the nuclear marker most commonly used for phylogeny reconstruction at low taxonomic levels has been the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA) cistron because the high number of repeats and availability of universal primers in the highly conserved flanking regions have greatly facilitated the procedure of in vitro amplification in a broad range of distantly related taxa (Baldwin et al. 1995; Álvarez and Wendel 2003; Small et al. 2004; Feliner and Rosselló 2007). The nrDNA cistron, formed by the 18S ribosomal gene, the ITS1, the 5.8S ribosomal gene, the ITS2, and the 26S ribosomal gene, is present in hundreds of copies distributed over one or more loci (Long and Dawid 1980). ITS polymorphisms have been instrumental in detecting patterns of reticulation in a large number of angiosperms (e.g., Sang et al. 1995; Campbell et al. 1997; Hughes et al. 2002; Aguilar and Feliner 2003; Devos et al. 2005, 2006; Mansion et al. 2005; Noyes 2006).

In the present study, we analyze newly sequenced chloroplast DNA (cpDNA) and ITS fragments of *Primula* sect. *Aleuritia* subsect. *Aleuritia* Duby (Primulaceae Vent.—hereafter called *Aleuritia*) with a combination of commonly used phylogenetic methods aimed at recovering divergent genealogies (maximum parsimony [MP] and Bayesian inference) and methods recently developed to identify incongruence among trees (consensus networks; Holland and Moulton 2003; Holland et al. 2005). In addition, extensive cloning of ITS allowed us to investigate infraspecific genetic polymorphisms via PCoAs, identify recombination between progenitor sequences, and detect shared polymorphic sites.

The circumboreal, polyploid *Aleuritia* complex is an ideal group with which to study reticulate patterns because speciation in *Aleuritia* was proposed as being driven by hybridization (Bruun 1932; Vogelmann 1956; Hultgård 1990, 1993; Kelso 1991, 1992). Indeed, ploidy levels in *Aleuritia* range from diploidy to 14-ploidy (Table 1), whereas morphological, karyological, and distributional data suggest hybrid origins for most polyploids, allowing for the identification of putative parents.

Speciation by hybridization in *Aleuritia* has been explained within the context of the secondary contact model (Stebbins 1984, 1985). According to this model, glacial advancement during the Pleistocene caused the fragmentation of diploid, allogamous populations, which may have survived in ice-free refugia. As glaciers retreated, the differentiated diploid populations came into contact again and hybridized, giving origin to autogamous, polyploid taxa (Bruun 1932; Vogelmann 1956; Hultgård 1990, 1993; Kelso 1991, 1992; Richards 2002). The higher success of the self-compatible, polyploid species at recolonizing habitats freed by glacial retreat might be explained in terms of selection for reproductive assurance (Guggisberg, Mansion, et al. 2006; Carlson et al. 2008). Indeed, all 8 diploid species are heterostylous, whereas 9 of the 10 polyploid species are homostylous, the exception being the tetraploid *Primula borealis*. Thus, *Aleuritia* may represent yet another example of the crucial role of allopolyploidy in generating species diversity in the Arctic (Brochmann et al. 2004).

Recently supported as monophyletic in chloroplast-based phylogenies (Mast et al. 2001, 2006; Guggisberg, Mansion, et al. 2006), *Aleuritia* includes 21 arctic–alpine species of small- to medium-sized plants, usually perennial and characterized by a single umbel of flowers, a yellow annulus at the center of the corolla, dense farina on the stem and calyx, syncolpate pollen, and a base chromosome number of $x = 9$ (Richards 2002). The main centers of diversity in *Aleuritia* are located in the major mountain systems of North America and Eurasia, except for the Himalayas, and the plains of North America (Table 1; Richards 2002). The only South American species of *Primula* described so far, *Primula magellanica*, also belongs to *Aleuritia* (Table 1; Richards 2002).

The *Aleuritia* complex comprises at least 5 different ploidy levels, including the diploids *Primula alcalina*, *Primula anvilensis*, *Primula exigua*, *Primula farinosa*, *Primula frondosa*, *Primula mistassinica*, *Primula modesta*, and *Primula specuicola* ($2n = 18$), the tetraploids *P. borealis*, *Primula halleri*, *Primula sachalinensis*, and *Primula yuparensis* ($2n = 36$), the hexaploids *Primula incana* and *Primula scotica* ($2n = 54$), the octoploids *Primula laurentiana*, *P. magellanica*, and *Primula scandinavica* ($2n = 72$), and the 14-ploid *Primula stricta* ($2n = \sim 126$; Table 1; Bruun 1932; Vogelmann 1956; Hultgård 1990, 1993; Kelso 1991; Richards 2002). Chromosome numbers are unavailable for 3 Asian endemics: *P. baldschuanica*,

TABLE 1. Distributional ranges and ploidy levels of the 21 species assigned to *Primula* sect

Primula species	Distributional range	Ploidy level
Primula alcalina[a] Cholewa & Henderson	NE Idaho	$2n = 2x = 18$
Primula anvilensis[a] Kelso	Seward Penins. (Alaska)	$2n = 2x = 18$
Primula baldschuanica B. Fedstch.	Tadzhikistan, E Afghanistan	NA
Primula borealis[a] Duby	NW Alaska, N Canada, NE Siberia	$2n = 4x = 36$
Primula capitellata Boiss.	Iran, Afghanistan, Pakistan	NA
P. egaliksensis[a] Wormsk. in Hornem.	N Iceland, W Greenland, N Canada, Alaska, Chukotsk Penins. (NE Siberia), a few sites in Colorado and NW Wyoming	$2n = 4x = 36, 40$
Primula exigua Velenovsky	SW Bulgaria	$2n = 2x = 18$
Primula farinosa[a] L	England, Denmark, Sweden to 64 °N, Finnish archipelago, Baltic states to 60 °N, Montes Universales (Spain), Pyrenees, Alps, Tatra	$2n = 2x = 18$
Primula frondosa Janka	Stara Planina (SE Bulgaria)	$2n = 2x = 18$
Primula halleri[a] Gmel.	E Alps, Tatra, Carpathians, ex-Yugoslavia, Albania, Rila and Pirin ranges (Bulgaria)	$2n = 4x = 36$
Primula incana[a] M. E. Jones	NW America	$2n = 6x = 54$
Primula laurentiana[a] Fernald	NE America	$2n = 8x = 72$
Primula magellanica[a] Lehm.	Tierra del Fuego, Patagonia, Falkland Is.	$2n = 8x = 72$
P. mistassinica[a] Michaux	Canada, central Alaska	$2n = 2x = 18$
Primula modesta[a] Bisset & Moore	Japan, Kurile I	$2n = 2x = 18$
P. nutans[a] Georgi	Gulf of Bothnia, N Fennoscandia eastward to NE Siberia, Kamchatka, Seward Penins. (Alaska), SW Yukon, N Altai, Baikal L., N Mongolia, Gansu Mtns. (China), NW Himalaya	$2n = 2x = 22$
Primula sachalinensis Nakai	E Siberia, Kamchatka and Sakhalin I	$2n = 4x = 36$
Primula scandinavica[a] (Bruun) Bruun	Norway, a few sites in Sweden	$2n = 8x = 72$
Primula schlagintweitiana Pax	N Pakistan, Kashmir and NW India	NA
Primula scotica[a] Hooker	N Scotland	$2n = 6x = 54$
Primula specuicola[a] Rydb.	SE Utah, N Arizona	$2n = 2x = 18$
Primula stricta[a] Hornem.	Scandinavia, eastward to Kola Penins. (Russia), Novaya Zemlya (Russia), N Iceland, W Greenland, NE Canada	$2n = 10 - 16x = \sim 88 - 136$
Primula yuparensis Takeda	Hokkaido (Japan)	$2n = 4x = 36$

Notes: Aleuritia subsect. Aleuritia (Aleuritia) and the 2 species of sect. Armerina (Primula egaliksensis and Primula nutans) supposed to form a hybrid complex with Primula mistassinica (Aleuritia). Abbreviations: E = East; I = Island(s); L = Lake; Mtns. = Mountains; N = North; NA = Not available; NE = Northeast; NW = Northwest; Penins. = Peninsula; SE = Southeast; SW = Southwest.

[a]Species included in the present study. Source: Bruun (1932), Hultgård (1990, 1993), Kelso (1991), Richards (2002), Vogelmann (1956), http://www-sbras.nsc.ru/win/elbib/atlas/flora/4129.html.

P. capitellata, and *P. schlagintweitiana* (Table 1). It has been suggested that most European polyploids of *Aleuritia* derive from a widespread, diploid *P. farinosa*-like ancestor (Bruun 1932; Hultgård 1990, 1993), whereas most American polyploids, including the South American octoploid *P. magellanica*, derive from a widely distributed, diploid *P. mistassinica*-like progenitor (Kelso 1991, 1992).

Although many studies were recently conducted on *Primula* (Hultgård 1990, 1993; Kelso 1991, 1992; Conti et al. 2000; Mast et al. 2001, 2006; Trift et al. 2002; Zhang and Kadereit 2004; Zhang et al. 2004; Guggisberg, Mansion, et al. 2006; Carlson et al. 2008), no investigations were yet aimed at elucidating patterns of hybrid speciation in the genus. Here, we intend to reconstruct the reticulate history of *Aleuritia* by using a combination of phylogenetic and consensus network analyses of cpDNA and ITS data, examination of polymorphic sites in ITS sequences, and identification of recombinant ITS clones. Specifically, we attempt to 1) identify the putative parental lineages of selected polyploids (i.e., *Primula egaliksensis*, 4*x*; *P. scotica*, 6*x*; *P. laurentiana*, 8*x*; *P. stricta*, 14*x*) and compare them with those previously proposed on the basis of chromosome numbers, morphology, and distribution (Hultgård 1990, 1993; Kelso 1991, 1992); 2) establish whether polyploid taxa formed recurrently; and 3) assess whether *Aleuritia* evolved within the framework of the secondary contact model. A more general goal of our study is to illustrate the use of different analytical approaches for discerning among the different possible sources of phylogenetic incongruence and provide a detailed scenario for the evolution of complex polyploid groups.

MATERIALS AND METHODS

Taxon Sampling

A total of 63 plant accessions were used for the present study (online Appendix 1, http://sysbio.oxfordjournals.org). Forty-four accessions represented 15 of the 21 species ascribed to sect. *Aleuritia* and 19 accessions represented 4 of the 14 species ascribed to sect. *Armerina* (hereafter called *Armerina*; Table 1; Richards 2002). The *Armerina* accessions were included because cytological, morphological, and biogeographic evidence suggested that *P. egaliksensis*, assigned to *Armerina* (Richards 2002), might have a hybrid origin involving a member of *Aleuritia* (i.e., *P. mistassinica*) and a member of *Armerina* (i.e., *Primula nutans*; Kelso 1991). Furthermore, recent phylogenies of *Primula* based on cpDNA markers supported the inclusion of selected members of

Armerina and *Aleuritia* in the same, largely unresolved clade and, more specifically, of *P. egaliksensis* within a well-supported *Aleuritia* subclade (Mast et al. 2001, 2006; Guggisberg, Mansion, et al. 2006). Finally, *Primula ioessa* (sect. *Sikkimensis*) was chosen to root the resulting trees because members of the mentioned section were sister to a large clade comprising *Aleuritia* and *Armerina* in the latest *Primula* phylogeny (Mast et al. 2006).

DNA Extraction, PCR Amplification, Sequencing, and Cloning

Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Basel, Switzerland). Polymerase chain reactions (PCRs) were performed in 20 μl volumes containing 1× buffer (including 1.5 mM $MgCl_2$), 2 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, 0.2 μM of each primer, and one unit Taq polymerase (Sigma-Aldrich, Basel, Switzerland). Amplifications were carried out on a thermocycler (Biometra, Goettingen, Germany) using the following conditions: a first cycle at 94 °C for 2 min; 35 cycles at 94 °C for 30 s, 52 °C for 1 min, and 72 °C for 1.75 min and a final cycle of 10 min at 72 °C, while lowering the ramp speed from 5.0 to 1.0 °C/s for the annealing step.

Data matrices were generated from both the chloroplast and the nuclear genomes. The maternal inheritance of the cpDNA has been demonstrated in many angiosperms, including *Primula* (Corriveau and Coleman 1988), whereas nuclear markers are biparentally inherited (Baldwin et al. 1995; Álvarez and Wendel 2003; Small et al. 2004). Five cpDNA markers were each targeted with independent PCRs. The *rpl*16 intron was amplified with primers rpL16F71 and rpL16R1516 (Small et al. 1998); the *rps*16 intron with primers rpS16F and rpS16R2 (Oxelman et al. 1997); the *trn*L-F region (comprising the *trn*L intron, the *trn*L exon, and the *trn*L-*trn*F intergenic spacer) with primers 5′trnL^{UAA}F (TabC) and trnF^{GAA} (TabF; Taberlet et al. 1991); the *trn*T-*trn*L intergenic spacer with primers trnT^{UGU}F (TabA) and 5′trnL^{UAA}R (TabB; Taberlet et al. 1991); and the *trn*D-T region (comprising 3 intergenic spacers and the *trn*Y and *trn*E exons located between the *trn*D and *trn*T genes) with primers trnD^{GUC}F and trnT^{GGU} (Demesure et al. 1995). The ITS region (comprising the ITS1, the 5.8S gene, and the ITS2) of the nrDNA cistron was amplified with primers ITS.LEU and ITS4 (Baum et al. 1998). Sequences of the *trn*D-T and ITS region, including 294 ITS clones (see below), were generated for this study, for a total of 428 new sequences; the remaining data sets (*rpl*16 intron, *rps*16 intron, *trn*L-F region, and *trn*T-L intergenic spacer) were published earlier (Guggisberg, Mansion, et al. 2006). The length of the amplified fragments was estimated by comparison with DNA ladders on 1.2% agarose gels stained with ethidium bromide. The absence of contamination was checked by running out the PCR products of a negative control on the same gel. Successfully amplified PCR products (amplicons) were cleaned with the GFX PCR DNA and Gel Band Purification Kit (Biosciences Amersham, Otelfingen, Switzerland).

Sequencing reactions were prepared with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Rotkreuz, Switzerland), using the same primers as in the PCR amplifications. Sequencing products were purified on 96-well multiscreen filtration plates (Millipore, Zug, Switzerland) to remove excess dye terminators and run on an ABI Prism 3100 automated sequencer (Applied Biosystems, Ann Arbor, MI). Sequencher 4.2 (Gene Codes Corp.) was used to check the quality of the electropherograms and compile the contiguous sequences (contigs) for each amplicon.

Direct sequencing of ITS amplicons produced electropherograms with double peaks and noncomplementarity between sequenced strands in the following accessions of *Aleuritia* and *Armerina*: borealis2, egaliksensis7, farinosa1/3/5/6/8, fasciculata, halleri2, incana 1/2/3, laurentiana4, mistassinica3/4/6/8/9, nutans 1/3/4/5/6/7/8/9, scandinavica2, scotica1, and stricta 1/2. To confirm the nucleotide heterogeneity detected via direct sequencing of ITS amplicons and search for rare repeats in apparently homogeneous samples, PCR products from the following 17 accessions were cloned into pCR®II-TOPO® (TOPO TA Cloning® Kit, Invitrogen, Basel, Switzerland) according to the manufacturer's instructions: alcalina, borealis2, egaliksensis7, farinosa1, halleri2, incana1, laurentiana1/4, mistassinica9, magellanica2, nutans7, scandinavica1/2, scotica1/2, and stricta1/3. Despite the presence of double peaks, the ITS amplicon of fasciculata was not cloned, for *Armerina* was not the focus of the present study. 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), 3 PCRs of each sample were pooled for use in ligations (Mason-Gamer 2004). Ten to 24 white colonies per cloning reaction were randomly selected, and their ITS inserts amplified prior to sequencing.

To estimate the percent of recombinant clones that might be attributed to PCR artifacts, total DNAs from *P. farinosa* (2*x*) and *P. halleri* (4*x*) were mixed at equal ratio for consecutive PCR and cloning (hereafter called "mixed cloning experiment"; see also Kovarík et al. 2005) and the resulting percent of recombinants was compared with the corresponding values obtained for *P. scotica* (6*x*), *P. scandinavica* (8*x*), and *P. stricta* (accessions stricta1; 14*x*) because the diploid (*P. farinosa*) and tetraploid (*P. halleri*) lineages were hypothesized to have contributed to the origin of the higher polyploids (see Results; Bruun 1932; Hultgård 1990, 1993; Arnold and Richards 1998; Richards 2002). To further distinguish between PCR artifacts and real recombinants, we checked whether cloning of products from a second PCR amplification of scandinavica2 yielded identical sequences to those obtained in the first amplification. To avoid potential technical biases, a different experimenter performed this verification.

Phylogenetic Analyses

The starting and ending points of the sequences generated from each PCR amplification were determined by comparison with the complete cpDNA sequence of *Nicotiana tabacum* (GenBank Z00044) and the partial nrDNA sequence of *Rhododendron kanehirai* (GenBank AF172290). Nucleotide sequences were aligned by eye, and 54 bp from the *trn*D-T region had to be eliminated due to ambiguity in the alignment. Unequivocal gaps in cpDNA alignments were coded as present or absent according to Simmons and Ochoterena (2000) with the software GapCoder (Young and Healy 2003) and added as binary characters to the end of the 4-state nucleotide matrix. All analyses of cpDNA sequences described below were performed on the gapped matrices. The aligned data matrices and resulting phylogenetic trees were submitted to TreeBASE (www.treebase.org; study accession number SN3797).

The matrices generated from each of the 5 cpDNA markers were first analyzed separately under MP optimization using the beta 10 version of PAUP* 4.0 (Swofford 1999; online Appendix 2). MP trees for each data set were generated by performing heuristic searches with character states weighted equally, gaps/polymorphisms treated as missing data/uncertainties, TBR branch swapping, Steepest Descent ON, Mulpars ON, and Collapse branches option ON for branches with a minimum length of zero. Two hundred searches were performed under these conditions, after randomizing the order of taxon addition. Five trees per replicate were saved and used as starting trees for a further round of branch swapping with TBR, now saving all the trees. Bootstrap support (BS) values (Felsenstein 1985) for individual branches of the resulting trees were obtained from heuristic searches of 1000 bootstrap replicates with 100 random sequence addition (holding 10 trees at each step), TBR branch swapping, Steepest Descent ON, and maxtrees set to 10.

Visual comparisons of the MP trees derived independently from each of the 5 cpDNA markers revealed no strongly supported (BS $>$ 68%) topological inconsistencies. Therefore, cpDNA data partitions were combined into a "global cpDNA matrix" that was analyzed using the same settings described above (online Appendix 2). Conversely, strongly supported (BS = 100%) topological inconsistencies were identified by comparing the global cpDNA tree (Fig. 1) with the tree derived from MP analyses of the nrDNA sequences (Fig. 2) performed as described above (cf. online Appendix 2), thus precluding the merging of cpDNA and ITS data sets for phylogenetic analysis.

Bayesian analyses of the cpDNA and nrDNA sequences were implemented in MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Prior to analysis, data sets were partitioned (Nylander et al. 2004) and the Akaike information criterion was used to select the best fit models of nucleotide substitutions for each defined partition with Modeltest version 3.6 (Posada and Crandall 1998; online Appendix 3). For the 5 cpDNA markers, the *trn*L exon, separating the *trn*L intron from the *trn*L-*trn*F intergenic spacer, was excluded because it was invariable, and the *trn*L-F region was split into 2 partitions: the *trn*L intron and the *trn*L-*trn*F intergenic spacer. Conversely, the 2 short exons (*trn*Y: 84 bp; *trn*E: 73 bp) separating the 3 spacers of the *trn*D-T region were retained in the data matrix because they varied at one nucleotide position. The cpDNA characters were divided into the following partitions: *rps*16 intron, *rpl*16 intron, *trn*L intron, *trn*L-*trn*F intergenic spacer, *trn*T-*trn*L intergenic spacer, *trn*D-T region, and coded gaps. A binary model (Lset coding = variable) was applied to all gaps coded in the 5 cpDNA markers. The ITS region data set was divided into the following categories: ITS1, 5.8S gene, and ITS2. The 5.8S gene, separating the ITS, was retained in the data matrix because it varied at 2 nucleotide positions.

Posterior probabilities of the trees and parameters for the selected substitution models were approximated by Monte Carlo Markov chain simulations using 1 cold and 3 incrementally heated chains (temperature = 0.2) over 5×10^6 generations (Nylander et al. 2004). Trees were sampled every 100 generations, resulting in 50 000 trees minus the trees sampled during the burn-in phase (i.e., before chains reached stationarity). Stationarity was determined by plotting the log-likelihood against the number of generations and ascertained for each parameter using Tracer v1.4 (Rambaud and Drummond 2007). Two independent runs (Nruns = 2), starting from different random trees, were performed to ensure that the individual runs had converged to the same result. Convergence of runs was asserted when the potential scale reduction factor provided by the sump output approached 1.0, and the resulting tree topologies, branch lengths, and clade credibility values were similar across runs (Huelsenbeck and Ronquist 2001; Huelsenbeck et al. 2002; Ronquist and Huelsenbeck 2003). Final inference of the trees and parameters was obtained from the concatenation of the 2 runs (online Appendix 4).

To test specific cases of incongruence between the cpDNA and the nrDNA trees, we used the likelihood-based Shimodaira–Hasegawa test (Shimodaira and Hasegawa 1999; Goldman et al. 2000; Lee and Hugall 2003). Given both the results of our phylogenetic analyses (see below) and previous hypotheses of relatedness (Hultgård 1990, 1993; Kelso 1991, 1992; Guggisberg, Mansion, et al. 2006), we defined the following topological constraints: 1) *P. egaliksensis* sister to *P. nutans* in the cpDNA tree; 2) *P. egaliksensis* sister to *P. mistassinica* in the nrDNA tree; 3) all accessions of *P. laurentiana* in the same clade in the cpDNA tree; and 4) all accessions of *P. stricta* in the same clade, both in the cpDNA and in the nrDNA trees. The significance levels of the differences in the likelihood scores for each comparison (Table 2), under the selected model of sequence substitution (online Appendix 4), were then checked on a null distribution obtained with the reestimated log-likelihoods (RELL) approximated with 1000 nonparametric bootstrap replicates. The RELL approximation was used to avoid reestimation of the parameters in the nonparametric bootstrap replicates (Shimodaira and Hasegawa 1999).

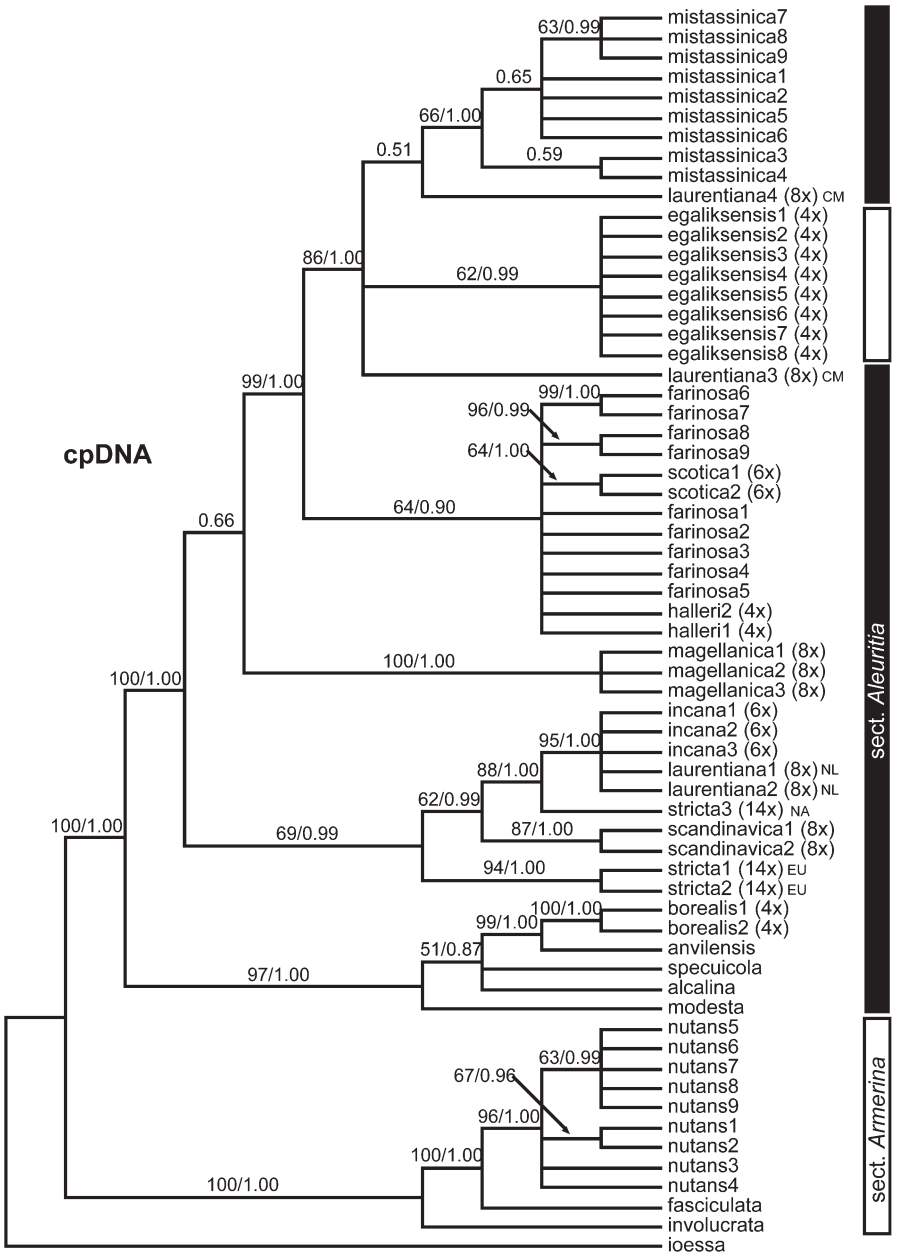

FIGURE 1. Fifty percent majority-rule consensus cladogram inferred by Bayesian analysis of the global cpDNA data set. Clades recovered also in the 50% majority-rule consensus of the 12 MP trees are highlighted by thicker branches. BS values greater than 50% and posterior probabilities, separated by a slash, are indicated above the branches of the cladogram. Abbreviations for terminals are given in online Appendix 1. CM, Canadian mainland; EU, Europe; NA, North America; NL, Newfoundland.

Consensus Networks

To facilitate comparison of the phylogenetic conflicts supported by the cpDNA and nrDNA sequences, we mirrored the respective 50% majority-rule consensus trees estimated by MP (cf. Figs 1 and 2) after editing them as follows (Fig. 3*a*): 1) for monophyletic species, multiple accessions were collapsed to a single terminal, whereas for nonmonophyletic species, accessions representing different clades in either tree were maintained (cf. Figs 1 and 2), and 2) nodes with BS lower than 70% were collapsed (cf. Figs 1 and 2), ensuring the comparison of only well-supported topological inconsistencies. The resulting trees are called "simplified 70% BS consensus trees" from now on (Fig. 3*a*).

Additionally, consensus networks were computed because they allow for the simultaneous identification of competing hypotheses of relationships supported by a group of trees (Holland and Moulton 2003; Holland et al. 2005). Consensus networks may vary between tree-like representations, when trees are fully congruent with each other, and net-like representations, when trees are strongly incongruent with each other (Holland

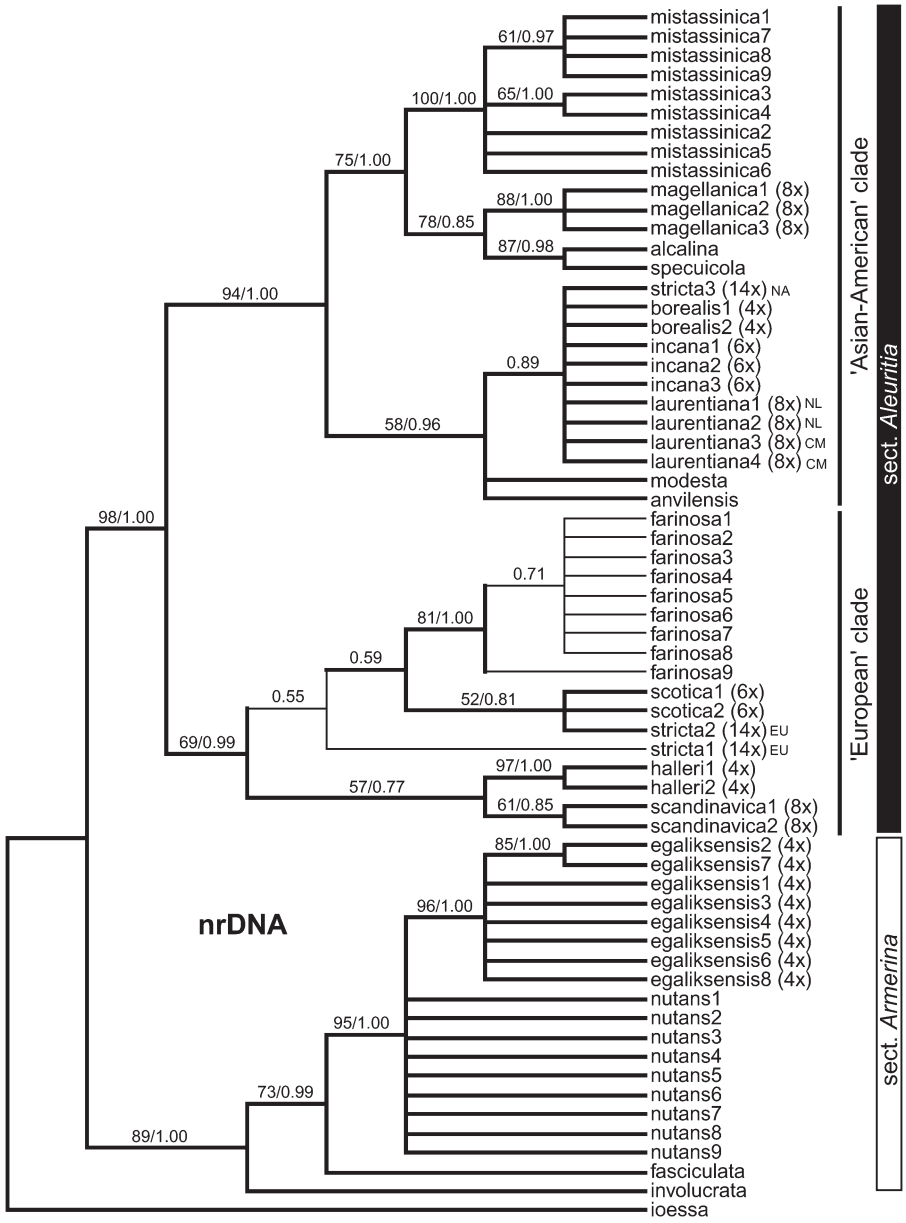

FIGURE 2. Fifty percent majority-rule consensus cladogram inferred by Bayesian analysis of the nrDNA data set. Clades recovered also in the 50% majority-rule consensus of the 349 MP trees are highlighted by thicker branches. BS values greater than 50% and posterior probabilities, separated by a slash, are indicated above the branches of the cladogram. Abbreviations for terminals are given in online Appendix 1. CM, Canadian mainland; EU, Europe; NA, North America; NL, Newfoundland.

and Moulton 2003; Holland et al. 2005). The complexity of a network also depends on the threshold x, representing the branches (or splits, σ) that occur in a certain proportion of the trees. For example, a network calculated by using $x = 0.1$ displays the splits occurring in at least 10% of the trees. Thus, lower or higher values of x, respectively, capture a greater or smaller proportion of the topological conflicts in a group of trees (McBreen and Lockhart 2006).

To identify the topological inconsistencies caused by diploid and polyploid accessions, respectively, consensus networks were computed from the simplified 70% BS consensus trees of the cpDNA and nrDNA matrices (cf. Fig 3*a*) by either excluding (using the Data Filter Taxa option in SplitsTree 4.6; Huson 1998; Huson and Bryant 2006; Fig. 3*d*) or including the polyploids in the analyses (Fig. 3*e*). We used the default value of x (0.1) because, when comparing only 2 trees, any x value between 0 and 0.5 will ensure representation of all topological conflicts.

Identification of Shared Nucleotides at Polymorphic Sites

We defined any position of the ITS alignment that varied among direct sequences from amplicons of different accessions as a "variable" site (e.g., site 21

TABLE 2. Results of Shimodaira–Hasegawa tests (Shimodaira and Hasegawa 1999; Goldman et al. 2000; Lee and Hugall 2003) on 4 topological constraints

	cpDNA				nrDNA			
Constraints	−lnL UTree	−lnL CTree	−lnL Diff	*P* value	−lnL UTree	−lnL CTree	−lnL Diff	*P* value
(*Primula egaliksensis–Primula nutans*) clade	8611.3	9005.1	393.8	<0.001***	NA	NA	NA	NA
(*P.egaliksensis–Primula mistassinica*) clade	NA	NA	NA	NA	2017.2	2137.6	120.4	<0.001***
(*Primula laurentiana*) clade	8611.3	8799.8	188.5	<0.001***	NA	NA	NA	NA
(*Primula stricta*) clade	8611.3	8636.8	25.5	<0.05*	2017.2	2077.4	60.2	<0.001***

Abbreviations −lnL = highest likelihood score; −lnL Diff = difference between likelihood scores of unconstrained tree (Utree) and constrained tree (Ctree); NA = not available. *significant at $P < 0.05$, ***significant at $P < 0.001$.

in online Appendix 5). A site was designated as "polymorphic" when more than one peak was present in the electropherogram derived from the direct sequence of an ITS amplicon (online Appendix 5) or when at least 2 different nucleotides were detected at the same position among the clones of a single ITS amplicon (online Appendix 6). In either case, the different nucleotides detected at the same position in the same individual are defined as polymorphic nucleotides, forming a single polymorphism (i.e., infraindividual variation). Given a rate of 2×10^{-4} errors/bp and an average length of 638 bp for each sequence, the 294 sequenced ITS clones may contain 38 point mutations resulting from Taq error. Hence, nucleotide positions differing in only one clone per amplicon were not defined as polymorphic. For example, whereas the direct sequence of an amplicon from sample scotica1 presented 6 polymorphic sites (e.g., position 11 of the ITS alignment; online Appendix 5), cloning of an amplicon from the same accession revealed 23 polymorphic sites, 17 of which were not detected by direct sequencing (e.g., position 12 of the ITS alignment; online Appendix 6). In addition, 18 positions that differed in only one clone were identified and, therefore, not defined as polymorphic (e.g., position 45, clone5; online Appendix 6).

Polymorphisms can lead to conflicting assessments of relatedness and are commonly treated as uncertainties in phylogenetic analyses. However, when intraindividual polymorphisms consist of nucleotides that are each identical to the diverged nucleotides at the same site of sequences from other accessions (i.e., shared nucleotides at polymorphic sites), they may help to identify the progenitors of hybrid species (Sang et al. 1995; Campbell et al. 1997; Aguilar and Feliner 2003). Therefore, the polymorphisms described above were identified and assembled in Table 3.

Analyses of Variation among ITS Clones

To investigate the infraindividual variation among cloned ITS sequences and visualize the positions of recombinant sequences of the higher polyploids in relation to lower polyploids and diploids, multivariate analyses were performed using NTSYSpc version 2.11S (Rohlf 2000). Pairwise distances were calculated with PAUP* 4.0b10 (Swofford 1999) for all possible pairs of clones using the best fit models of nucleotide substitutions selected by the Akaike information criterion implemented in Modeltest version 3.7 (Posada and Crandall 1998). The generated triangular dissimilarity matrices were then used for PCoA (Gower 1966).

Initially, all 294 sequenced ITS clones (179, if excluding duplicates) were analyzed by PCoA (online Appendix 7a). Because this first analysis revealed that clones from the "European" and "Asian-American" clades (cf. Fig. 2) did not form tight clusters, the respective subsets of 134 (91) and 133 (68) clones were each analyzed separately to increase resolution (Fig. 4*a* and online Appendix 7b). Because this second PCoA showed that ITS sequences from the European high polyploids (i.e., *P. scotica* [6*x*], *P. scandinavica* [8*x*], and sample stricta1 of *P. stricta* [14*x*]) occupied intermediate positions between those of *P. farinosa* (2*x*) and *P. halleri* (4*x*; Fig. 4*a*), we further investigated the origin of the mentioned polyploids by mapping the base composition at the 10 polymorphic sites that differ between *P. farinosa* and *P. halleri* (i.e., positions 32, 43, 61, 85, 105, 167, 208, 231, 235, and 514 of the ITS alignment; see Table 3) onto diagrams of the ITS sequences of *P. scotica*, *P. scandinavica*, and *P. stricta* (accession stricta1; Fig. 4*b*). A recombination event was inferred each time there was a switch (identified by a cross in Fig. 4*b*) from a nucleotide specific to *P. farinosa* to another specific to *P. halleri* or vice versa (see online Appendix 6; Buckler et al. 1997).

RESULTS

Assessment of PCR-Mediated Recombination in ITS Clones

A total of 294 ITS sequences (179, if excluding duplicates) were generated from cloned PCR products, comprising 20 (6) clones for *P. alcalina*, 16 (9) for *P. borealis*, 19 (8) for *P. egaliksensis*, 24 (13) for *P. farinosa*, 21 (16) for *P. halleri*, 18 (12) for *P. incana*, 41 (23) for *P. laurentiana*, 9 (6) for *P. magellanica*, 9 (7) for *P. mistassinica*, 8 (7) for *P. nutans*, 41 (27) for *P. scandinavica*, 34 (27) for *P. scotica*, and 34 (18) for *P. stricta* (online Appendix 6).

The mixed cloning experiment recovered 36% (33 of 92) PCR-mediated recombinant clones. The fact that 88% (30 of 34), 71% (29 of 41), and 86% (12 of 14) recombinants were found in *P. scotica*, *P. scandinavica*,

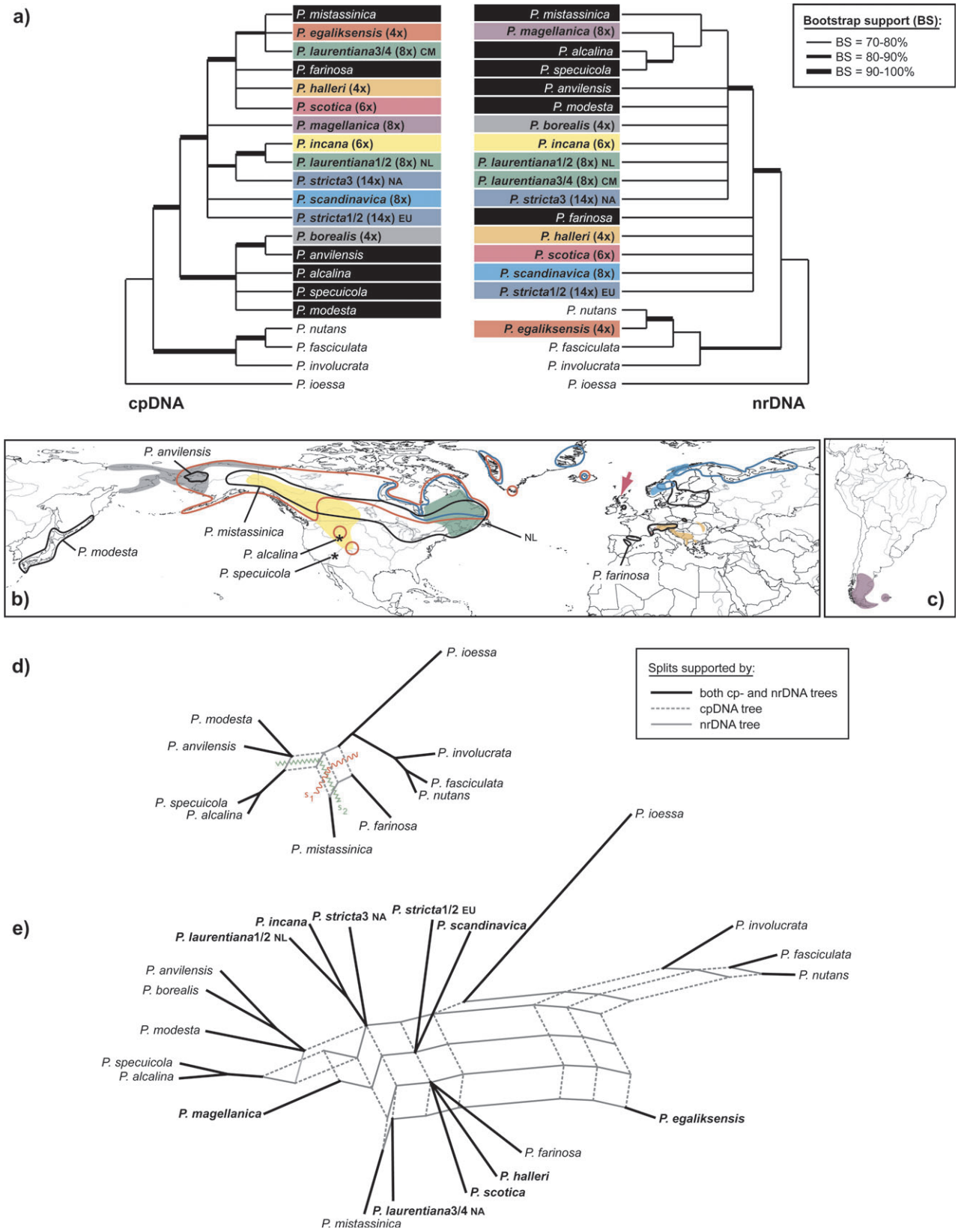

FIGURE 3. Continued.

and sample stricta1 of *P. stricta*, respectively (Fig. 4*b*), suggests that a nonnegligible proportion of recombinant clones in these polyploids does not result from PCR artifacts. This conclusion is corroborated by 2 additional findings. First, for a majority (79%) of the recombinant clones from the mixed cloning experiment only one recombination was necessary to explain the observed patterns of nucleotide composition (data not shown), whereas a minority (3%) of all the recombinant clones obtained from *P. scotica*, *P. scandinavica*, and *P. stricta* were explained by a single recombination event (see scotica1 clone9 and scandinavica2 clone21; Fig. 4*b*). Second, 50% (3 of 6) of the recombination events inferred for the European polyploids *P. scotica*, *P. scandinavica*, and *P. stricta* are shared across clones, individuals, and ploidy levels (see recombination events inferred between sites 208 and 231, 231 and 235, and 235 and 514; Fig. 4*b*).

The additional 14 ITS cloned sequences obtained from the second PCR amplification of scandinavica2 confirmed the results from the first amplification. Ten clones (71%) were fully homogenized toward the *P. farinosa* ITS repeat type at the 10 nucleotide sites distinguishing *P. farinosa* and *P. halleri* (see below); they corresponded to the sequences obtained previously for clones4/5/6/7/11/13/14/15/18/20/22/23 (52% of 23 total clones; Fig. 4). The remaining 4 clones (29%) were composed of 3 nucleotides shared with *P. farinosa*, 7 shared with *P. halleri*, and 1 autapomorphy at the 10 positions differing between *P. farinosa* and *P. halleri* (data not shown) and corresponded to the repeat type found earlier for clones1/2/8/9/17/19 (26% of 23 total clones; Fig. 4).

Altogether, results from the mixed cloning experiment and the repeated cloning of scandinavica2 suggest that 6 recombinant clones (scandinavica1 clone6 and scandinavica2 clones3/10/12/16/21) may result from PCR artifact; they are highlighted by an asterisk in Figure 4 and in subsequent mentions. This conclusion relies upon the observation that recombination events inferred between sites 32 and 43 (see scandinavica2 clone12*), 43 and 61 (see scandinavica1 clone6*), and 167 and 208 (see scandinavica2 clones3/10/12/16/21*) are not shared across individuals or ploidy levels (Fig. 4*b*) and corroborates the failure of reamplifying the aforementioned clones for scandinavica2.

Combined Evidence on the Origin of Selected Polyploids

The nrDNA tree strongly supports (BS 94%) a clade comprising all taxa from Asia and America ("Asian-American clade") and moderately supports (BS 69%) a clade formed by all European taxa ("European clade") sampled within *Aleuritia* (Figs 2 and 3*a*,*b*). Conversely, in the cpDNA tree, strongly supported, smaller clades formed by American taxa (i.e., [*P. mistassinica, P. egaliksensis, P. laurentiana*]; *P. magellanica*; [*P. incana, P. laurentiana*, stricta3]) are members of polytomies that include European taxa (Figs 1 and 3*a*,*b*).

The 6 diploid taxa of *Aleuritia* (*P. alcalina*, *P. anvilensis*, *P. farinosa*, *P. mistassinica*, *P. modesta*, and *P. specuicola*) present only 8% (6 of 71) shared nucleotides at the polymorphic sites, whereas the 9 polyploid taxa present 92% (65 of 71) shared nucleotides at the polymorphic sites (Table 3). Accordingly, the inclusion of polyploid lineages in the consensus networks computed from the simplified 70% BS consensus cpDNA and nrDNA trees causes most of the conflicts (Fig. 3*e*), whereas diploid taxa only yield less incongruence (Fig. 3*d*). An additional important result of our phylogenetic analyses is that all species for which multiple accessions were available are supported as either monophyletic or potentially so in both trees (Figs 1 and 2), with 2 notable exceptions: the North American octoploid *P. laurentiana* ($2n = 72$) and the amphi-Atlantic 14-ploid *P. stricta* ($2n = 126$; Fig. 3*a*,*b*). Below we focus on results from different lines of evidence that allow us to elaborate on the origin of selected polyploids.

Primula egaliksensis.—A clear incongruence between the cpDNA and the nrDNA trees concerns the placement of the mainly North American *P. egaliksensis* ($2n = 4x = 36, 40$; Fig. 3*a*,*b*), which falls in an unresolved polytomy with the North American *P. mistassinica* ($2n = 2x = 18$) and *P. laurentiana* (accessions laurentiana3/4; $2n = 8x = 72$) in the cpDNA tree (BS 86%; Fig. 1) and with the circumboreal *P. nutans* ($2n = 2x = 22$) in the nrDNA tree (BS 95%; Fig. 2). The analyses of topological constraints indicate that the likelihood of the data is significantly lower when *P. egaliksensis* is forced to group with either *P. nutans* in the cpDNA tree or *P. mistassinica* in the nrDNA tree (Table 2). The inclusion of *P. egaliksensis* results in a 2.14-fold increase in

FIGURE 3 (previous page). *a*) Simplified 70% BS consensus trees supported by cpDNA (left) and nrDNA (right) data sets. *b*, *c*) Distributional ranges of the tree terminals represented with matching colors. Source: Hultgård (1990; 1993), Kelso (1991), and Richards (2002). *d*, *e*) Consensus networks computed from the 2 simplified cpDNA and nrDNA 70% BS consensus trees to identify topological inconsistencies caused by diploid (*d*) and polyploid accessions (*e*), respectively. Splits supported by both cpDNA and nrDNA trees are indicated by thick black lines, those supported by the cpDNA tree by gray dashed lines, and those supported by the nrDNA tree by plain gray lines. *d*) By cutting through the splits supported by the cpDNA tree (e.g., σ_1: red wavy line) and the nrDNA tree (e.g., σ_2: green zigzag line), respectively, it is apparent that incongruence among the diploid species arises from the placement of *Primula mistassinica* with either *Primula farinosa* in the former or (*Primula alcalina, Primula specuicola*) in the latter (cf. *a*). *e*) The topological conflicts caused by polyploid taxa mainly arise from the differential placement of *Primula egaliksensis* and accessions of *Primula incana*, *Primula laurentiana*, *Primula magellanica*, *Primula scandinavica*, and *Primula stricta* between the nrDNA and cpDNA trees (cf. *a*). Polyploid accessions are in bold; abbreviations as in online Appendix 1. Abbreviations: CM = Canadian mainland; EU = Europe; NA = North America; NL, Newfoundland.

complexity of the consensus network of Figure 3*e*, from initially 7 to a total of 15 splits involved in net-like structures. Finally, multivariate analyses of cloned PCR products show that all ITS sequences from *P. egaliksensis* cluster with *P. nutans* (online Appendix 7a), confirming that they are homogenized toward a single repeat type.

Primula scotica.—A weakly supported incongruence between the cpDNA and nrDNA trees concerns the placement of the hexaploid *P. scotica* ($2n = 54$), endemic to northern Scotland and the adjacent Orkney Islands (Fig. 3*b*). In the cpDNA tree (Fig. 1), the 2 accessions fall in a weakly supported (BS 64%) polytomy with the European *P. farinosa* ($2n = 2x = 18$) and *P. halleri* ($2n = 4x = 36$). In the nrDNA tree (Fig. 2), the same accessions form a weakly supported (BS 52%) clade with a European sample of *P. stricta* (stricta2) and are members of the larger European clade, which includes also *P. farinosa* and *P. halleri*. The inclusion of *P. scotica* yet creates no additional net-like structures in the consensus network (Fig. 3*e*). The analysis of ITS polymorphisms prove more informative than phylogenetic and network analyses, revealing that *P. scotica* shares polymorphic nucleotides with *P. farinosa* and *P. halleri* at the 10 sites (32, 43, 61, 85, 105, 167, 208, 231, 235, and 514) that differ between the diploid and tetraploid species (Table 3). The detailed analysis of 16 (14, if excluding duplicates) ITS clones from scotica1 and 18 (12) ITS clones from scotica2 suggests that 2 clones (scotica1 clones2/16) are completely homogenized toward the *P. farinosa* ITS type and 2 other clones (scotica1 clones5/12) toward the *P. halleri* ITS type (Fig. 4*b*). The remaining 30 (22) ITS clones of *P. scotica* show evidence of recombination between the 10 sites that distinguish *P. farinosa* from *P. halleri* (Fig. 4*b*) but share most of the nucleotides with *P. farinosa* (Fig. 4*b*), thus clustering with the "farinosa" group in the PCoA scatterplot (Fig. 4*a*).

Primula scandinavica.—The 2 accessions of the Norwegian endemic octoploid *P. scandinavica* ($2n = 72$; Fig. 3*b*), while being sister to each other in both trees, show inconsistent relationships between the cpDNA and nrDNA phylogenies (Figs 1 and 2). In the cpDNA tree, *P. scandinavica* is weakly supported (BS 62%) as sister to a clade formed by the North American hexaploid *P. incana* ($2n = 54$), the octoploid *P. laurentiana* ($2n = 72$), and the amphi-Atlantic 14-ploid *P. stricta* ($2n = 126$; Fig. 1), whereas in the nrDNA tree, *P. scandinavica* is weakly supported (BS 61%) as sister to the European tetraploid *P. halleri* ($2n = 36$; Fig. 2). Furthermore, *P. scandinavica* does not add to the complexity of the consensus networks (Fig. 3*e*). However, detailed analyses of ITS sequences prove more informative than phylogenetic inference. *Primula scandinavica* shares 10 nucleotide polymorphisms with the hexaploid *P. scotica* ($2n = 54$) at sites 32, 43, 61, 105, 167, 208, 235, 378, 445, and 594 (Table 3). Two nucleotides at positions $378_{(T)}$ and $445_{(A)}$ are exclusively shared with *P. scotica*, whereas polymorphisms at the remaining 8 sites are shared with the

TABLE 3. Thirty-two polymorphic sites from the ITS region of *Primula* sect. *Aleuritia*: the nucleotides shared between different accessions are highlighted in gray; the 10 sites at which *Primula farinosa* and *Primula halleri* differ are designated by arrows; site 378 belonging to the 5.8S gene and separating the ITS1 from ITS2 is italicized

Clade	Accessions	1 2	1 2	3 3	4 3	5 4	6 1	↓ 8 5	↓ 1 5	↓ 1 5	1 2	1 2	1 5	↓ 1 6	1 6	1 7	1 8	1 9	2 0	2 0	2 1	2 3	2 3	↓ 2 3	↓ 3 7	4 4	4 5	4 6	5 0	5 4	5 0	5 1	5 7	5 9	6 0	6 1
Asian-American	*Primula mistassinica*	T	G	C	A	A	T	G	C	C	G	G	C	C	G	C	G	A	C/T	T	C	G	C	T	C	C	C	C	A	T	A/G	A	T
	Primula specuicola	T	A	C	A	A	C	G	C	C	G	G	C	C	G	C	G	A	C	T	C	G	C	C	C	C	C	C	A	C	G	A	C
	Primula alcalina	T	A	C	A	A	C	G	C	C	T	G	C	C	G	C	G	A	C	T	C	G	C	C	C	C	C	C	A	C	G	A	C
	Primula arvilensis	C	A	C	A	A	C	G	C	C	G	G	C	C	G	C	G	A	C	T	C	G	C	C	C	C	C	C	A	C	G	A	C
	Primula modesta	T	A	C	A	A	C	G	C	C	C	G	C	C	G	C	G	A	C	T	C	G	C	C	C	C	C	C	A	C	G	A	C
	Primula borealis (4*x*)	A	A	C	A	A	C	G	C	C	C	G	C	C	G	C	G	A	C	T	C	G	C	C	C	C	C	C	A	C/T	G	A	C/T
	Primula incana (6*x*)	A	A	C	A	A	C	G	C	C/T	C/G	G	C	C	G	C/T	G	A	C	T	C	G	C	C	C	C	C	C	A	T	G	A	C
	Primula laurentiana (8*x*)	A	A	C	A	A	C	G	C	C	G	G	C	C	G	C	G	A	C	T	C	G	C/G	C	C	C	C	C	A	T	G	A	C
	Primula magellanica (8*x*)	T	A	C	A	A/G	C	G	C	C	G	G	C	C	G	C	G	A	C	T	C	G	C	C	C	C	C	C	A	C	G	A	C
	Primula stricta3 (14*x*)	A	A	C	A	A	C	G	C	C	G	G	C	C	G	C	G	A	C	T	C	G	C	C	C	C	C	C	A	T	G	A/G	C
European	*P. farinosa*	T	A	T	A	A	T	G	C	A/T	G	G/-	C	C	A	T	A/T	A	C	T	C	G	G	C	C	C	C	C	A	T	G	A/G	C
	P. halleri (4*x*)	T	A	C	C	A	C	C	T	T	G	G	C/T	C	G	T	A	A	C/T	A	C	T	C	C	C	C	C	C	T	T	A/G	A	C
	Primula scotica (6*x*)	A/G/T	A/C	C/T	A/C	A	C/T	C/G	C/T	T	G	A/G	C	A/C	A/G	T	A	A/C	C	A/T	C/T	G/T	C/G	C/T	A/C	C/T	C	C/T	A/T	T	A/G	A	C
	Primula scandinavica (8*x*)	T	C/G	C/T	A/C	G	C/T	A/G	C/T	T	G	G	C	C	A/G	T	A	A	C/T	A/T	C	G	C/G	C/T	A/C	C	C/T	C	A	T	A/G	A	C
	P. stricta1/2 (14*x*)	A/T	A/G	C/T	A/C	A	C/T	A/G	C/T	T	G	G/-	C/T	A/C	A/G	T	A	A/C	C/T	A/T	C/T	G	C/G	C	C	C/T	C/T	C/T	A	T	G	A	C

Note: Clade affiliation as in Figure 2.

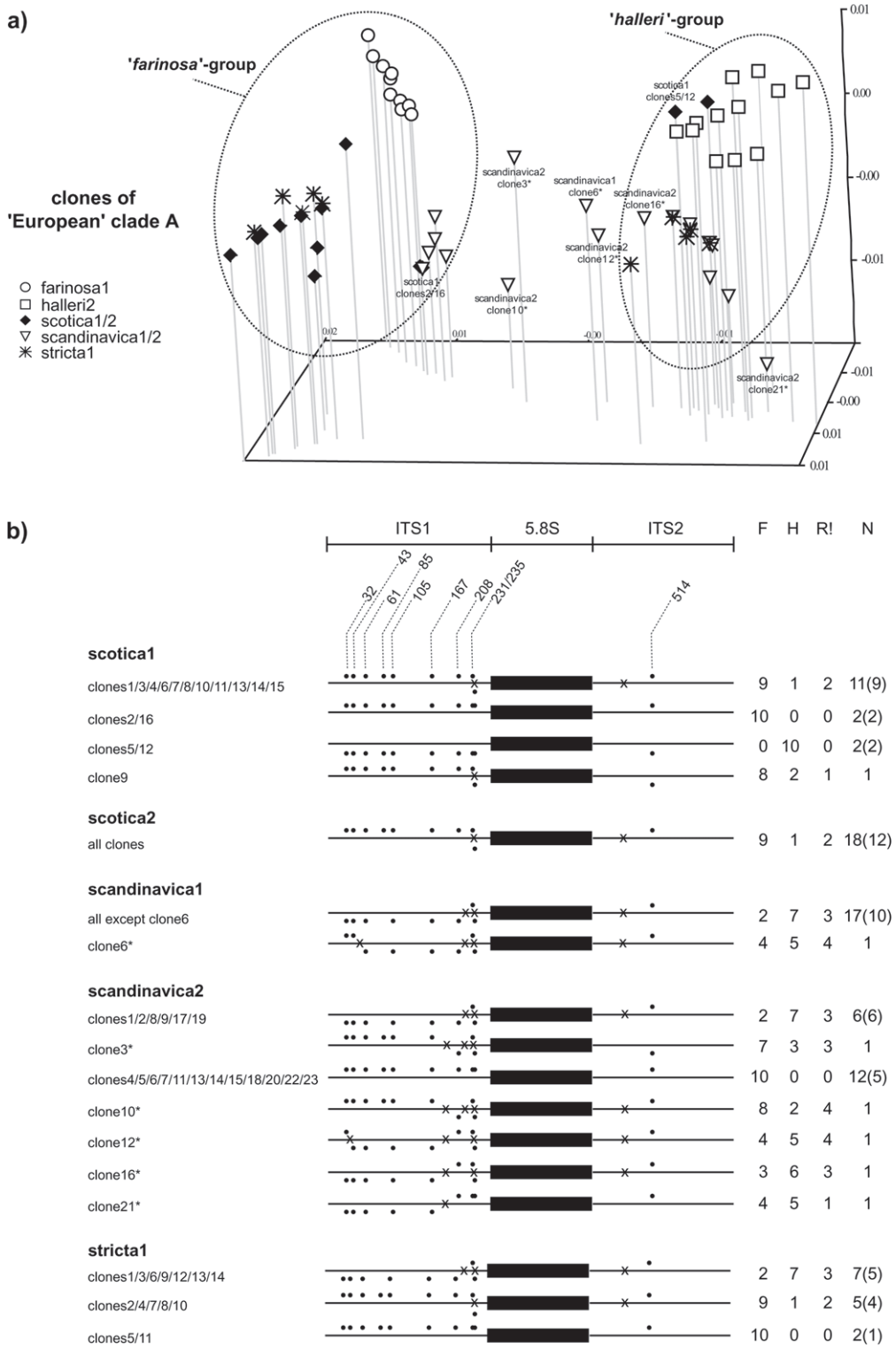

FIGURE 4. Continued.

diploid *P. farinosa* ($2n = 18$) and the tetraploid *P. halleri* ($2n = 36$; Table 3). The analysis of 41 (27, if excluding duplicates) cloned ITS sequences from 2 accessions of *P. scandinavica* revealed that 12 (5) clones are completely homogenized toward the *P. farinosa* ITS type (Fig. 4*b*), thus clustering with the "farinosa" group in the PCoA scatterplot (Fig. 4*a*). The remaining 29 (22) ITS clones show varying degrees of recombination. Depending on the proportion of nucleotides shared with *P. farinosa* and *P. halleri*, respectively (Fig. 4*b*), these recombinant clones either fall in intermediate positions between the "farinosa" and the "halleri" group (see scandinavica1 clone6* and scandinavica2 clones3/10/12/21*) or cluster with the "halleri" group in the PCoA scatterplot (Fig. 4*a*).

Primula laurentiana.—In the cpDNA tree (Fig. 1), the 2 accessions of *P. laurentiana* from Newfoundland (laurentiana1-2; designated by NL) form a strongly supported clade (BS 95%) with the North American hexaploid *P. incana* ($2n = 54$), whereas the 2 accessions from the Canadian mainland (laurentiana 3-4; designated by CM) fall in a strongly supported (BS 86%) polytomy with the North American diploid *P. mistassinica* ($2n = 18$) and the tetraploid *P. egaliksensis* (of sect. *Armerina*; $2n = 36, 40$), as seen above. Conversely, in the nrDNA tree (Fig. 2), the 4 accessions of *P. laurentiana* form a weakly supported (BS 58%) clade with North American diploid (*P. modesta* and *P. anvilensis*) and polyploid taxa (*P. borealis*, 4*x*; *P. incana*, 6*x*; stricta3, 14*x*). Likewise, all 41 (23, if excluding duplicates) cloned ITS sequences fall along those of *P. borealis*, *P. incana*, and stricta3 in the PCoA scatterplot (online Appendix 7b). The analyses of topological constraints support the conclusion that the likelihood of the cpDNA data is significantly lower when the 4 accessions of *P. laurentiana* are forced to be monophyletic (Table 2). Accordingly, the inclusion of *P. laurentiana* results in a 1.25-fold increase in the complexity of the consensus network, from initially 12 to a total of 15 splits involved in net-like structures (Fig. 3*e*).

Primula stricta.—The 3 accessions of the amphi-Atlantic *P. stricta* ($2n = 14x = 126$) do not form a monophyletic group either in the cpDNA or in the nrDNA tree (Fig. 3*a*,*b*). The analyses of topological constraints support the conclusion that the likelihood of the data is significantly lower when the 3 accessions of *P. stricta* are forced to be monophyletic either in the cpDNA or in the nrDNA tree (Table 2). Accordingly, the inclusion of *P. stricta* results in a 1.25-fold increase in the complexity of the consensus network, from initially 12 to a total of 15 splits involved in net-like structures (Fig. 3*e*).

The 2 European accessions of *P. stricta* (stricta1-2; designated by EU) form a strongly supported clade (BS 94%) in the cpDNA tree (Fig. 1), but their relationships are poorly resolved both in the cpDNA and in the nrDNA phylogenies (Fig. 3*a*). Again, detailed analyses of the 14 (10, if excluding duplicates) ITS clones from stricta1 prove more informative. Seven (5) clones are completely (clones5/11) or almost completely (clones2/4/7/8/10) homogenized toward the *P. farinosa* ITS type at the 10 sites differing between *P. farinosa* and *P. halleri* and cluster along ITS clones of the "farinosa" group in the PCoA scatterplot (Fig. 4). The remaining 7 (5) clones (clones1/3/6/9/12/13/14) share 7 (of 10) nucleotides with *P. halleri* and cluster along ITS clones of the "halleri" group in the PCoA scatterplot (Fig. 4). The tight clustering of cloned ITS sequences of stricta1 along those of *P. scotica* ($2n = 6x = 54$) in the "farinosa" group (Fig. 4*a*) reflects the 5 nucleotides exclusively shared with the hexaploid at position $160_{(A)}$, $198_{(C)}$, $219_{(T)}$, $446_{(T)}$, and $504_{(T)}$(Table 2 and online Appendix 6). Likewise, the tight clustering of ITS clones of stricta1 along those of *P. scandinavica* ($2n = 8x = 72$) in the "halleri" group (Fig. 4*a*) reflects the 3 nucleotides exclusively shared with the octoploid at position $12_{(G)}$, $85_{(A)}$, and $500_{(T)}$ (Table 2 and online Appendix 6).

In the cpDNA tree, the North American accession of *P. stricta* (stricta3; designated by NA) is a strongly supported (BS 88%) sister to the North American clade formed by *P. incana* ($2n = 6x = 54$) and *P. laurentiana* ($2n = 8x = 72$; Fig. 1), whereas it clearly (BS 94%) falls in a polytomy comprising all sampled Asian-American taxa of *Aleuritia* in the nrDNA tree (Fig. 2). Accordingly, multivariate analyses of ITS clones reveal that the cloned sequences of stricta3 cluster very tightly with those of *P. borealis* ($2n = 4x = 36$), *P. incana*, and *P. laurentiana* (online Appendix 7b).

Discussion

The use of multiple approaches including classic phylogenetic and more recently developed consensus network methods, the identification of shared nucleotides at polymorphic sites, the multivariate analysis of cloned

FIGURE 4 (previous page). Analyses of variation among ITS clones. *a*) Three-dimensional scatterplot for the first 3 axes obtained by PCoA of 134 (91, if excluding duplicates) cloned ITS sequences from accessions of the "European" clade of *Aleuritia* (see Fig. 2). *b*) Recombination in cloned ITS sequences of *Primula scotica* (6*x*), *Primula scandinavica* (8*x*), and accession stricta1 of *Primula stricta* (14*x*). Lines represent ITS1 and ITS2 and filled boxes the 5.8S ribosomal gene. Site numbering follows the ITS alignment. The 10 polymorphic sites that differ between *Primula farinosa* and *Primula halleri* (see Table 3) were mapped on diagrams of the cloned sequences. Filled circles placed above and below the lines denote synapomorphies with *P. farinosa* and *P. halleri*, respectively. A recombination event was inferred each time there was a switch (identified by a cross) from a nucleotide specific to *P. farinosa* to another specific to *P. halleri* or vice versa. Recombinant clones most likely resulting from PCR artifacts are highlighted by an asterisk (see Results). Abbreviations: F = number of nucleotides shared with *P. farinosa*; HN = number of nucleotides shared with *P. halleri*; R = minimum number of recombinations necessary to explain the observed nucleotides; N = number of clones with the shown haplotype (excluding duplicates). Abbreviations of samples as in online Appendices 1 and 6.

ITS sequences, and the detection of recombination in ITS sequences provided new insights into the complex reticulate history of *Aleuritia*. Specifically, we identified the likely parental lineages of selected polyploids and compared our hypotheses with those previously proposed on the basis of karyological, morphological, and distributional evidence (Bruun 1932; Vogelmann 1956; Hultgård 1990, 1993; Kelso 1991, 1992; Richards 2002). We also established that in at least 2 cases the polyploids likely formed recurrently, a conclusion that is congruent with the secondary contact model (Stebbins 1984, 1985) earlier invoked to explain speciation in *Aleuritia*. Below, we elucidate the origin of an intersectional hybrid involving a species of *Aleuritia* and summarize the evidence supporting the secondary contact model. A general theme emerging from our multifaceted study is that each analytical method provided complementary insights that allowed us to disentangle different aspects of phylogenetic incongruence and, ultimately, reticulation in the complex.

The Arctic flora has recently been recognized as a model for the study of polyploidy in plants (Brochmann et al. 2004) because the repeated cycles of habitat fragmentation, range expansion, and reunion of the Arctic flora during the Pleistocene glaciations (Stebbins 1984, 1985; Abbott and Brochmann 2003) seem to have favored the evolution of many intricate allopolyploid complexes (e.g., in *Cerastium*, *Draba*, *Poa*, or *Saxifraga*; reviewed in Brochmann et al. 2004; Brysting et al. 2007). The observed increase in the frequency of polyploids with increasing degree of glaciation (Löve A. and Löve D. 1957; Stebbins 1971; Brochmann et al. 2004) can be attributed to the higher success of polyploids over diploids at recolonizing newly deglaciated (i.e., disturbed) habitats (Stebbins 1971; Ehrendorfer 1980). Alternatively, polyploids may be more prevalent at higher latitudes because the environmental instability encountered at these latitudes may promote the production of unreduced gametes (Hagerup 1932; Felber 1991; Mable 2004), which constitute the predominant mode of polyploidization in plants (Bretagnolle and Thompson 1995).

Our data on *Aleuritia* provide another example of an alpine–arctic polyploid complex (see discussion below). Previous investigations (Kelso 1992; Guggisberg, Mansion, et al. 2006) further suggest that the parallel switch in breeding system and ploidy level, from diploid allogamous (heterostylous) to polyploid autogamous (homostylous) populations, may have represented a selective advantage in the colonization of barren postglacial habitats because polyploid homostyles were independent of both mate density and pollinator activity to find new populations (Baker 1955; Stebbins 1957; Fausto et al. 2001; Kalisz et al. 2004). Hence, the present work not only proposes a way to handle complex reticulation patterns but also advances our understanding of the processes shaping the organismal diversity of arctic plants. Further studies addressing the evolutionary history of the Arctic flora in space and time (e.g., Scheen et al. 2004; Schneeweiss et al. 2004) are though necessary to tackle the general question of whether bursts of polyploidization are associated with climatic oscillations.

Intersectional Allopolyploidization

The mainly North American tetraploid *P. egaliksensis* ($2n = 36, 40$; Fig. 3*b*) was taxonomically ascribed to sect. *Armerina* owing to its entire petiolate leaves, narrow elongate capsules, and the absence of farina (Kelso 1991, 1992). The proposed hybrid origin of *P. egaliksensis* from the North American diploid *P. mistassinica* ($2n = 18$) of *Aleuritia* and the circumboreal diploid *P. nutans* ($2n = 22$) of sect. *Armerina* relied primarily on the additivity of chromosome numbers, the intermediacy of gland types, pollen sizes, colpi numbers, and exine reticulation patterns, and the overlapping distribution of the mentioned diploids in northwestern Canada, along the border with Alaska (Kelso 1991, 1992). The incongruent placement of *P. egaliksensis* between the cpDNA and nrDNA trees (Fig. 3*a*,*e*) and the analyses of topological constraints (Table 2) support the hypothesis of an intersectional origin for *P. egaliksensis*. Additionally, the maternal inheritance of the chloroplast genome in Primulaceae (Corriveau and Coleman 1988) and the homogenization of the ITS clones of *P. egaliksensis* toward the *P. nutans* repeat type (online Appendix 7a) suggest that a *P. mistassinica*-like and a *P. nutans*-like ancestor likely provided the maternal and paternal parent, respectively, in the hybridization event. Our conclusions on the parental origins of *P. egaliksensis* are consistent with the results of the latest molecular cytogenetic investigations (Guggisberg et al. 2008).

The mean ITS sequence divergence between the putative progenitors of *P. egaliksensis* (0.06205 ± 0.00032) falls within the average range of genetic distances calculated for other allopolyploid species complexes (0.06249 ± 0.00695; Chapman and Burke 2007), suggesting that the intersectional allopolyploidization event discussed above is not an unusual case. Indeed, a recent survey by Chapman and Burke (2007) indicates that the likelihood of polyploid hybrid speciation may increase with increasing evolutionary divergence between the hybridizing species (but see Buggs et al. 2008). Several hypotheses may be put forward to explain this trend. Fertilization between unreduced gametes is thought to be the major mode of polyploid emergence (Bretagnolle and Thompson 1995), and the production of unreduced gametes may be positively correlated with the genetic divergence between hybridizing species (Ramsey and Schemske 1998). Alternatively, allopolyploids formed from distantly related parents may be more fertile and may establish more easily than those formed upon hybridization of closely related species because pairing between homologous chromosomes would be largely prevented (Darlington 1937).

Support for the Secondary Contact Model in Aleuritia

It has been repeatedly suggested that the *Aleuritia* polyploid complex originated within the framework

of the secondary contact model (Stebbins 1984, 1985) through hybridization events that involved differentiated populations of 2 widespread diploids, a *P. mistassinica*-like ancestor in America and a *P. farinosa*-like ancestor in Europe (Bruun 1932; Vogelmann 1956; Hultgård 1990, 1993; Kelso 1991, 1992; Richards 2002). The DNA-based data presented here are congruent with the hypothesis that cycles of habitat fragmentation and reconnection driven by glacial advancement and retreat during the Pleistocene promoted active speciation within the *Aleuritia* polyploid complex.

Our molecular data are compatible with previously proposed hybrid origins and putative parental lineages of selected polyploids (Bruun 1932; Vogelmann 1956; Hultgård 1990, 1993; Kelso 1991, 1992; Richards 2002). For example, a general hypothesis of a hybrid origin for *P. scotica* ($2n = 6x = 54$) had been proposed based on the high levels of intrapopulation allelic variation revealed by allozyme markers (Glover and Abbott 1995). Different lines of evidence from molecular analyses of cpDNA and nrDNA sequences (Figs 3 and 4) lend support to the assumption of an allopolyploid origin of *P. scotica* involving a diploid ($2n = 18$) *P. farinosa*-like and a tetraploid ($2n = 36$) *P. halleri*-like ancestor. Interestingly, *P. farinosa* currently occurs in northern England (Fig. 3*b*), and seeds probably belonging to *P. halleri* were discovered in Quaternary deposits of Cambridegshire, along with seeds attributed to *P. scotica* (Dovaston 1956). Likewise, the high levels of crossfertility between *P. scandinavica* ($2n = 8x = 72$) and *P. farinosa* ($2n = 2x = 18$; Arnold and Richards 1998) in conjunction with the shared presence of dense farina on vegetative parts and perforate pollen ornamentations in *P. scandinavica* and *P scotica* ($2n = 6x = 54$; Hultgård 1990, 1993) had prompted the hypothesis of an allopolyploid origin of the octoploid involving the respective diploid and hexaploid species. Our data from cpDNA and nrDNA phylogenies (Fig. 3) together with the analyses of ITS clones (Fig. 4) appear to support this conclusion.

One of the assumptions supported by the secondary contact model is that polyploids might form recurrently in different locations, as differentiated populations of the progenitors come into contact. At the phylogenetic level, this expectation should translate into polyphyletic origins of the polyploids. Our phylogenetic results from the chloroplast and nuclear genomes support this scenario in 2 cases. The polyphyletic placement of the accessions ascribed to *P. laurentiana* ($2n = 8x = 72$) in the cpDNA tree (Fig. 1) suggests that this octoploid might have originated repeatedly in different locations. The strongly supported relationships in the available cpDNA phylogeny (Fig. 1) suggests that a maternal hexaploid ($2n = 54$) similar to *P. incana* may have contributed to the origin of the Newfoundland populations of *P. laurentiana*, although their extant distributions do not overlap (Fig. 3*b*). Interestingly, Vogelmann (1960) reported both hexaploid and octoploid counts for *P. laurentiana* from Newfoundland, implying that 2 cytotypes may be recognized. Large ice masses covered most of North America during the last glacial maximum (ca. 18 000 BP; Dyke et al. 2002), but the simultaneous survival of a hexaploid lineage in 2 disjunct glacial refugia (e.g., in Beringia and along the North Atlantic coast of Newfoundland, respectively; see, e.g., Abbott and Comes 2003; Jaramillo-Correa et al. 2004) may have lead to the formation of 2 geographically and genetically disparate entities. Conversely, the Canadian mainland populations of *P. laurentiana* may stem from a hybridization event involving a maternal ancestor similar to the widespread diploid *P. mistassinica* ($2n = 18$; Fig. 1). The overlapping distributions of these 2 taxa in North America are consistent with this hypothesis (Fig. 3*b*). In theory, it is also possible that the origin of the mainland accessions of *P. laurentiana* may have involved the tetraploid *P. egaliksensis* of sect. *Armerina* (Fig. 1), for some populations were reported to have a chromosome number of $2n = 36$ (Table 1; Kelso 1991, and references therein) that would be compatible with the base number of *P. laurentiana*. However, recent cytogenetic investigations did not confirm these counts (Guggisberg et al. 2008). Clearly, the clarification of the parental ancestry of *P. laurentiana* requires more chromosome counts from its distributional range and sequences from additional populations and nuclear markers.

Based on additivity of chromosome numbers, crossfertility experiments, and distributional patterns, 2 possible progenitor pairs were proposed for *P. stricta* ($2n = 14x = 126$): the North American *P. incana* ($2n = 6x = 54$) and *P. laurentiana* ($2n = 8x = 72$) and the European *P. scotica* ($2n = 6x = 54$) and *P. scandinavica* ($2n = 8x = 72$; Kelso 1991, 1992; Arnold and Richards 1998). Combined evidence from different analyses of chloroplast and DNA sequences (Figs 3 and 4) supports the polyphyletic origins of the amphi-Atlantic *P. stricta* and suggests that the North American and European accessions of *P. stricta* probably originated independently from different progenitor pairs on the 2 sides of the Atlantic. Additional sampling of European and North American populations of *P. stricta* and putative relatives are though necessary to confirm the proposed polyphyletic origins of this taxonomic entity.

Identifying Causes of Phylogenetic Incongruence

The comparison between cpDNA and nrDNA phylogenies of *Aleuritia* (Figs 1 and 2) revealed several cases of topological inconsistencies, and the analysis of consensus networks computed from the simplified 70% BS consensus trees (Fig. 3*a*) suggested that the polyploids are responsible for most of the observed incongruence (Fig. 3*e*). Considering the previously published hypotheses of hybrid origins for the *Aleuritia* polyploids, based on karyological, morphological, and distributional evidence (Bruun 1932; Vogelmann 1956; Hultgård 1990, 1993; Kelso 1991, 1992; Richards 2002), it seems reasonable to point to hybridization as a cause of phylogenetic incongruence in *Aleuritia*. However, the evolutionary causes of phylogenetic incongruence may

also include paralogy, lineage sorting, concerted evolution, and recombination (Doyle 1992; Maddison 1997; Doyle and Davis 1998; Wendel and Doyle 1998; Álvarez and Wendel 2003; Bailey et al. 2003; Linder and Rieseberg 2004). Below we illustrate selected examples of how different analytical methods induced us to favor reticulation as the major source of incongruence in the *Aleuritia* complex.

Phylogenetic incongruence can occur when gene duplication events precede cladogenetic divergence. In this case, discordance between cpDNA and nrDNA trees may be caused by the use of paralogous, rather than orthologous gene sequences for phylogenetic inference (Doyle 1992; Maddison 1997; Doyle and Davis 1998; Álvarez and Wendel 2003; Bailey et al. 2003; Linder and Rieseberg 2004). Barring extinction of gene copies, it is ideally necessary to sample all repeats to reduce the probability of erroneous phylogenetic comparisons. Whereas our sampling of infraspecific ITS variation may not be exhaustive, the analysis of 179 ITS clones (excluding duplicates) allowed the additional detection of 23 shared polymorphisms that had not been identified by direct sequencing of PCR products and that provided crucial details into the evolutionary history of *Aleuritia* (see Results). For example, ITS cloning revealed 16 additional polymorphisms that linked *P. halleri* (4*x*) with *P. scotica* (6*x*), solidifying the suggestion that the tetraploid contributed to the origin of the hexaploid.

Both lineage sorting, the stochastic sorting of alleles following divergence from a polymorphic ancestor, and reticulation, the merging of differentiated genomes in a single species, may cause alleles from one species to be more closely related to alleles from a different species than to alleles from the same species (Doyle 1992; Maddison 1997; Wendel and Doyle 1998), potentially causing phylogenetic incongruence (Maureira-Butler et al. 2008). In the nrDNA and cpDNA consensus trees of *Aleuritia* (Figs 1 and 2), all sequences from multiple accessions of the same species were monophyletic or members of polytomies, with the exception of sequences from the octoploid *P. laurentiana* and the 14-ploid *P. stricta*, which were polyphyletic in at least 1 tree and topologically incongruent between the 2 trees (Fig. 3*a*). These results could be interpreted as evidence for either lineage sorting or reticulation in these 2 species. Whereas our data are not fully conclusive on the causes of phylogenetic incongruence in the case of *P. laurentiana*, detailed analyses of ITS sequences proved more informative for *P. stricta*. More specifically, the observation that cloned ITS sequences of the European accession stricta1 cluster with either of the previously proposed parental lineages (i.e., *P. scotica* and *P. scandinavica*; Fig. 4*a*; Kelso 1991, 1992; Arnold and Richards 1998), reflecting recombination and different degrees of homogenization toward the parental repeats (Fig. 4*b*), seems to favor the reticulation hypothesis for the observed incongruence between the cpDNA and nrDNA consensus trees (Fig. 3*a*).

Phylogenetic incongruence also arises when processes of genetic homogenization in a multigene family of the nuclear genome cause the conversion of all copies in a given species to a single repeat type that differs in its relationships from those of the cpDNA topology (Doyle and Davis 1998; Wendel and Doyle 1998; Álvarez and Wendel 2003). For example, in the tetraploid *P. egaliksensis*, all ITS sequences obtained directly from PCR products of 8 individuals form a single clade with ITS sequences of the diploid *P. nutans* (Fig. 2), a topology that differs from the placement of *P. egaliksensis* accessions with the diploid *P. mistassinica* in the cpDNA tree (Fig. 1). Multivariate analyses of cloned PCR products showed that all ITS sequences from *P. egaliksensis* clustered with *P. nutans*, confirming that they had homogenized toward a single repeat type (online Appendix 7).

Finally, recombination between different repeat types, by producing sequences that possess a mixture of parental characteristics, often leads to conflicting hypotheses of relationships, resulting in a lack of phylogenetic resolution (Doyle and Davis 1998; Wendel and Doyle 1998; Álvarez and Wendel 2003; Linder and Rieseberg 2004). In the cpDNA and nrDNA consensus trees of *Aleuritia*, the relationships of the polyploid *P. scotica* are slightly inconsistent, for direct ITS sequences of this species form a poorly resolved clade with *P. stricta* (i.e., sample stricta2) and *P. farinosa* (Fig. 2), whereas cpDNA sequences form a polytomy with *P. farinosa* and *P. halleri* (Fig. 1). The analysis of cloned ITS sequences from *P. scotica* revealed that 2 clones each were completely homogenized toward the *P. farinosa*-(scotica1 clones2/16) and *P. halleri*-repeat type (scotica1 clones5/12), respectively, whereas 22 clones (excluding duplicates) showed evidence of recombination between sites differentiating *P. halleri* and *P. farinosa*, with partial homogenization toward the *P. farinosa* type (Fig. 4*b*), thus suggesting that phylogenetic inconsistencies can be explained by recombination between parental ITS repeat types. The high rate of recombination detected in *P. scotica*, *P. scandinavica*, and accession stricta1 of *P. stricta* may be the hallmark of ongoing processes of concerted evolution that ultimately lead to the homogenization of divergent ITS repeats in allopolyploids, for concerted evolution acts via intra- and interlocus recombination (Wendel et al. 1995; Kovaríik et al. 2004, 2005).

To summarize, phylogenetic incongruence may be suggestive of hybrid origins, but it is insufficient to eliminate other possible causes of discrepancies between phylogenies derived from differentially inherited genomes. Therefore, the use of multiple analytical approaches (i.e., generation of cpDNA and nrDNA trees and their comparison via consensus networks, identification of shared nucleotides at polymorphic sites, multivariate analysis of cloned ITS sequences, and analysis of recombination in ITS) is fundamental to achieve a comprehensive understanding of evolutionary relationships in polyploid complexes. In the study presented

here, each line of evidence contributed to clarifying different details of the evolutionary history of *Aleuritia*. In combination with preexisting knowledge from morphology, karyology, and distribution, our multifaceted approach allowed us to propose an integrative scenario of speciation via reticulation congruent with the secondary contact model in a polyploid, arctic–alpine group of plants. The next step will consist of investigating more nuclear regions to further distinguish between the different processes causing phylogenetic incongruence in our data set and verify the conclusions presented in this paper.

Supplementary Material

Supplementary material can be found at: http://www.sysbio.oxfordjournals.org/.

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