EVOLUTION AND POLYPLOID ORIGINS IN NORTH AMERICAN ARCTIC Puccinellia (Poaceae) BASED ON NUCLEAR RIBOSOMAL SPACER AND CHLOROPLAST DNA SEQUENCES

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The proportion of polyploid plant species increases at higher latitudes, and it has been suggested that original postglacial Arctic immigrants of some large groups, including grasses, were polyploid. We analyzed noncoding nuclear and chloroplast DNA of all North American diploid Puccinellia (Poaceae) and a subset of arctic polyploids to hypothesize evolutionary relationships among diploids and to evaluate the parentage of polyploids. Diploids formed three lineages: one unifying arctic species P. arctica and P. banksiensis; a second comprising arctic species P. tenella, P. alaskana, P. vahliana, and P. wrightii; and a third unifying the two temperate species P. lemmoneii and P. parishii. The arctic species P. angustata (hexaploid) and P. andersonii (primarily octoploid) apparently derive from the P. arctica-P. banksiensis lineage based on ITS and chloroplast sequences, and share an ancestor with arctic triploid/tetraploid P. phryganodes based on nrDNA sequences. Sequence comparisons also suggest tetraploid P. bruggemannii evolved from two arctic lineages: P. vahliana-P. wrightii and P. arctica-P. banksiensis. These patterns and the predominance of arctic rather than temperate diploid species support the idea that diploid Puccinellia recolonized the Arctic from northern glacial refugia like Beringia, and also formed stabilized polyploid hybrids during these refugial events or subsequently during postglacial colonization.

Key words: allopolyploidy; cpDNA; ETS; ITS; North American Arctic; phylogenetic network; Puccinellia; restriction sites; rpl16; rpoB-trnC

In many plant groups, the proportion of polyploid taxa increases northward from the tropics to the Arctic (Stebbins, 1950; Brochmann et al., 2004). Polyploid events may be more likely in colder regions like the Arctic because cold treatment of flowering plants can result in increased production of unreduced gametes (Raymond and Schemske, 1998), which are involved in most polyploidization events (deWet, 1980). Recent studies have shown that some polyploid species evolved from Arctic species [Saxifraga opalensis A. Blytt and S. svalbardensis D. O. Ovstedal, Steen et al., 2000; Dupontia fisheri R.Br., Brysting et al., 2004; hexaploid Draba lactea Adams, Grundt et al., 2004; Silene involucrata (Cham. & Schltldl.) Bocquet, S. sorensenis (B. Boivin) Bocquet, and S. ostenfeldii (A. E. Porsild) J. K. Morton, Popp et al., 2005, Popp and Oxelman, 2007; Saxifraga rivularis L., Jørgensen et al., 2006; Cerastium spp., Brysting et al., 2007]. Abbott and Brochmann (2003) stated that in some large groups (grasses, sedges, and willows) the original immigrants were already polyploid (Murray, 1995). This generalization is supported by the fact that some polyploid Arctic species are also found in alpine zones in temperate regions and that all species in some arctic genera are polyploid (e.g., Dupontia, Phippsia). However, the grass genus Puccinellia has both diploid and polyploid species in the Arctic (Consaul et al., 2008a, 2008b), and as such, provides an excellent system to explore the evolutionary relationships of arctic diploid species and the origins of their polyploid relatives.

Puccinellia is a genus of pioneering grasses that colonize coastal and harsh alkaline areas worldwide and is one of the largest genera of grasses in the Arctic. Over half of the 14 recognized North American arctic species are polyploid (Davis and Consaul, 2007). Our recent phenetic studies showed that there are five or six diploid species in the North American Arctic and two diploid taxa in temperate North America (Consaul et al., 2008a). We also demonstrated that three polyploid species, P. bruggemannii T. J. Sorensen, P. angustata (R.Br.) E. L. Rand & Redfield, and P. andersonii Swallen, likely had P. arctica (Hook.) Fernald & Weath. and P. vahliana (Liebm.) Scribn. & Merr. as parental ancestors (Consaul et al., 2008b), and that P. phryganodes (Trin.) Scribn. & Merr. is an additional possible parent of P. angustata and P. andersonii, while P. banksiensis Consaul is another possible parent for P. andersonii. Phylogenetic work on Puccinellia to date has been limited to chloroplast DNA (cpDNA) restriction site analysis, which has shown very little resolution for 14 temperate (Choo et al., 1994) and eight arctic species (Gillespie and Soreng 2005), plus five ITS and trnF-trnF sequences (Gillespie et al., 2008), which separated P. vahliana from the other taxa.
Additional molecular analyses are needed to understand the evolution of arctic species within this group. DNA sequences from these studies have been indispensable to show parentage in allopolyploid plant species worldwide and in the Arctic (Steen et al., 2000; Brysting et al., 2004, 2007; Grundt et al., 2004; Popp et al., 2005; Popp and Oxelman, 2007). Nuclear ribosomal DNA has been used in this way, with cloning (e.g., Popp and Oxelman, 2007), with restriction site analysis (Soltis and Soltis, 1991; Brochmann et al., 1992), or by examining mutations in direct sequences (Whittall et al., 2000; Aguilar and Felder, 2003). The tandem repeats of the nuclear ribosomal DNA region, including the internal transcribed spacer (ITS), can retain multiple copies from parental species (Buckler et al., 1997) so they can be used to detect possible parentage (maternal, paternal, or both), or they may be homogenized through concerted evolution or lineage sorting into uniform repeats. If mutations from parental species can be detected from differential cutting by restriction enzymes, then hybrid parentage can be inferred more readily by using restriction digest analysis of amplifications that contain multiple sequences rather than sequencing clamped fragments from these amplifications. This technique was recently shown to be useful to resolve a hybrid question in Carex by Smith and Waterway (2008). Chloroplast DNA (cpDNA) sequences are inherited maternally, so evidence of maternal evolution can be shown by using these genetic markers. If hybridization happens in both directions, chloroplast data from several individuals may reveal both parental genotypes of hybrid specimens (Gillespie and Soreng 2005).

Scientists studying whole genome duplication argue that additional natural auto- and allopolyploid species for which the parental constituency can be clearly characterized will help them to close the gap between evolutionary models (where origins are known) and genetic models (where processes are known) (Buggs, 2008). By investigating the parental origins of polyploid Puccinellia, one of the largest grass genera in the Arctic, we are addressing this, which will help increase our understanding of polyploid complexes in the Arctic, and in general (Popp and Oxelman, 2007; Buggs, 2008).

In this paper, we investigate whether original postglacial immigrants to the Arctic were polyploid by using a combination of direct sequencing of several nrDNA and cpDNA regions and restriction enzyme analysis. Our goals are to compare DNA sequences and restriction site data of arctic Puccinellia: (1) to test whether groups delimited by sequence data are congruent with groups obtained through studies of morphology, ploidy, and AFLP patterns (Consaul et al., 2008a, b); (2) to infer the phylogeny of the diploid species; and (3) to determine whether we can use consistent diagnostic mutations in the diploid taxa to discover the parentage and geographical origins of the polyploid taxa.

**MATERIALS AND METHODS**

**Specimen sampling**—Puccinellia samples were collected from across the North American Arctic as described in Consaul et al. (2008b) and are a subset of those analyzed in that paper (Appendix 1). We investigated the evolutionary relationships among the North American diploid species, including Greenland: P. alaskana Scribn. & Merr., P. arctica, P. banksiensis, P. lemnonii (Vasey) Scribn., P. parishii Hitchc., P. tenella (Lange) Holmb. subsp. langeana (Berlin) Tzvelev, P. vahlana, and P. wrightii (Scribn. & Merr.) Tzvelev, plus the eastern Russian P. tenella subsp. tenella. *Puccinellia agrostidea* T. J. Sørensen and *P. poacea* T. J. Sørensen have been placed in synonymy under *P. arctica* (Consaul et al., 2008a; Davis and Consaul, 2007). We

| DNA sequence data | DNA was extracted as described in Consaul et al. (2008a). We analyzed a set of 65 (35 diploid and 34 polyploid) specimens of *Puccinellia*, plus two outgroup specimens (*Phippisia algida*), for each of two nrDNA regions (ITS and ETS). We screened 10 *Puccinellia* specimens representing different diploid and polyploid species and the outgroup taxon *Phippisia algida* for variability in six noncoding cpDNA regions and analyzed the same specimens used for the nrDNA regions for the two most variable of these cpDNA regions (69 accessions for rpoB-trnC; 60 accessions for rpl16) (Appendix 2). The entire internal transcribed spacer region (ITS1/5.8S/ITS2) was amplified using forward primer N18L18 (Wen and Zimmerman, 1996) and reverse primer ITS4 (White et al., 1990). We included dimethyl sulfoxide (DMSO) in the reaction mix to avoid preferential amplification of paralogous gene copies or non-functional pseudogenes (Buckler et al., 1997; Alvarez and Wendel, 2003).

Primers for the 5′ end of the ETS1 region for the Poaceae have been developed for tribe Paniceae (Duvall et al., 2003) and tribe Triticeae (Sallares and Brown, 2004), but these primers did not amplify *Puccinellia* DNA ( tribe Poaceae). To design a primer, we amplified a test group of specimens coded IGS in Appendix 1 (*P. alaskana*, *P. arctica*, *P. banksiensis*, *P. tenella*, *P. vahlana*, and *Phippisia algida*) for the entire intergenic spacer region (IGS) of nuclear ribosomal DNA (nrDNA), using the primers in coding regions 26S and 18S (26S-F and 18S-R; Starr et al., 2003). The amplifications of the entire IGS region gave single bands at ~3000 bp for most of the specimens; the bands of *P. tenella* and *P. banksiensis* were ~5000 bp and 4000 bp, respectively. From aligned sequences of test samples for the reverse strand (sequenced by 18S-R), conserved regions were selected. The primer ETS1P (5′-TCCAGTGGAGGAACTT-3′) amplifies the area approximately 800 bp upstream from the start of the 18S-R primer.

Primers ETS1P and 18S-R were used with the same reaction mixture as for ITS and PCR conditions as defined in 3 min at 94°C, 25× (1 min at 94°C, 1 min at 54°C, 1 min at 72°C), and 7 min at 72°C to amplify this ETS region.

Amplification and sequencing of the chloroplast regions used primers from Taberlet et al. (1995) for trnL-F (including trnL intron) and trnL-F; for trnH-psB-A, we used the primer from Tate and Simpson (2003) for trnH and from Sang et al. (1997) for psbA; for trnD-F, we used the primer from Demesure et al. (1995) for trnD and the primer from Sun et al. (2002) for trnT. We used primers from Shaw et al. (2005), modified from Ohnishi and Ohsako (2000), for rpoB-trnC and primers from Small et al. (1998) for rpl16.

After purification, the sequences were run on an ABI3730XL with GenBank Quebec or an ABI310 in our laboratory. Sequences were edited and aligned with the programs Vector NTI 6.0 (Invitrogen, Carlsbad, California, USA) or Sequencher 4.5 (Gene Codes, Ann Arbor, Michigan, USA) and CLUSTAL_X 1.81 (Thompson et al., 1997), with visual examination and manual editing when necessary. Indels were coded as additional characters using the simple indel coding method of Simmons and Ochoterena (2000).

Two sequences were found per ITS amplification product in the hexaploid and octoploid samples. One sequence had peaks of normal height. The second sequence had smaller peaks and was lacking a four-nucleotide deletion near the beginning of the forward sequence, meaning that it was shifted by the four nucleotides for the rest of the sequence. It was straightforward to score the “secondary” peaks, owing to this consistent shift. All specimens of *P. angustata* and *P. andersonii* had the same pattern of double peaks, but only three were actually scored, done manually base by base. Assembling contigs using these “secondary” sequences and the reverse strand of a specimen of *P. arctica* was successful and unambiguous; convincingly, as the “secondary” sequence matched the *P. arctica* sequence. We used these contigs in the analyses as *angustata2* and *andersonii2*. Thus, although this does not take the place of confirming multiple sequences by cloning, we were confident that *angustata2* and *andersonii2* would be informative in our analyses. In other words, polymorphic bases for the
hexaploid and octoploid specimens were not coded as such in the data matrix, because the shift in nucleotides allowed us to construct two distinct sequences for analysis instead. There were some small secondary peaks in the ETS sequences as well, but scoring secondary sequences was too unreliable because there was not a large indel to provide an obvious shift; therefore, we scored the highest peaks, which were easy to discern. *Puccinellia phygyanodes* had six polymorphic sites, with subequal peaks. We could not infer primary and secondary peaks for these, so we used IUPAC/UBI ambiguity codes (Cornish-Bowden, 1985). We also tested the analyses with the polymorphic nucleotide characters excluded, to determine whether these characters were informative in other parts of the analysis, or uninformative (= noisy) and better removed (Hillis, 1991).

**Phylogenetic analyses**—We combined data from compatible DNA regions to construct trees based on maximum parsimony (MP) and Bayesian analysis (BA). To determine which regions could be combined, we measured the level of incongruence among the data sets using the incongruence length difference (ILD) test (Farris et al., 1994) implemented as the partition homogeneity test in the program PAUP* 4.0b10 (Swofford, 2002) with 100 replicates and also examined compatibility between trees by testing for conflict among well-supported clades (those at the 95% level in the majority-rule Bayesian trees) with the help of the program compat.py (Kauff and Lutzoni, 2003).

Phylogenetic analyses of sequence data were performed using PAUP* for MP analysis and the heuristic search option. 1000 random addition-sequence replicates, and tree-bisection-reconnection (TBR) branch swapping. All minimal trees were saved during the search, and branches were collapsed (creating polytomies) if maximum branch length was zero. Reliability of MP clades was assessed in PAUP* using nonparametric bootstrapping (BS) (Felsenstein, 1985), with 2000 bootstrap replicates and 1000 random-addition sequences per bootstrap replicate.

Models for BA were selected using the program MrModeltest 2.2 (Nylander, 2005). We analyzed the diploid data set with model HKY (Hasegawa et al., 1985) for ITS and ETS and model F81+I (Felsenstein, 1981) for *rpoB-trnC* and *rpl16*. For the diploid/polyploid data sets, we used the models GTR+I+F (general time reversible model with site-specific rate heterogeneity estimated by the gamma distribution; Yang, 1993) for ITS, HKY+F (Hasegawa et al., 1985) for ETS, GTR for *rpoB-trnC*, and F81+I for *rpl16*. When data sets were combined, we partitioned the data and used the appropriate model for each partition. We analyzed the data sets using Bayesian inference in MrBayes (version 3.1.2; Huelsenbeck and Ronquist, 2001). Two independent runs were performed in each analysis. We ran the Markov chain Monte Carlo simulation for 1 000 000 generations (depending on when the average standard deviation of split frequencies reached 0.01 or below, and the potential scale reduction factor of all parameters approached 1.0), sampling every 1000 generations. To ensure convergence of the trees to stationarity, we discarded the first 25% of the trees before constructing the majority-rule consensus tree from the remaining trees. We constructed phylogenetic networks for diploid + polyploid data sets of nrDNA and cpDNA sequence data using the program SplitsTree (version 4.3; Huson and Bryant, 2006) with models chosen by the hierarchical likelihood ratio tests in the program MrModeltest (Nylander, 2005); HKY for the nrDNA data and F81 for the cpDNA data set, and HKY for the combined nrDNA + cpDNA data because there is no multiple-model option in SplitsTree. For comparative analysis, we produced an nrDNA tree (Fig. 2A) and a cpDNA tree (Fig. 2B). However, the ILD test among all four regions (four partitions) was marginally insignificant at P = 0.07, and the compatibility test showed these trees were incompatible, so we did not combine nrDNA and cpDNA regions for the diploid + polyploid BA and MP analyses.

**RESULTS**

**Tree congruence**—For the diploid species, in the ILD tests between ITS and ETS, between *rpoB-trnC* and *rpl16*, and between nrDNA and cpDNA, none of the tests were significant (P ≥ 0.99 for all data sets), and all were compatible for Bayesian clades of 95% probability or above. Thus we produced a total DNA diploid tree (Fig. 1). For the diploid + polyploid species, the tests between the two nrDNA regions (P = 0.99) and between the two cpDNA regions (P = 0.40) again were insignificant and compatible for Bayesian clades of 95% probability or above, so we produced an nrDNA tree (Fig. 2A) and a cpDNA tree (Fig. 2B). However, the ILD test among all four regions (four partitions) was marginally insignificant at P = 0.07, and the compatibility test showed these trees were incompatible, so we did not combine nrDNA and cpDNA regions for the diploid + polyploid BA and MP analyses.

**Relationships among diploid taxa**—We coded each diploid taxon group with a single-letter code to refer to them more easily. The codes are: *P. alaskana* = A, *P. arctica* = R, *P. banksiensis* = U, *P. lemnonis* = M, *P. parishii* = S, *P. tenella* = T, *P. vahliana* and *P. wrightii* = V (Fig. 1). Although there is minor sequence variation among the individual accessions of *P. vahliana* and *P. wrightii*, they do not resolve as two separate clades; therefore we refer to them together as *P. vahliana* s.l.

**nrDNA**—The ETS region had more variable sites than the ITS region (81 vs. 56 variable sites, Table 1). Maximum parsimony (MP) analysis of the ITS region gave a single most parsimonious tree, and the ETS gave four most parsimonious trees, both having the same clades as shown in the total DNA BA tree (Fig. 1).

**cpDNA**—Four of the cpDNA regions (*psba-trnH*, *trnD-trnT*, *trnF-trnL*, and *trnL-trnF*) tested for variability ranged in size from 630 to 1110 bp but had little variability, with four or fewer variable sites. The six variable sites in *rpl16* and 10 variable sites in *rpoB-trnC* were distributed across the main clades of a MP strict consensus tree (not shown), and therefore we chose to use these two regions in the analysis of the larger data set of 69 diploid and polyploid *Puccinellia* accessions. In the cpDNA MP strict consensus and BA trees, the arctic species (genomes R, U, T, V, and A) formed a clade separate from the southern species (genomes M and S) with moderate to low support. In contrast to the results with nrDNA data, the individuals with the V genome (*P. vahliana, P. wrightii*) did not form a well-supported clade in either parsimony or Bayesian analysis when cpDNA was used alone.

**Comparing nrDNA and cpDNA sequences**—Each diploid species had 1–12 unique nrDNA mutations and 0–4 cpDNA mutations. The combined nrDNA + cpDNA BA (Fig. 1) tree had the same clades as the total DNA MP tree. Bootstrap values were high for all of the major clades. Combining the nrDNA and cpDNA data increased support for the southern M+S clade, and placed *P. arctica* 2808-14 within the R clade, whereas in the nrDNA tree this specimen formed a polytomy with the R+U clades. Although tests indicated that the nrDNA and cpDNA data sets were congruent and compatible in this analysis of diploids, branches with low support placed *P. lemnonis* and *P. parishii* sister to the T+R+V clade in the nrDNA tree (as in Fig. 1, the combined tree), but sister to all of the arctic species as a group in the cpDNA tree.
Fig. 1. Phylogenetic hypothesis for diploid species of *Puccinellia*. Tree based on both nrDNA and cpDNA regions from Bayesian analysis. Restriction sites defining the genomes of the diploids (Table 3) shown; restriction site *Sau96I* is found in all *Puccinellia* taxa except *P. alaskana*. The diploid genome codes are: R = *P. arctica*; U = *P. banksiensis*; T = *P. tenella*; V = *P. vahliana* and *P. wrightii*; A = *P. alaskana*; M = *P. lemmonii*; S = *P. parishii*. Scale bar units are substitutions/site.
Polyplod taxa—nrDNA—In the trees resulting from analysis of the 46 unique ITS and ETS Puccinellia sequences from the 69 diploid and polyploid accessions, P. bruggemannii and P. vahliana are in a strongly supported clade, as are the primary sequences of P. angustata and P. andersonii (Fig. 2A). Direct comparison of the sequences revealed that the primary sequences of octoploid P. andersonii are distinguished from P. angustata by one mutation in the ITS that it also shares with P. arctica.

The primary ITS sequences for P. angustata and P. andersonii were similar to each other but not closely related to any diploid species. The secondary ITS sequences for P. angustata2 and P. andersonii2 were similar to those of P. arctica and P. banksiensis, including a four-nucleotide deletion diagnostic of the R+U clade. Four of the five P. phryganodes specimens, from the northern, southwestern, and southeastern Arctic, were consistent in having six single nucleotide sites that had two equally strong bases in the ITS electropherogram, indicating more than one sequence in the amplification product. At each of the polymorphic sites, both bases were mostly shared with P. angustata/P. andersonii while the second base was often shared with P. tenella and most of the other species (Table 2). However, for base pair 79, G was only also present in P. tenella and P. parishii; for base pair 125, T was only also present in P. angustata and P. andersonii, and for base pair 528, T was only present in P. phryganodes itself. These four P. phryganodes specimens were in the same clade as P. andersonii and P. angustata, while the fifth specimen (lacking polymorphisms) groups with P. tenella in the BA and MP trees (Fig. 2A). When the six polymorphic nucleotide characters were excluded, three of the P. phryganodes accessions were in a polytomy at the base of the Puccinellia clade, while the remaining two were basal to the P. angustata clade (tree not shown), suggesting that phylogenetically informative mutations were present at these alignment positions.

cpDNA—Puccinellia bruggemannii, P. angustata, and P. andersonii are all in the same clade with the R and U diploid species (Fig. 2B). This clade also includes the other two polyploid species we sampled for comparison, P. pumila and P. sp 2. Individuals of the diploids P. tenella, P. alaskana, and P. lemmonii each form species clades, but these clades are part of a large basal polytomy with individuals of the other sampled diploids (P. vahliana, P. parishii) and triploid/tetraploid P. phryganodes. Constructing the MP cpDNA tree using all six chloroplast regions for one representative of each species did not increase the resolution of the main clades (not shown).

Phylogenetic networks—In the phylogenetic networks (Figs. 3–5), the main groups are separated as in the described trees. The network based on nuclear data alone (not shown) is similar to that based on nrDNA and cpDNA (Fig. 4), except that four specimens of P. phryganodes are grouped more directly at the base of P. andersonii and P. angustata. In contrast, the chloroplast network (Fig. 3) has a two-group structure, with the diploids P. alaskana, P. lemmonii, P. parishii, P. tenella, and P. vahliana, as well as P. phryganodes at one end while the diploids P. arctica s.l. and P. banksiensis and the other polyploids are at the opposite end, similar to the groups in the cpDNA tree (Fig. 2B). Also in the chloroplast network (Fig. 3), P. bruggemannii and P. angustata are distinct. The network based on all genes (Fig. 4) places P. andersonii and P. angustata close together, between P. phryganodes and P. arctica/P. banksiensis, and places P. bruggemannii and P. vahliana s.l. in close proximity. When the six polymorphic nucleotide positions in P. phryganodes were removed from the data matrix, the network was similar in structure to the network based on all genes (Fig. 4), but the P. phryganodes specimens were in a more tightly knit group. The network based on AFLP data (Fig. 5) differs by placing all taxa in separate groups, not grouping P. bruggemannii with P. vahliana and not grouping P. phryganodes with P. angustata and P. andersonii. The AFLP network is most similar to the chloroplast network, with a shorter connecting line between the two main groups, of interest because we would expect the AFLP network to be closest to the nuclear network, given that AFLPs are likely mostly nuclear.

Restriction enzyme analyses—Patterns of cutting by restriction enzymes in the ITS region help to distinguish among the diploid genomes. BanII cuts only P. arctica and P. banksiensis to distinguish the R and U genomes from the others; Alul cuts only P. banksiensis to distinguish it (U-genome) from all other species; BbvI cuts only P. tenella to distinguish it (T-genome) from all other species (Table 3). Sau96I cuts the ITS region twice, cutting all diploid species except P. alaskana at the site referred to as Sau96I-1 in Table 3. It cuts P. alaskana ITS at a different site, Sau96I-2, to distinguish the A-genome; it cuts the ITS region of P. vahliana s.l. at both sites, the double cut indicating the V-genome. DpnII does not cut the ITS region in any of the diploid species, but does so in P. andersonii, P. angustata, and P. phryganodes. The genome distinguished by this DpnII cut has not yet been found in a diploid species; we refer to this genome as the Z-genome. In the chloroplast genome (rpoB-trnC region), Sau96I cuts all but P. vahliana s.l., distinguishing it from the other diploid species (Table 3).

Among the polyploids, P. bruggemannii has only the V-genome pattern for ITS. Puccinellia angustata and P. andersonii have both the R-genome and the Z-genome in each individual, with most of the fragments in the ITS amplification product from each specimen cutting for the Z-genome. Puccinellia phryganodes has the Z-genome and no diploid genome. Puccinellia pumila and most of the specimens (75%) in the population P. sp 2 have only the R-genome. The remainder (25%) of the specimens in population P. sp 2 cut with the same pattern as P. angustata and P. andersonii (R-genome and Z-genome). For the chloroplast patterns, the rpoB-trnC amplification products of all of the polyploid species are totally cut by Sau96I; none of the products had uncut fragments that would indicate the V-genome.

DISCUSSION

Delimited groups—The DNA sequence-based analyses presented here support the Puccinellia species defined on the basis of patterns of morphology, ploidy, and AFLP data by Consaul et al. (2008a, b). The diploid genomes R, U, T, A, V, M, and S correspond to five arctic (P. arctica [including synonyms P. agrostidea and P. poacea], P. banksiensis, P. tenella, P. alaskana, and P. vahliana s.l.) and two temperate (P. lemmonii, P. parishii) Puccinellia species, each distinguished by nrDNA and cpDNA mutations (Fig. 1). As in Consaul et al. (2008a), which focused on AFLP data, P. wrightii is indistinguishable from P. vahliana based on sequence data, but more specimens need to be analyzed before we propose formal synonymy of these two species. Puccinellia arctica, P. agrostidea, and P. poacea together formed a monophyletic group, so the placement in synonymy of the latter two in P. arctica (Consaul et al., 2008a; Davis and
Consaul, 2007) is supported. The three higher polyploid species that we studied in detail, *P. bruggemannii*, *P. angustata*, and *P. andersonii*, had variable degrees of branch support in the trees, but were more clearly distinguished in phylogenetic networks.

**Diploid phylogenetic relationships**—Sequencing and restriction site data revealed three evolutionary lineages (1: *P. arctica* and *P. banksiensis*; 2: *P. alaskana*, *P. tenella*, *P. vahliana*, and *P. wrightii*; and 3: *P. lemmonii* and *P. parishii*) that are also supported by AFLP data (Consaul et al., 2008a). Morphologically, however, only the second lineage has a diagnostic character (glabrous branches) (Consaul and Gillespie, 2001).

**Diploid mutations to track polyploid evolution**—We investigated the origins of the putative polyploid series *Puccinellia phryganodes*, *P. bruggemannii*, *P. angustata*, and *P. andersonii*. Our previous work has shown that *P. phryganodes* is triploid and tetraploid in North America, *P. bruggemannii* is tetraploid, *P. angustata* is hexaploid, and *P. andersonii* is mostly octoploid with some hexaploid plants and that hybrids...
between this species and *P. angustata* occur (Consaul et al., 2008b). Direct sequencing and restriction digest analyses gave several lines of evidence that these species arose from arctic lineages.

The evolution of *P. phryganodes* is complex and not well understood. One specimen of *P. phryganodes* in this study lacked polymorphic sites, had an ITS sequence most similar to the arctic species *P. tenella* (Table 2, Fig. 2). Given the resolution of *P. phryganodes* in the *P. tenella* clade in the cpDNA tree and cpDNA network, evidence suggests that an ancestor of *P. tenella* is the maternal parent of *P. phosphanodes* and that in this specimen only a single ITS sequence amplified. The sequences of four of the *P. phryganodes* specimens had polymorphic bases (Table 2), suggesting incomplete homogenization of the ITS copies. Many of these polymorphic nucleotides were shared with diploid arctic *Puccinellia* species, potentially indicating their involvement in the origin of this polyploid. A similar interpretation was made from superimposed nucleotide additivity patterns in direct sequencing of polyploid species of *Sidalcea* by Whittall et al. (2000). *Puccinellia phryganodes* appears to share a parent with *P. angustata* and *P. andersonii*, with which it shares the Z-genome, characterized by several different bases not shared with any other species (at sites 70, 125, 128, and 424, Table 2). Because *P. phryganodes* is of lower ploidy than *P. angustata* and *P. andersonii* in North America (Consaul et al., 2008b), it may be involved in the evolution of the hexaploid and octoploid species, but it does have two bases (G at site 79 and T at site 528, Table 2) that are not found in these higher polyploids. Additional nuclear evidence and cloning will be necessary to place *P. phryganodes* more accurately.

Evidence suggests that *P. bruggemannii* is allopolyploid, with both parents from arctic lineages. The female parent appears to share a common ancestor with *P. arctica* and *P. banksiensis*, because *P. bruggemannii* has sequences that are sister to, but not identical to, the R- and U-genomes in cpDNA (Fig. 2B). In the phylogenetic network based on cpDNA data, *P. bruggemannii* specimens group with *P. angustata* on the same main “arm” as both *P. arctica* and *P. banksiensis* (Fig. 3). Evidence also suggests that the male parent was *P. vahliana*. *Puccinellia bruggemannii* and *P. vahliana* form a clade in the nrDNA tree (Fig. 2A), with no secondary sequences visible in the electropherograms nor any multiple cutting patterns in the restriction digest gels. This nrDNA pattern of *P. vahliana*, which appears to be maintained relatively unchanged in *P. bruggemannii*, suggests that the ITS DNA has homogenized by concerted evolution to such an extent that sequences of only the male parent are now present.

Our data did not provide any evidence that *P. bruggemannii* formed more than once. The nr+cpDNA phylogenetic network shows specimens of *P. bruggemannii* at the base of the circled “*P. vahliana*/P. wrightii” branch (Fig. 4), implying that speciation of *P. bruggemannii* could have occurred before subsequent mutations in *P. bruggemannii* and *P. vahliana*. In addition, branching within *P. bruggemannii* in the cpDNA tree and network occurs after the branching of *P. bruggemannii* itself (Figs. 2B, 3). Moreover, neither *P. arctica* nor *P. banksiensis* is

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**Table 1.** Summary of DNA sequence characteristics for ITS, ETS, *rpoB-trnC*, *rpl16*, nrDNA combined, and cpDNA combined data sets, for the ingroup alone, and including the outgroup. If the latter values differed, they are given in square brackets following the values for the ingroup. *N* = 71 accessions (*69 Puccinellia + 2 Phippsia*) for all except *rpl16*, for which *N* = 62 (*60 Puccinellia + 2 Phippsia*).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ITS+ 585–1752</th>
<th>ETS</th>
<th><em>rpoB-trnC</em></th>
<th><em>rpl16</em></th>
<th>nrDNA</th>
<th>cpDNA</th>
</tr>
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<tr>
<td>Length range (bp)</td>
<td>592–596</td>
<td>778–780</td>
<td>153–1197</td>
<td>794–836</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G+C content range (%)</td>
<td>60.1–60.2</td>
<td>56.7–57.1</td>
<td>30.4–30.7</td>
<td>31.8–32.6</td>
<td>58.2–58.4</td>
<td>30.9–31.5</td>
</tr>
<tr>
<td>Number of indels</td>
<td>1</td>
<td>2</td>
<td>6[10]</td>
<td>6</td>
<td>3</td>
<td>12[16]</td>
</tr>
<tr>
<td>Number of variable sites</td>
<td>56</td>
<td>81[84]</td>
<td>26[34]</td>
<td>12[21]</td>
<td>137[140]</td>
<td>38[55]</td>
</tr>
</tbody>
</table>

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**Table 2.** Positions in ITS sequences of *Puccinellia phryganodes* where more than one base is represented in a single amplification product, seen as subequal multiple peaks on the *P. phryganodes* ITS electropherogram. Ambiguity codes in parentheses. Nucleotides found in other species show that some bases found in *P. phryganodes* are not represented in the other taxa, while some nucleotides are found only in *P. phryganodes*, *P. angustata*, and *P. andersonii*. Ploidy level and diploid genome code follow the species names. Ang1 and and1 = primary sequences in *P. angustata* and *P. andersonii* (large peaks), coded as the Z-genome. Ang2 and and2 = secondary sequences in *P. angustata* and *P. andersonii* (small peaks), aligned with the R-genome. Additional tiny peaks in electropherograms are given in square brackets. The mutation for nucleotide “A” at base 128 was detected by the *DpnII* restriction cuts.

<table>
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<tr>
<th>Taxon</th>
<th>70</th>
<th>79</th>
<th>88</th>
<th>125</th>
<th>128</th>
<th>424</th>
<th>528</th>
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<tbody>
<tr>
<td><em>P. phryganodes</em> (3x/4x)</td>
<td>A,G (R)</td>
<td>G,T (K)</td>
<td>G.T (K)</td>
<td>A,T (W)</td>
<td>A.C (M)</td>
<td>T.G (K)</td>
<td>C.T (Y)</td>
</tr>
<tr>
<td><em>P. phryganodes</em> (3x, specimen lacking polymorphic sites)</td>
<td>G</td>
<td>T</td>
<td>T</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>C</td>
</tr>
<tr>
<td><em>P. angustata</em> (6x) (ang1, ang2)</td>
<td>A.G</td>
<td>T.T</td>
<td>G.T</td>
<td>T[+A],G</td>
<td>A.C</td>
<td>T.G</td>
<td>C.C</td>
</tr>
<tr>
<td><em>P. andersonii</em> (6x, 8x) (and1, and2)</td>
<td>A[+G],G</td>
<td>T.T</td>
<td>G.[+C]</td>
<td>T[+A],G</td>
<td>A.C</td>
<td>T.G</td>
<td>C.C</td>
</tr>
<tr>
<td><em>P. bruggemannii</em> (4x)</td>
<td>G</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>C</td>
</tr>
<tr>
<td><em>P. alaskana</em> (2x, A)</td>
<td>G</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>C</td>
</tr>
<tr>
<td><em>P. arctica</em> (2x, R)</td>
<td>G</td>
<td>T</td>
<td>T</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
</tr>
<tr>
<td><em>P. banksiensis</em> (2x, U)</td>
<td>G</td>
<td>T</td>
<td>T</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
</tr>
<tr>
<td><em>P. lemmonii</em> (2x, M)</td>
<td>G</td>
<td>T</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
</tr>
<tr>
<td><em>P. parishii</em> (2x, S)</td>
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<td>T</td>
<td>G</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
</tr>
<tr>
<td><em>P. tenella</em> (2x, T)</td>
<td>G</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>C</td>
</tr>
<tr>
<td><em>P. vahlia</em> (2x, V)</td>
<td>G</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>C</td>
</tr>
</tbody>
</table>
currently found in the main geographical region of *P. bruggemannii* in the central Canadian Arctic (Consaul et al., 2008a, b), suggesting that it may have formed in a glacial refugium with subsequent postglacial colonization of other areas in the Canadian Arctic.

In *P. angustata*, one parent appears to be an ancestor of the arctic species *P. arctica* and *P. banksiensis* because the “secondary” ITS sequence is similar to that of these species, including the presence of a four nucleotide deletion found in the R+U clade (Fig. 2A). Furthermore, in the cpDNA BA tree, *P. angustata* is in a well-supported clade that includes these two diploids, but none of the other diploids (Fig. 2B). We expected to see evidence in the sequence data that *P. vahliana* was also a parent because, morphologically, *P. angustata* resembles *P. vahliana* in several aspects, such as thick, strongly curled roots and relatively long glumes (Davis and Consaul, 2007). There are also two AFLP bands in *P. angustata* that are diagnostic for *P. vahliana* among the diploids (Consaul et al., 2008b). However, the sequence data suggest that the second parent is shared with *P. phryganodes*, given that the Z-genome is shared by *P. angustata* and *P. phryganodes* (Table 2, Fig. 2). The diploid (R/U genome) plus the tetraploid *P. phryganodes* genome would almost account for the *P. angustata* genome. However, there are at least two bases in the *P. phryganodes* ITS sequence not found in *P. angustata*, which may be explained if these bases have been lost through homogenization of the ITS in *P. angustata*, that is, one of the *P. phryganodes* parental copies is lost. Alternatively, there may be an as yet undiscovered or extinct parent for both of these species. Although *P. angustata* and *P. bruggemannii* have often been confused in the field, these direct ITS sequences suggest that *P. angustata* did not form simply by *P. bruggemannii* hybridizing with a diploid, given that *P. bruggemannii* has nrDNA sequences similar to those of *P. vahliana* (Fig. 2A).

_Puccinellia andersonii_ is very closely related to *P. angustata*. It has the same nrDNA and cpDNA pattern in general as *P. angustata* (Table 3), and the octoploid specimens can be distinguished by one ETS mutation that they also share with *P. arctica*. Some of the hexaploid specimens (coded D6) may lack this mutation, reinforcing their possible hybrid status suggested in a previous paper (Consaul et al., 2008b) and requiring further study. _Puccinellia andersonii_ has the same primary ITS sequence as *P. angustata*, and a secondary sequence, that, although not identical, also has the four-nucleotide deletion diagnostic of the R+U clade. One hypothesis for the origin of _P. andersonii_ is that it may have evolved from a hybridization of *P. angustata* with *P. phryganodes* or *P. banksiensis*. There are extra AFLP bands of _P. andersonii_ that are also characteristic of *P. phryganodes* (Consaul et al., 2008b), but, as in *P. angustata*, there are bases in *P. phryganodes* not present in _P. andersonii_. The contribution of *P. banksiensis* is suggested because _P. andersonii_ and *P. banksiensis* were situated on the same arm in the cpDNA network (Fig. 3). Moreover one of the diagnostic _P. banksiensis_ AFLP bands is found in all populations of _P. andersonii_ (Consaul et al., 2008b). An ancestor of both *P. arctica* and *P. banksiensis* may have been an original parent of both polyploid species, with *P. phryganodes* or *P. banksiensis* contributing the fourth genome to the octoploid.

_Puccinellia_ sp. 2 was a single population examined as a putative species because it was statistically different from the other polyploid species for some morphological characters (Consaul et al., 2008b). Our results here show that this species has an nrDNA pattern that is different from those of *P. angustata* and _P. andersonii_, but similar to those of _P. pumila_. _Puccinellia_
parental lineages found in *P. bruggemannii* clearly support this hypothesis, as do the arctic parental lineages found in *P. andersonii*, *P. angustata*, and *P. phryganodes*, but these polyploids also had sequences not found in the other species sampled, so more global sampling is required to fully understand their origins.

**Discussion of techniques and future investigations**—Different sources of DNA data were helpful in this study because the resolution attained by sequencing the different regions of DNA was variable. Sequences from chloroplast regions gave low resolution within *Puccinellia*. The number of mutations was also low in the ITS sequences, but these mutations distinguished the diploid species reliably, thus being appropriate for using restriction site mutations to test for multiple copies of ITS sequences originating from different parents in a single direct amplification. We did not clone ITS sequences, but examined the polymorphic sites individually, to compare multiple base pairs with the diploid species and determined that information can be gained to answer some of our questions in the absence of cloning. Most importantly, with an ITS amplification and *Sau* 96I restriction enzyme, one can easily distinguish *P. bruggemannii* from the other two polyploids in this study. We found increased resolution in the ETS region over ITS, consistent with *Starr et al. (2003)* in *Uncinia* (Cyperaceae) and *Beardsley and Olmstead (2002)* in the Phrymaceae, although *Kelch and Baldwin (2003)* found the level of variation was similar between ITS and ETS in *Cirsium* (Asteraceae). Restriction enzyme analysis to detect the nucleotide mutations diagnostic for the diploid taxa allowed us to screen larger numbers of samples more easily than would be
possible with cloning and showed us when multiple sequences were present.

Several intriguing questions remain that could be investigated using the methods in this paper, coupled with additional molecular markers and techniques that have proven useful in other grasses (e.g., granule-bound starch synthase I [GBSSI] [Mason-Gamer et al., 1998; Ingram and Doyle, 2003]; microsatellites [Provan et al., 2004], and in situ hybridization [Brysting et al., 2000]). What are the diploid origins of *P. phryganodes*? Are there other diploid parents of the hexaploid *P. angustata* and octoploid *P. andersonii*? Did arctic species arise from temperate species or vice versa? Additional worldwide sampling will also be required to answer these questions.

**Table 3.** Frequencies of restriction site presence/absence in 12 arctic *Puccinellia* species used to define genomes of diploids and infer multiple genomes of polyploids. Restriction sites given for nrDNA (ITS, to detect several diploid genomes in polyploids) and cpDNA (*rpoB-trnC*, to detect the V genome when uncut). Letter codes refer to the diploid genomes defined in Fig. 1; Z = genome particular to some polyploid species only. Letter codes in round brackets at the top of each enzyme column indicate the genomes that are cut by the associated restriction enzyme. Genome codes in square brackets are found in very low frequencies. *N* = sample size.

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<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>A</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>R</td>
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<td>R</td>
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<tr>
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<td>1</td>
<td>0</td>
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<td>0</td>
<td>U</td>
<td>1</td>
<td>U</td>
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<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>V</td>
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<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>V</td>
<td>0</td>
<td>V</td>
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<td>T</td>
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<td>0</td>
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<td>V</td>
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<td>not V</td>
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<td>0</td>
<td>1</td>
<td>R, Z</td>
<td>1</td>
<td>not V</td>
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<tr>
<td><em>P. andersonii</em> (25)</td>
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<td>0.25</td>
<td>R [Z]</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>R</td>
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<td>not V</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>R</td>
<td>1</td>
<td>not V</td>
</tr>
</tbody>
</table>
LITERATURE CITED


Diploid species

P. alaskana Scribn. & Merr. USA. Alaska, Bogoslof Island, Aleutian Islands. R. Meehan #Bog (CAN), R = 3, IGS. #Bog-01 GB = GQ283111 / GQ283182 / GQ283249 / GQ283312 / GU082421 / GU082453 / GU082442 / GU082431; #Bog-02 GB = GQ283112 / GQ283183 / GQ283250 / GQ283313; B.F. Friedman 80-147 (ALA), R = 1; Kiska Quad, Rat Islands, Buddia Island, M. Dick 84 (ALA).


Appendix I. Collections studied in this paper, with voucher information for each collection. The last four GenBank sequences in the accession lists are for only 11 samples; “na” indicates no sequence available for that accession. Number of individuals analyzed by restriction site digests follows “R = “. “IGS” indicates populations sampled for IGS sequence for ETS primer design. Samples are at MTMG unless stated otherwise.

Taxon. Locality, Voucher (herbarmium), R, GenBank accessions (GB) = ITS/ETS/trpB-trnC/trnD-I/trnL-F/trnD-I/trnH-psbA]
Polyploid species

P. angustata (R. Br.) E. L. Rand & Redfield. NWT, Banks I, top of ridge, north of Mansik R. LLG, and H. Bickerton 2807, 2807-3 R = GB = GQ283134 & GQ283135 & GQ283205 & GQ283270 & GQ283331; \\
NWT, Banks I, Worth Point, LLG, and H. Bickerton 2850, 2809-3 R = GB = GQ283134 & GQ283135 & GQ283205 & GQ283270 & GQ283331;