DIVERSITY, PHYLOGENY, AND EVOLUTION IN THE MONOCOTYLEDONS
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Phylogeny and Reticulation in Subtribe Poinae and Related Subtribes (Poaceae) Based on nrITS, ETS, and *trnTLF* data

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Abstract—The worldwide temperate to arctic distributed subtribe Poinae comprises the largest grass genus *Poa* (500+ species) and 14 smaller genera. Phylogenetic analyses of combined nuclear ribosomal ITS and ETS and chloroplast *trnT-trnL-trnF* sequence data for Poinae and the closely related subtribes Alopecurinae, Cinninae, Miliinae, and Puccinelliinae resolve three strongly supported major clades: 1) *Poa*; 2) all Poinae taxa except *Poa*, and including *Alopecurus*, *Beckmannia* (both Alopecurinae), and *Cinna*; and 3) subtribe Puccinelliinae. *Phleum* (Alopecurinae) and *Milium* resolve as two separate lineages, but with low support. Plastid, ITS, and ETS data partitions were not significantly incongruent, with some exceptions in *Poa*, and combining these datasets resulted in stronger support for many clades. Our results indicate that subtribe Poinae, as currently circumscribed, is paraphyletic, and suggest that subtribe Alopecurinae may be polyphyletic since none of its three sampled genera were resolved as sister taxa. Analyses with all three Alopecurinae genera constrained as monophyletic resulted in trees considerably longer than those from unconstrained analyses. Placements of *Arctopoa* and *Nicoraepoa pugionifolia* were incongruent between plastid and nrDNA trees suggesting hybrid origins involving members of the *Poa* and Poinae (minus *Poa*) clades. The presence of two distinct ITS sequences in *Aniselytron* also suggest evolution involving hybridization between these same two major clades.

Keywords—Alopecurinae, *Aniselytron, Arctopoa*, hybridization, phylogeny, reticulate evolution

The grass subtribe Poinae (Poaceae, Pooideae, Poeae) currently includes 15 genera and an estimated 550 species distributed worldwide, mostly in cool temperate regions (Soreng et al. 2007; Gillespie et al. 2008). *Poa* is by far the largest genus with about 500 species. The number and circumscription of Poinae genera have changed considerably since the subtribe was established by Dumortier (1829), particularly over the past two decades as a result of new molecular studies combined with morphological data (Soreng et al. 1990; Soreng and Davis 2000; reviewed in Gillespie et al. 2008). For the current circumscription of subtribe Poinae and the arrangement of genera and subtribes in tribe Poeae see Soreng et al. (2003, classification version 27 Oct 2008). In the most recent modification Gillespie et al. (2008) tentatively placed the three genera of subtribe Cinninae (*Cinna, Cyathopus, Limnodea*) within Poinae, which, if accepted, would bring the total number of Poinae genera to 18.

Plastid and nuclear ribosomal DNA (nrDNA) ITS data have inferred nine Poinae genera to be nested within the *Poa* clade (Hunter et al. 2004; Gillespie and Soreng 2005; Gillespie et al. 2007, 2008; Refulio-Rodriguez 2007). Consequently, *Anthochloa* Nees & Meyen, *Austrofestuca* (Tzvelev) E. B. Alexeev, *Dasypoa* Pilg., *Eremopoa* Roshev., *Neuropoa* W. D. Clayton, and *Parodiochloa* C. E. Hubb. have been subsumed within *Poa* (Gillespie and Soreng 2005; Gillespie et al. 2007, 2008), and combinations within *Poa* are pending for *Aphanelytrum*, *Dissanthelium*, and *Tovarochloa* (Refulio-Rodriguez 2007). *Poa* subg. *Andinae* Nicora was determined not to be part of the *Poa* clade, and the new Poinae genus *Nicoraepoa* was described for this group of Patagonian species (Gillespie et al. 2007, 2008; Soreng and Gillespie 2007). With regard to other Poinae genera, *Festucella* E. B. Alexeev was recently synonymized under *Hookerochloa*, both endemic to Australia (Gillespie et al. 2008; Jacobs et al. 2008). *Ventenata* and *Gaudiniopsis*, sometimes separated as subtribe Ventenatinae (nom. inval., and traditionally allied to *Avena* L. and *Trisetum* Pers. [Aveninae]), have been added into the Poinae mix of genera based on recent DNA studies (Döring et al. 2007; Quintanar et al. 2007; Gillespie et al. 2008).

Phylogenetic relationships among Poinae and related subtribes were investigated most recently using nrDNA ITS and plastid *trnT-trnL-trnF* (TLF) sequence data (Gillespie et al. 2008). Separate ITS and TLF analyses provided evidence for *Poa* as a lineage distinct from all other Poinae genera. Both analyses resolved a clade of subtribes Alopecurinae, Miliinae, Poinae, and Puccinelliinae (PPAM clade, Gillespie et al. 2008), but with very low bootstrap support. Five lineages were resolved in the PPAM clade: Puccinelliinae, *Poa*, Poinae (minus *Poa*) (this clade also includes two Alopecurinae genera), *Phleum*, and *Milium* (with evidence in ITS of a possible broader Miliinae lineage). The plastid trees provided strong support for a sister-group relationship between Puccinelliinae and an Alopecurinae-Miliinae-Poinae clade (PAM, Gillespie et al. 2007), but apart from this, relationships among PPAM lineages were poorly supported in plastid and nrDNA analyses. Resolution and support within PPAM lineages varied consider-

ably between ITS and TLF trees. For example, relationships within the Poinae (minus *Poa*) and Puccinelliinae clades were more resolved in ITS than TLF, but the reverse was true for the *Poa* clade.

Multiple plastid and ITS analyses suggest that subtribe Alopecurinae (currently defined as including: *Alopecurus, Beckmannia, Cornucopiae, Limnas, Phleum, Pseudophleum,* and *Rhizocephalus*) may be polyphyletic (Soreng and Davis 2000; Davis and Soreng 2007; Döring et al. 2007; Gillespie et al. 2007; Quintanar et al. 2007; Soreng et al. 2007; Gillespie et al. 2008). Three of seven genera have been examined so far: *Alopecurus* (ca. 37 spp., widespread), *Beckmannia* (2 spp., boreal), and *Phleum* (ca. 13 spp., widespread). The other four genera have one or two species each, and are of more limited distribution (Mediterranean, Turkey, NE Asia [*Limnas*]). In the most recent analysis *Alopecurus* and *Beckmannia* are part of the Poinae (minus *Poa*) clade, but do not resolve as sister taxa, and *Phleum* resolves as a separate lineage (Gillespie et al. 2008). These molecular results appear to contradict the close morphological similarity of Alopecurinae members. These seven genera share a unique combination of morphological characters in the PPAM clade, including dense spicate panicles and one-flowered spikelets that disarticulate below the glumes (Soreng and Davis 2000; Soreng et al. 2007; Gillespie et al. 2008).

Conflicts between plastid and ITS trees suggest that three Poinae taxa may have originated from hybridization between *Poa* and other Poinae (including Cinninae) genera (Gillespie et al. 2008). All collections (eight) and species (three) examined of *Arctopoa* have ITS DNA placing it in the Poinae (minus *Poa*) clade, while plastid data places the genus in the *P*oa subg. *Sylvestres* clade, with which it bears little morphological resemblance (Gillespie et al. 2008). Though generally treated as a subgenus within *Poa* (subg. *Arctopoa* (Griseb.) Prob.), *Arctopoa* was resurrected based on these molecular data and morphology (Gillespie et al. 2008). Similarly, but with reversed placements, the south-east Asian genus *Aniselytron* was positioned in the *P*. subg. *Sylvestres* (V.L. Marsh ex Soreng) Soreng & L.J. Gillespie clade in ITS analyses, but in the Poinae (minus *Poa*) clade in plastid analyses. However, this finding needs to be verified since it was based on different samples for ITS and TLF. *Nicoraepoa pugionifolia* also resolved with conflicting placements. Plastid and morphological data placed the species in the Poinae (minus *Poa*) clade consistent with all other species examined of the newly described Poinae genus *Nicoraepoa* (Soreng and Gillespie 2007; Gillespie et al. 2008), whereas ITS analyses placed it within *Poa* in the primarily sub-Antarctic island section *Parodiochloa* (C. E. Hubb.) Soreng & L. J. Gillespie clade.

Since the number of parsimony informative characters was limited and character consistency was lower in ITS (compared to TLF, which had 10% more characters and a substantially higher overall consistency index, 0.633 versus 0.322, Gillespie et al. 2008), we decided to increase the size of the nuclear ribosomal DNA (nrDNA) dataset by sequencing part of the nrDNA external transcribed spacer (ETS) (Baldwin and Markos 1998). ETS has been successfully combined

with ITS to enhance resolution and support of nrDNA phylogenies (e.g., Baldwin and Markos 1998; Markos and Baldwin 2001; Li 2002; Urbatsch et al. 2003; Acevedo-Rosaset al. 2004; Razafimandimbisona et al. 2005).

In this paper we use TLF, ITS, and ETS sequence data to infer phylogenetic relationships in the PPAM clade with a focus on genera of subtribes Poinae and Alopecurinae. Our goals are to: 1) examine congruence among the three datasets; 2) generate more robust phylogenetic hypotheses based on combined nrDNA ITS and ETS data, and on combined plastid TLF and nrDNA ITS/ETS data; 3) explore relationships among Alopecurinae genera and investigate the monophyly of the subtribe; and 4) further investigate the origin and evolution of three putative intergeneric hybrid taxa, *Aniselytron*, *Arctopoa*, and *Nicoraepoa pugionifolia*.

Materials and Methods

Taxon Sampling—Seventy-two accessions representing 38 Poinae species, 14 other PPAM species, and three outgroup species were included in this study (Appendix 1). New ETS sequence data were collected for 64 accessions including 37 Poinae species and 10 other PPAM species. TLF and ITS sequences were previously published for many of these accessions (Gillespie et al. 2007, 2008); new TLF and ITS sequences were generated for 12 and 16 collections, respectively. Complete TLF sequences were obtained for *Aniselytron* where we previously had only partial (*trnL* intron and *trnLF*) sequences. For each operational taxonomic unit (OTU) in the combined data matrix TLF, ITS, and ETS sequences were from the same collection, except for *Cinna latifolia* and *Milium effusum* (Appendix 1).

In this paper we included only those species/accessions for which we had 1) both ITS and ETS sequences (50 species, 64 accessions), and 2) sequences of all three DNA regions TLF, ITS, and ETS (49 species, 61 accessions) (Appendix 1). Among PPAM clade members this included eight Poinae and three Alopecurinae genera, plus *Cinna* (Cinninae), *Milium* (Miliinae), and *Catabrosa* and *Puccinellia* (both Puccinelliinae). We included a reduced set of *Poa* species similar to Analysis I of Soreng et al. (2010): diploids (2n = 14) plus *P.* subg. *Sylvestres* (species sampled are tetraploid), with one accession of each species. Two additional species of *Poa*, *P. flabellata* (tetraploid; also included in Analysis II of Soreng et al. 2010) and *P. kerguelensis* (chromosome number unknown), were added in an attempt to narrow down the affinities of one of the putative hybrid taxa. Three outgroup genera were included as representatives of the other two major Poeae s.l. clades: *Deschampsia* P. Beauv. (subtribe Airinae), *Festuca* L.(Loliinae), and *Helictotrichon* Besser ex Schult. & Schult. f. (Aveninae) (Quintanar et al. 2007, Gillespie et al. 2008). This selection was in part based on outgroup taxa for which we were able to generate ETS sequence data.

DNA Sequences—Methods of DNA extraction, amplification, and direct sequencing of TLF and ITS were outlined in Gillespie et al. (2008). A region of approximately 500 base pairs (bp) at the 3' end of the nrDNA external tran-

scribed spacer (ETS) was amplified and sequenced using the same reaction conditions as ITS and primers 18S-R (Starr et al. 2003) and RETS4-F, a primer newly designed for this study (5'-TGGCTACGCGAGCGCATGAG-3'). Although designed specifically for use in *Poa*, the RETS4-F primer was also found to successfully amplify other Poinae genera and more widely in Poeae.

Sequence assembly, alignment, and editing were performed using Sequencher vers. 4.7 (Gene Codes Corp., Ann Arbor, Michigan), ClustalX vers. 1.83 (Jeanmougin et al. 1998), and BioEdit vers. 5.0.9 (Hall 1999), respectively, as outlined in Gillespie et al. (2008). Obvious and unambiguous nucleotide variants within a sequence (i.e., double peaks on electropherogram trace of approximately equal strength, or at least unambiguous double peaks in an otherwise clean sequence) were coded using standard IUB ambiguity codes. Within sample insertion-deletion (indel) variants were inferred via direct sequencing. Minor indel variants that were not parsimony informative were coded for the dominant variant, or where variants were of approximate equal intensity the longest. Where possible, more substantial, parsimony informative indel variation within a sample was coded as separate variant sequences.

Analyses—TLF, ITS, and ETS alignments were merged into a single nexus format data matrix. Indel characters were not included in this matrix. To determine if it was appropriate to analyze combined plastid and nuclear ribosomal data partitions, we used the Incongruence Length Difference (ILD) test of Farris et al. (1995) as implemented with the Partition-Homogeneity Test in PAUP* 4.0b10 (Swofford 2002) with 100 –1000 replications (depending on run times). We determined the extent of conflict between 1) the two nrDNA data partitions, ITS and ETS, and 2) the plastid and nrDNA partitions, TLF, ITS, and ETS. Collections resulting in substantial incompatibility between the TLF and ITS/ETS partitions were subsequently each treated as two separate OTUs: plastid and nrDNA.

Following exploratory parsimony analyses of TLF, ITS, ETS, ITS/ETS and TLF/ITS/ETS data partitions, three final parsimony analyses were run (Table 1). Analysis I included ITS and ETS data partitions and 65 OTUs. This included all OTUs for which we had ITS and ETS data that, *Poa* aside, were not significantly incompatible. Included here, but not in the following TLF/ITS/ETS analyses (II and III), were *Bellardiochloa polychroa* and one additional collection each of *Arctagrostis latifolia* (*A14*) and *Nicoraepoa robusta* (*S7359*) for which we had ITS and ETS data but not TLF data (Appendix 1). The putative hybrid taxa *Arctopoa* and *Nicoraepoa pugionifolia* were included, as were three collections of *Aniselytron* (four OTUs, see Results). We excluded *Poa infirma* and *P. supina*, two sister species on long branches whose positions conflicted considerably between ITS and ETS in exploratory analyses. Analysis II included TLF, ITS, and ETS data partitions and 49 OTUs. The three putative hybrid taxa (*Arctopoa*, *Aniselytron*, and *Nicoraepoa pugionifolia*) that in previous analyses had resolved in highly incongruent positions between TLF and ITS trees (Gillespie et al. 2008) were not included

Analysis	TLF	ITS	ETS	ITS/ETS	ITS/ETS*	ITS/ETS* + hybrid I – Fig. 1	TLF/ITS/ ETS II – Fig. 2	TLF/ITS/ ETS + hybrid III – Fig. 3
OTUs	49	49	49	49	50	65	49	74
No. of characters	1972	629	517	1146	1146	1146	3118	3118
No. of parsimony informative char.	161	147	184	331	298	303	492	511
% parsimony informative char.	8.2%	23.4%	35.6%	28.9%	26.0%	26.4%	15.8%	16.4%
Tree Length	477	545	694	1258	1099	1138	1761	1821
Number of trees	453	673	105	36	35	4483	12	8300
CI	0.705	0.475	0.530	0.496	0.495	0.489	0.530	0.524
RI	0.848	0.742	0.745	0.734	0.763	0.792	0.748	0.779

Table 1. Summary statistics for maximum parsimony analyses of separate TLF, ITS, and ETS datasets, and combined datasets (Analyses I –III). Consistency index does not include parsimony uninformative characters The ITS/ETS* taxon set was the same as that of ITS/ETS* + hybrid (Analysis I) but excluding the three putative hybrid taxa.

in this analysis. Analysis III was the same as Analysis II, with the addition of the three putative hybrid taxa. TLF and ITS/ETS sequences of *Arctopoa* and *N. pugionifolia* were not combined; each accession was treated as two OTUs, one containing only the TLF sequence, the other the combined ITS/ETS sequence. *Aniselytron* OTUs are explained under Results. The total number of OTUs was 74, including three species of *Arctopoa* (eight collections, 17 OTUs), one species of *Aniselytron* (three collections, four OTUs), and *N. pugionifolia* (two collections, four OTUs).

Maximum parsimony (MP) and bootstrap analyses were performed as in Gillespie et al. (2008). Complete heuristic searches were run in PAUP* 4.0b10 (Swofford 2002) for Analyses I and II, with MULPARS and TBR branch swapping. Two heuristic search strategies were used for Analysis III: 1) maximum tree setting of 90,000, no replication; and 2) 100 random addition replicates, 900 trees saved per replicate. Monophyly constraint analyses were performed in PAUP* using the constraints command and the monophyly option. Bootstrap analyses were performed with 1000 replicates, TBR swapping, and the MULTREES setting on for Analyses I and II, and with 1000 replicates, 10 addition sequences per replicate, TBR swapping, and the MULTREES setting turned off for Analysis III due to long search times (DeBry and Olmstead 2000). Following the suggestion of Starr et al. (2004), bootstrap support (BS) for clades was characterized as very poor (<55%), poor (55%–64%), moderate (65%–74%), good (75%–84%), very good (85%–94%), or strong (95%–100%). Consistency index (CI) is calculated on the basis of parsimony informative chaacters only. Clade support refers to bootstrap support, unless otherwise indicated.

We conducted Bayesian analyses using MrBayes v. 3.1 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). To choose the DNA substitution model we used the Akaike Information Criterion (AIC) as implemented in ModelTest ver. 3.7 (Posada and Crandall 1998). For datasets of all three final analyses the optimal model of evolution was GTR + Γ + I. For each analysis, two parallel sets of four chains (three heated and one cold) were run simultaneously, with each Markov chains starting from a random tree. Runs were stopped after the average standard deviation of split frequencies (a convergence diagnostic) was less than 0.01; the number of generations run for each data partition was 1 million. Trees were sampled every 100 generations and the first 25% of the trees were discarded as burn-in. For each analysis trees from each run were pooled and a majority-rule consensus tree was computed in PAUP* to estimate posterior probabilities (pp.) of individual clades. Clades were considered well defined if their pp. values were 95% or above.

Results

Sequence Characteristics and ILD Tests—Characteristics of the TLF, ITS, and ETS data matrices considered separately and combined are provided in Table 2.

For details on TLF and ITS sequences and alignments refer to Gillespie et al. (2008). The aligned ETS data matrix comprised a total of 642 characters. Eleven insertions (either unique or those shared by few OTUs and lacking parsimony informative nucleotide characters), one to 64 bases in length, were deleted from the data matrix prior to analysis, as was a 12 bp region that could not be unambiguously aligned, for a total of 517 analyzed base positions. Length of the sequenced ETS region for individual accessions ranged from ca. 500 bp (numerous species) to 567 bp (*P. infirma*). *Poa infirma* and *P. supina* were both characterized by numerous, mostly shared indels (the largest, a 64 bp insertion, was unique to *P. infirma*). In addition, these two species shared one very large insertion (222 bases in length in *P. infirma*, incomplete at 194 bases in *P. supina*) at the 3' end of ETS, 15 bases downstream of the 18S-R primer, which was located just outside the segment of ETS included in the final data matrix.

Intra-sample variation was detected in some TLF, ITS, and ETS sequences, suggesting the presence of two (or possibly more) sequence variants within a sample (in the nrDNA data these variants may occur on different arrays, and are thus not polymorphisms in the strict sense). Single nucleotide differences represented most of this variation, while single base indel variants were less frequent. ITS variants were discussed previously in Gillespie et al. (2008); one collection was coded differently here. *Arctopoa tibetica* (*OL03-7*) had both indel and base variants and was previously coded only as polymorphic for the base variants; the sample was reinterpreted here as including two variant ITS sequences, 1 and 2. One sample of *Aniselytron treutleri* (*S5229*) newly seqeunced here also contained two variant ITS sequences (discussed further below). A second collection of *Nicoraepoa pugionifolia* (*P17128*) had the same two ITS indel variants found in the sample previously analyzed (Gillespie et al. 2008: *S7336*). Indel variants in the TLF region were detected in only two species sampled here:

Taxa constrained as monophyletic		ITS	ITS/ETS + hybrid	TLF/ITS/ETS	TLF/ITS/ETS + hybrids
	Analysis		I	II	III
	OTUs	54	65	49	74
None	Length	586	1138	1761	1821
Alopecurus + *Beckmannia*	Length increase	1	3	4	4
Alopecurus + *Beckmannia* + *Phleum*	Length increase	6	10	15	15

Table 2. Monophyly constraint analyses for subtribe Alopecurinae. For each of the four datasets number of operational taxonomic units (OTUs) and tree length (L) are given for the unconstrained analysis, analysis constraining *Alopecurus* and *Beckmannia* as monophyletic, and the analysis constraining *Alopecurus*, *Beckmannia*, and *Phleum* as monophyletic. For the constrained analyses number of steps longer than the unconstrained analysis are given.

Aniselytron treutleri and *Nicoraepoa pugionifolia.* Both species (two collections each) had the same poly-A indel variant in the *trnT-L* section of the sequence.

ETS sequences included only minor variants, and there was no evidence of highly divergent sequences within a sample in our data generated using the direct sequencing approach. Single 1 bp indel variants were present in ETS sequences of *Aniselytron treutleri* (both collections), *Nicoraepoa pugionifolia* (both collections), *N. robusta*, and *Poa ligulata*. The sample of *Catabrosa werdermannii* contained two indel and numerous nucleotide variants resulting in a messy but interpretable sequence; only the dominant sequence variant was coded.

Based on ILD tests ITS and ETS data partitions were determined to be incompatible for all 65 OTUs (49 taxa) included in Analysis I (p=0.1 –0.2). Likewise, all combinations of the three data partitions were found to be incompatible for all 49 OTUs (45 taxa) included in Analysis II (p=0.1 –0.2). Exploratory analyses and ILD tests showed that much of this incompatibility can be attributed to the *Poa* clade. Removing various combinations of *Poa* species (other than *P.* subg. *Sylvestres* species) resulted in data partitions that were not significantly incompatible for all partition combinations (p = 0.15 –0.79). Removing seven of the 20 *Poa* species (*P. diaphora, P. flabellata*, *P. infirma*, *P. kerguelensis*, *P. lettermanii*, *P. pseudoabbreviata*, and *P. supina*) resulted in the highest p value for the ITS/ETS and TLF/ITS partitions (0.79 for each). Retaining only *P.* subg.. *Sylvestres* species (*P. autumnalis*, *P. saltuensis*, *P. sylvestris*, and *P. wolfii*) resulted in the highest p value for the TLF/ETS (0.51) and TLF/ITS/ETS partitions (0.15). Excluding the three outgroup taxa, in addition to all *Poa* except for *P.* subg. *Sylvestres*, gave an even higher p value (0.25) for the TLF/ITS/ETS partition, possibly due to exclusion of *Deschampsia,* which has previously been shown to be unstable in position between plastid and nuclear analyses (Quintanar et al. 2007; Soreng et al. 2007; Gillespie et al. 2008).

There appeared to be no significant incompatibility between any combination of the TLF, ITS, and ETS partitions for taxa in the Poinae (minus *Poa*) clade, or more broadly for taxa in the PPAM clade with the exception of *Poa* based on the above ILD tests. Given that we are primarily interested here in relationships among Poinae and Alopecurinae genera, and not in relationships within *Poa*, we feel that combining datasets is justified for the purposes of this paper. The significant incompatibilities between the two nrDNA datasets, ITS and ETS, when all taxa in our dataset were included was surprising, and is an issue that will be addressed in a future paper focusing on relationships within *Poa*.

Previous analyses showed that the positions of *Arctopoa*, *Aniselytron,* and *Nicoraepoa pugionifolia* were highly incongruent between TLF and ITS trees (Gillespie et al. 2008). ILD tests performed on TLF/ITS/ETS, TLF/ITS, and TLF/ETS data partitions for the taxon set excluding *Poa* members except for *P.* subg. *Sylvestres* plus either *Arctopoa* or *Nicoraepoa pugionifolia* indicated that these partitions were significantly incompatible when either taxon was included (p=0.01). In

contrast, ITS/ETS data partitions for the same set of taxa were not significantly incompatible (p=0.20, 0.24, 0.12 for the same taxon set including *Arctopoa*, *N. pugionifolia,* and both taxa, respectively). Therefore ITS and ETS sequences were combined, but TLF and ITS/ETS sequences were not combined for *Arctopoa* and *N. pugionifolia*. Each accession of these two taxa was treated as two OTUs, one containing only the TLF sequence, the other the ITS/ETS sequence.

The situation with *Aniselytron* was more complex than previously thought (Gillespie et al. 2008). Preliminary analyses suggested that TLF and ETS sequences may be compatible, and the ILD test confirmed this (p=0.34 for the Analysis II taxon set including *Aniselytron* and excluding all *Poa* except for *P.* subg. *Sylvestres*). The one additional collection sequenced for ITS (*S5229*) comprised two divergent sequences, which differed in three indel variants and numerous base variants. Although indel variants result in garbled electropherogram traces downstream of the variant, by careful reading of multiple sequences obtained from end and internal primers, we were able to decipher two consensus sequences. The first variant sequence was identical to that previously obtained for *S5264* (Gillespie et al. 2008), whereas the second appeared most similar to sequences of Poinae genera such as *Nicoraepoa* and *Hookerochloa*. ILD tests confirmed that the TLF/ITS/ETS data partitions were not significantly incompatible when the combined *S5229* sequence with ITS variant 2 was included (p=0.07 [borderline], 0.19 with taxon set excluding outgroups). In contrast, data partitions for the taxon set including the combined *S5229* sequence with ITS variant 1 were significantly incompatible (p=0.01). Thus, for accession *S5229* ITS variant 1 was analyzed as a separate OTU, while variant 2 was combined with TLF and ETS data.

Analyses—Summary tree statistics for MP analyses of separate TLF, ITS, and ETS datasets and of combined ITS/ETS and TLF/ITS/ETS datasets are provided in Table 1. ETS, the shortest DNA region, had the highest number and percentage of parsimony informative (PI) characters. Plastid TLF, the longest region, had by far the lowest percentage, but an intermediate number of PI characters, whereas ITS had the lowest number of PI characters. MP trees resulting from the separate TLF analysis had the highest consistency index (CI). ETS MP trees had a higher CI than ITS trees, a somewhat surprising result given the greater number of variable and PI characters in the ETS dataset. Consistency indices of trees resulting from analyses of combined datasets were all greater than those from the ITS analysis, and not substantially lower than the average of the CIs from the separate analyses.

ANALYSIS I—Maximum parsimony analysis of the combined ITS/ETS data matrix with putative hybrid taxa resulted in 4483 trees, 1128 steps long, with a CI of 0.490 (Table 1). A phylogram depicting one of the most parsimonious trees, with branches found in the strict consensus tree shown by bold lines, is illustrated in Fig. 1.

The Bayesian majority rule consensus tree was for the most part identical in structure to the MP tree shown in Fig. 1. The major differences were *Milium* and

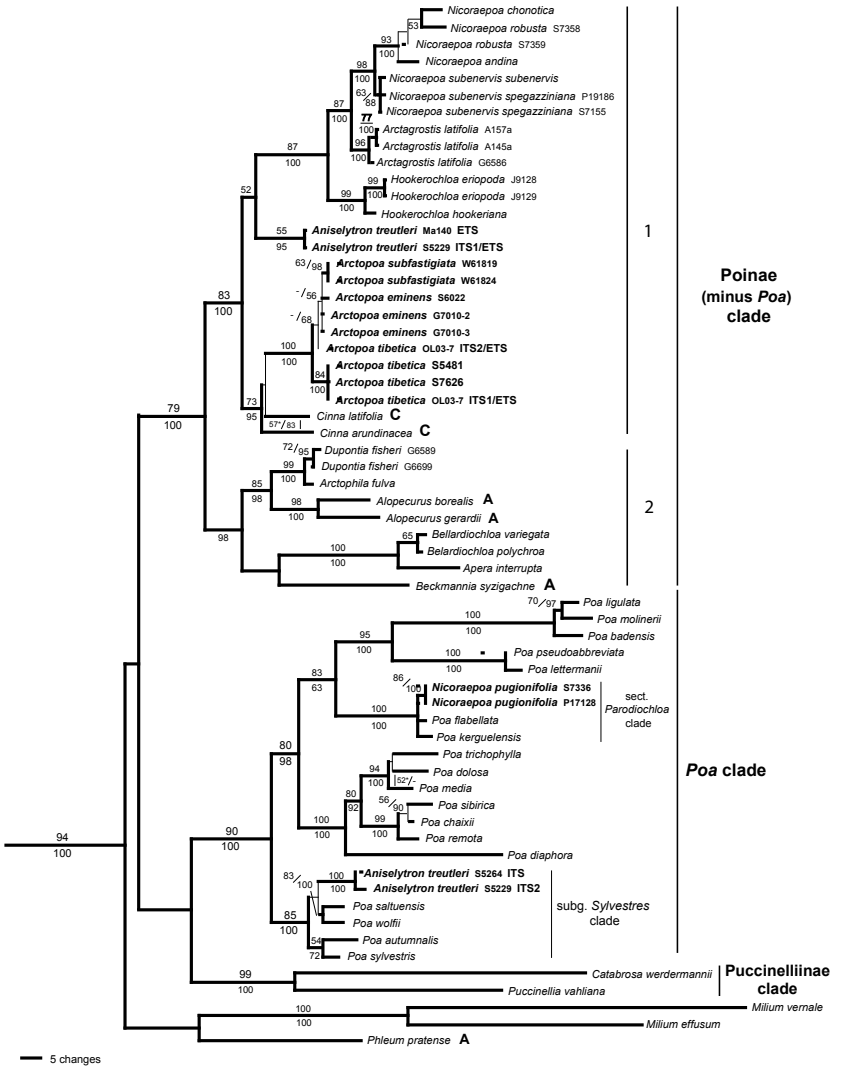

Fig. 1. Phylogram with strict consensus tree shown in bold lines resulting from maximum parsimony (MP) analysis of combined ITS and ETS data for collections of subtribes Poinae, Alopecurinae, Cinninae, and Puccinelliinae (4483 trees, 1138 steps, CI = 0.495). MP bootstrap values >50% are given above branches, Bayesian posterior probability values below branches. Collection information is given only for species represented by multiple collections; 'ITS' and/or 'ETS' are provided only for OTUs having variant sequences or where only one of the two regions was sequenced; variant ITS sequences are indicated as 'ITS1' or 'ITS2'; 'A' indicates species belonging to subtribe Alopecurinae, 'C' those belonging to Cinninae. Outgroup taxa are not shown.

Phleum forming a clade with *Poa* with high posterior probability (pp. =100, not shown on Fig. 1), and *Beckmannia* as sister to the *Alopecurus-Arctophila-Dupontia* clade (pp. =68).

Three major PPAM clades are inferred with good to strong support and high posterior probability: 1) *Poa* (including *Nicoraepoa pugionifolia* and some *Aniselytron* ITS OTUs) (BS=90%, pp. =100); 2) Poinae (excluding *Poa*) with *Alopecurus*, *Beckmannia*, and *Cinna* (referred to here as the Poinae (minus *Poa*) clade) (BS=79, pp. =100); and 3) Puccinelliinae (BS=99, pp. =100). Relationships among these three clades and the two separate *Milium* and *Phleum* lineages are not supported (BS<50). Support for the monophyly of genera ranges from poor (*Cinna;* BS=57), moderate (*Bellardiochloa*, *Dupontia*; BS=65 –72), very good (*Poa*; BS=90), to strong (*Alopecurus*, *Arctagrostis*, *Arctopoa*, *Hookerochloa*, *Nicoraepoa* [excluding *N. pugionifolia*]; BS=96 –100). Within the Poinae (minus *Poa*) clade the *Apera-Bellardiochloa* subclade is strongly supported (BS=100), while the *Arctophila-Alopecurus-Dupontia* subclade (BS=85) and the large subclade comprising *Arctagrostis*, *Arctopoa*, *Aniselytron*, *Cinna*, *Hookerochloa*, and *Nicoraepoa* (subclade 1: BS=83) are well supported. Relationships among these clades plus *Beckmannia* receive no support (BS<50). Within the *Poa* clade subgenus *Sylvestres* resolves with very good support as a clade (BS=85) and with good support as sister to a clade comprising all other *Poa* species included in the analysis (BS=80). *Aniselytron* resolved in two different positions, with *P.* subg. *Sylvestres* in the *Poa* clade (two ITS sequences) and in subclade 1 of the Poinae (minus *Poa*) clade (one ETS and one combined ETS/ITS sequence).

ANALYSIS II—Parsimony analysis of the combined TLF/ITS/ETS data matrix resulted in 12 trees, 1761 steps long, with a CI of 0.530 (Table 1; Fig. 2). The Bayesian majority rule consensus tree was identical in branching structure to the MP phylogram shown in Fig. 2, and thus slightly more resolved than the MP strict consensus tree (shown by bold lines in Fig. 2). However, the two clades that resolved in the Bayesian tree, but not in the MP strict consensus tree (monophyly and position of *Cinna* as shown in Fig. 2), were present in the bootstrap tree with moderate to good support. Several large clades had high posterior probablity in the Bayesian tree, but low support in the MP tree, e.g., *Alopecurus-Apera-Arctophila-Bellardiochloa-Beckmannia-Dupontia* (clade 2, pp. =100 versus BS=58) and *Milium-Phleum-Poa* (pp. =100, BS=65).

The TLF/ITS/ETS analysis strongly supports the same three major PPAM clades as in Analysis I: 1) *Poa* (BS=99, pp. =100); 2) Poinae (excluding *Poa*) plus *Alopecurus*, *Beckmannia*, and *Cinna* (BS=96, pp. =100); and 3) Puccinelliinae (BS=100, pp. =100). As in Analysis 1 *Milium* and *Phleum* form two additional separate lineages; here they resolve together in a clade with *Poa* (BS=65, pp. =100). There is strong support (BS=98 –100) for the monophyly of all genera (where more than one species was included), with the exception of *Dupontia* (BS=89) and *Cinna* (BS=60). The Puccinelliinae clade resolves as sister to all other PPAM members with good support (BS=83).

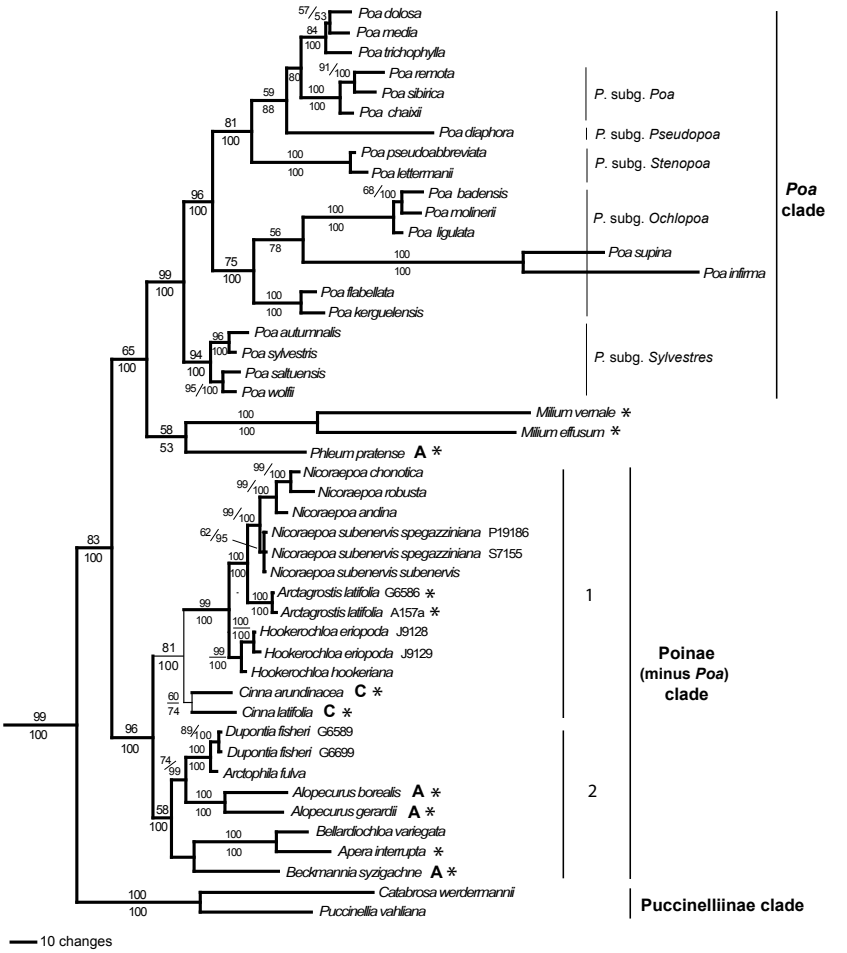

Fig. 2. Phylogram with strict consensus tree shown in bold lines resulting from MP analysis of combined TLF, ITS and ETS data for subtribe Poinae, excluding putative hybrid taxa, plus representative members of subtribes Alopecurinae, Cinninae, and Puccinelliinae (12 trees, 1761 steps, CI = 0.530). MP bootstrap values >50% are given above branches, Bayesian posterior probability values below branches. Collection information is given only for species represented by multiple collections; 'A' indicates species belonging to subtribe Alopecurinae, 'C' those belonging to Cinninae; '*' indicates taxa with one-flowered spikelets, all others have multi-flowered spikelets. Outgroup taxa are not shown.

Within the *Poa* clade, subgenus *Sylvestres* resolves with strong support as a clade (BS=94) and as sister to a clade comprising all other *Poa* species included in the analysis (BS=96). Species in this latter clade form two well supported clades corresponding for the most part to subgenus *Ochlopoa* (Asch. & Graebn.) Hyl. (BS=75), and subgenera *Poa* (*P. chaixii, P. remota*, *P. siberica*), *Pseudopoa* (K. Koch.) Stapf (*P. diaphora*), and *Stenopoa* (Dumort.) Soreng & L.J. Gillespie (*P. badensis*, *P. ligulata*, *P. molinerii*) (BS=81); the exception is *P. media* (Ochlopoa). This species, *P dolosa*, and *P. trichophylla* are discussed in Soreng et al. (2010).

Strongly supported clades in the Poinae (minus *Poa*) clade include: *Arctagrostis* and *Nicoraepoa* (BS=100); *Arctagrostis*, *Hookerochloa*, and *Nicoraepoa* (BS=99); *Apera* and *Bellardiochloa* (BS=100); and *Arctophila* and *Dupontia* (BS=100). *Alopecurus* is sister to *Arctophila-Dupontia* (BS=74), and *Cinna* is sister to *Arctagrostis-Hookerochloa-Nicoraepoa* (BS=81), with moderate to good support, respectively.

ANALYSIS III—Parsimony analysis of the combined TLF/ITS/ETS data matrix including putative hybrid taxa resulted in 8300 trees, 1821 steps long, with a CI of 0.524 (Table 1, Fig. 3). The Bayesian majority rule consensus tree was very similar to the MP tree shown in Fig. 3, differing mostly in being somewhat more resolved. The additional clades in the Bayesian tree had mostly low posterior probablity, and the only one of interest here was an *Aniselytron* (ITS OTUs)-*Arctopoa* (TLF OTUs) clade (pp. =72). As in analysis II several large clades had high posterior probability, but low bootstrap support (pp. =99 –100 versus BS<65), while one clade strongly supported in the MP tree was not detected in the Bayesian tree (e.g., *Arctopoa* TLF OTUs, BS=95).

Tree structure is congruent with that in Analysis II, but with less resolution among *Arctagrostis*, *Hookerochloa*, and *Nicoraepoa* members. Although not present in the MP strict consensus tree, bootstrap analysis provided moderate to good support for the following clades: *Arctagrostis* and *Nicoraepoa* (excluding *N. pugionifolia*) (BS=81); and this clade plus *Hookerochloa* and *N. pugionifolia* (BS=74). The putative hybrid taxa each resolved as two clades, plastid and nrDNA (except see below for partition of *Aniselytron* OTUs), with very good (*N. pugionifolia*, BS=85) to strong support (*Arctopoa*, *Aniselytron*, BS=95 –96). *Arctopoa* TLF OTUs resolved together (BS=95) in the *Poa* clade (BS=93) with subg. *Sylvestres* (BS=62), whereas ITS/ETS OTUs resolved in the Poinae clade (minus *Poa*) (BS=89) within subclade 1 (BS=58). *Nicoraepoa pugionifolia* TLF OTUs resolved in the same Poinae (minus *Poa*) subclade, but ITS/ETS OTUs resolved in the *Poa* subg. *Ochlopoa* sect. *Parodiochloa* clade (BS=100). Two ITS OTUs of *Aniselytron* resolved in the *Poa* subg. *Sylvestres* clade (*S5229* ITS variant 1 and *S5264*). The single complete TLF/ITS/ETS sequence (*S5229* including ITS variant 2) and the TLF/ETS sequence (*Ma 140*; ITS not successfully sequenced) of *Aniselytron* formed a clade (BS=96) in subclade 1 in the Poinae (minus *Poa*) clade.

ALOPECURINAE MONOPHYLY ANALYSES—Constraint analyses testing for the monophyly of subtribe Alopecurinae are summarized in Table 2. None of the three Alopecurinae genera examined here, *Alopecurus*, *Beckmannia*, and *Phleum*,

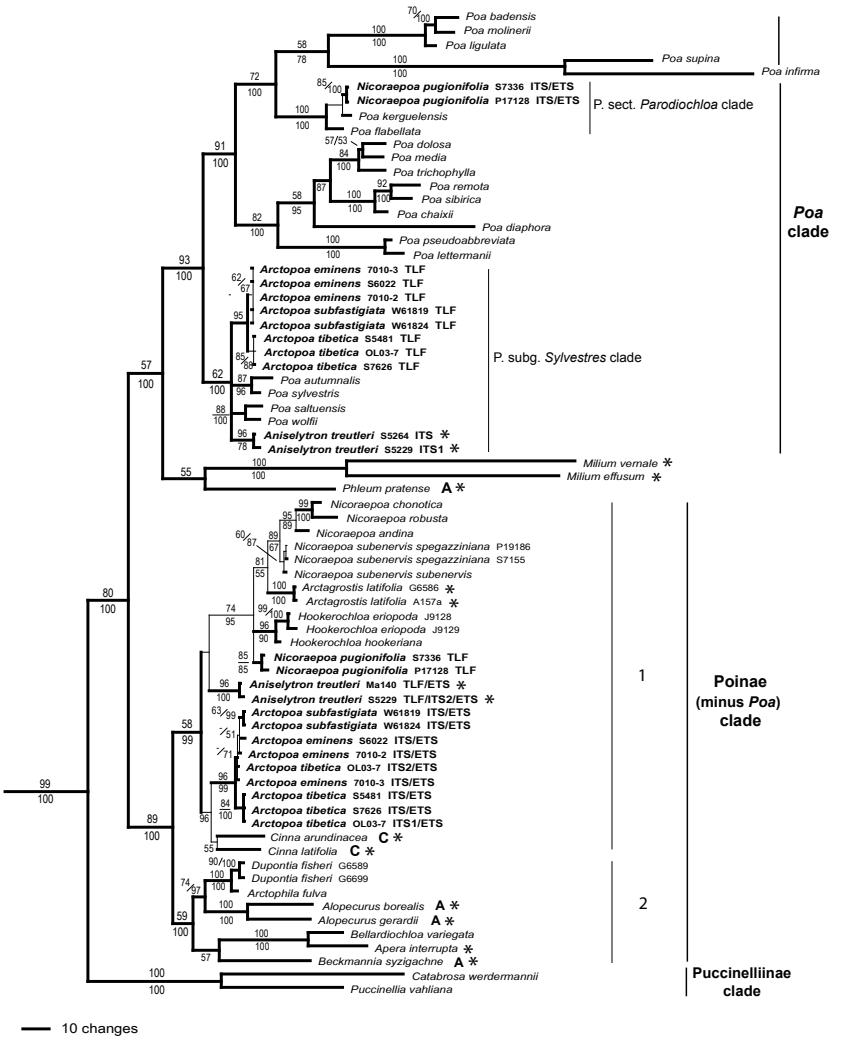

Fig. 3. Phylogram with strict consensus tree shown in bold lines resulting from parsimony analysis of combined TLF, ITS and ETS data for subtribe Poinae, including putative hybrid taxa, plus representative members of subtribes Alopecurinae, Cinninae, and Puccinelliinae (8300 trees, 1821 steps, CI = 0.524). MP bootstrap values >50% are given above branches, Bayesian posterior probability values below branches. Collection information is given only for species represented by multiple collections; variant ITS sequences are indicated as 'ITS1' or 'ITS2'; 'A' indicates species belonging to subtribe Alopecurinae, 'C' those belonging to Cinninae; '*' indicates taxa with one-flowered spikelets, all others have multi-flowered spikelets. Putative hybrid taxa are indicated in bold. TLF and ITS/ETS data were not combined for *Arctopoa* and *Nicoraepoa pugionifolia*; each accession is represented by two OTUs indicated as 'TLF' and 'ITS/ETS'. *Aniselytron* OTUs are indicated by their constituent DNA regions described in detail in the text. Outgroup taxa are not shown.

formed a clade in TLF, ITS, ITS/ETS, or TLF/ITS/ETS unconstrained analyses. However, *Alopecurus* and *Beckmannia* did form a clade in the ETS analysis (not shown), sister to the *Arctophila-Dupontia* clade in the Poinae (minus *Poa*) major clade, the same position that *Alopecurus* occupied in the ITS (not shown), ITS/ETS (Fig. 1) and TLF/ITS/ETS trees (Figs. 2-3). Constraining *Alopecurus* (two species and collections) and *Beckmannia* (one collection) as monophyletic in the combined TLF/ITS/ETS datasets resulted in MP trees only four steps longer than trees from the unconstrained analyses (Figs. 2-3), whereas constraining all three genera, *Alopecurus*, *Beckmannia*, and *Phleum* (one collection), resulted in MP trees 15 steps longer. Strict consensus trees were identical to those in the unconstrained analyses, except for the positions of *Beckmannia* and *Phleum,* and the more or less resolved position of *Cinna* (e.g., more resolved in TLF/ITS/ETS as sister to the *Arctagrostis-Hookerochloa-Nicoraepoa* clade). In all analyses *Beckmannia* and *Phleum* (where constrained) resolved with *Alopecurus*, in a clade sister to the *Arctophila-Dupontia* clade. Since five additional ITS sequences were available for Alopecurinae (Gillespie et al. 2008), we also ran a constraint analysis only on the ITS data matrix. In the unconstrained analysis each of the three genera were monophyletic: *Alopecurus* (four species, five collections: *A. geniculatus* and *A. vaginatus* added); *Beckmannia* (two species and collections: *B. eruciformis* added); and *Phleum* (two species and collections: *P. phleoides* added). Constraining *Alopecurus* and *Beckmannia* as monophyletic in the ITS analysis resulted in MP trees one step longer than those from the unconstrained analysis, whereas constraining all three genera, *Alopecurus*, *Beckmannia*, and *Phleum*, resulted in a tree seven steps longer. In both analyses the constrained genera resolved as a clade sister to the *Arctophila-Dupontia* clade, as in the TLF/ITS/ETS constrained analysis.

Discussion

Analyses of combined plastid TLF and nrDNA ITS and ETS data resulted in a much more robust phylogenetic hypothesis for subtribes Poinae and related Alopecurinae, Cinninae, Miliinae, and Puccinelliinae (PPAM clade) (Figs. 2-3) compared with those obtained from previous separate analyses of plastid and ITS data (Gillespie et al. 2007, 2008). Likewise combining ETS and ITS data resulted in a more strongly supported nrDNA phylogeny (Fig. 1) than that obtained with ITS data alone. TLF, ITS, and ETS data partitions were for the most part congruent. Although all three data partitions were incongruent for all taxa, this conflict was determined to lie in the *Poa* clade. Data partitions for all other genera, with only the *Poa* subg. *Sylvestres* clade included to represent *Poa*, were not significantly incongruent.

Surprisingly, conflict within the *Poa* clade between ETS and ITS partitions was as high as between either of these nrDNA partitions and the plastid partition. Positions of several diploid species of *Poa* (in particular *P. infirma*, *P. let-*

termanii, *P. pseudoabbreviata*, and *P. supina*) were determined to be incongruent between ETS and ITS trees. This finding will be explored in more detail in a future paper focusing on the phylogeny of *Poa*. Incongruence between ITS and ETS data is unusual, but has been recorded previously for several other taxa (Bena et al. 1998; Cubas et al. 2006; Mitsui et al. 2008) and may be due to concerted evolution operating at different rates and/or with different outcomes in the ETS and ITS regions.

Nucleotide and indel sequence variants within a sample were detected in both diploid and polyploid Poinae taxa, in putative hybrid and non-hybrid taxa, and in all three DNA regions. The more complex variants, including the only case of divergent paralogous sequences, were found in the polyploid taxa *Aniselytron* and *Nicoraepoa pugionifolia*, providing further evidence for their putative hybrid status.

PPAM–major clades—The phylogeny of the PPAM clade based on combined plastid and nrDNA data is mostly consistent with, but more strongly supported than, trees from previous separate analyses of plastid and ITS data (Gillespie et al. 2007, 2008). Likewise, the combined ITS/ETS phylogeny is mostly consistent with and more strongly supported than those based only on ITS or ETS data (Gillespie et al. 2008; and analyses performed here). All of these analyses focusing on the PPAM clade resolve three major clades: *Poa*; subtribe Poinae (minus *Poa*) including *Alopecurus*, *Beckmannia* (both Alopecurinae) and *Cinna*; and subtribe Puccinelliinae; plus two separate lineages, *Milium* and *Phleum*. The three major clades have good to strong support in the combined trees presented here (Figs. 1-3), whereas the *Poa* and Poinae (minus *Poa*) clades had bootstrap support less than 55% in ITS analyses and moderate to very good support in TLF analyses (Gillespie et al. 2007, 2008). The Puccinelliinae clade was well supported as sister to all remaining members of the PPAM clade in the combined TLF/ITS/ETS and ITS/ETS trees, consistent with previous trees based only on plastid data, but not with ITS alone. This supports recognition of a PAM clade (first recognized and named in Gillespie et al. 2007) comprising subtribes Alopecurinae, Miliinae, and Poinae as detected in several earlier analyses of plastid data alone (Soreng and Davis 2000 [note: *Puccinellia stricta* in error]; Davis and Soreng 2007; Döring et al. 2007; Gillespie et al. 2007, 2008; Soreng et al. 2007). Our revised classification of the PPAM clade is presented in Table 3 and modifications are discussed below.

Poa resolves as a strongly supported clade in combined ITS/ETS and TLF/ITS/ETS trees, compared to only poorly to moderately supported in previous separate TLF and ITS analyses (Gillespie and Soreng 2005; Gillespie et al. 2007, 2008). We now have strong support for the genus as monophyletic. Recent taxonomic and nomenclatural changes based on molecular and morphological evidence have fine tuned its circumscription to exclude some species (e.g., *P.* subg. *Andinae* = *Nicoraepoa*) and submerge genera that resolved within it (reviewed in Introduction). *Poa* subg. *Sylvestres* is very well supported as a clade compared to

1. Subtribe Alopecurinae: *Alopecurus* L., *Beckmannia* Host, *Cornucopiae* L.^, *Limnas* Trin.^
2. Subtribe Miliinae: *Colpodium* Trin., *Milium* L., *Zingeria* P.A. Smirn.
3. Subtribe Phleinae: *Phleum* L., *Pseudophleum* Dogan^, *Rhizocephalus* Boiss.^
4. Subtribe Poinae:

i. *Poa* clade: *Poa* (including *Aphanelytrum* Hack., *Dissanthelium* Trin., and *Tovarochloa* T. D. Macfarl. & But, plus genera now subsumed within, see Introduction)
ii. Poinae (minus *Poa*) clade: *Aniselytron* Merr.[H], *Apera* Adans., *Arctagrostis* Griseb., *Arctophila*, (Rupr.) Anderss., *Arctopoa* (Griseb.) Prob.[H], *Bellardiochloa* Chiov., *Cinna* L.[C], *Cyathopus* Stapf[C], *Dupontia* R. Br., *Gaudiniopsis* (Boiss.) Eig, *Hookerochloa* E. B. Alexeev, *Nicoraepoa* Soreng & L. J. Gillespie, *Ventenata* Koeler
iii. genera lacking DNA data: *Libyella* Pamp.^, *Limnodea* Dewey[C]^, *Lindbergella* Bor^, *Nephelochloa* Boiss.^

5. Subtribe Puccinelliinae: *Catabrosa* P. Beauv., *Catabrosella* (Tzvelev) Tzvelev, *Hyalopoa* (Tzvelev) Tzvelev, *Oreopoa* H. Scholz & Parolly^, *Paracolpodium* (Tzvelev) Tzvelev, *Phippsia* (Trin.) R. Br., *Pseudosclerochloa* Tzvelev^, *Puccinellia* Parl., *Sclerochloa* P. Beauv.

Table 3. Modified classification of subtribes and genera in the PPAM clade.[C]= subtribe Cinninae genera now placed in Poinae; [H]= genera of putative hybrid origin; ^= no DNA data.

being only poorly supported in separate analyses, and its position as sister to all other *Poa* species is now strongly supported. Relationships within *Poa* based on combined TLF/ITS/ETS analyses will be explored in a separate paper. We remain in doubt as to the sister group to *Poa*. TLF/ITS/ETS analyses suggest that *Milium* and *Phleum* may form the sister clade, but support for this clade is poor and its sister relationship to *Poa* has only poor to moderate bootstrap support (but high posterior probability in the Bayesian analyses). ITS/ETS MP analyses are even less conclusive, with relationships among the three major clades, *Milium,* and *Phleum* all having no bootstrap support although a *Milium-Phleum-Poa* clade does have high posterior probability in the Bayesian analysis.

Our results indicate that subtribe Poinae, as currently circumscribed, is paraphyletic. While the majority of Poinae genera form a strongly supported clade, two of three Alopecurinae genera (*Alopecurus* and *Beckmannia*) and the single genus of Cinninae examined here are firmly embedded in this clade. A second Cinninae genus, *Cyathopus*, also resolved with very good support in a clade equivalent to our Poinae (minus *Poa*) clade in the plastid *matK* analysis of Döring et al. (2007). Even if Cinninae is submerged within Poinae (as recommended by Gillespie et al. 2008), Alopecurinae are sufficiently distinct morphologically to tentatively retain as a separate subtribe. Our results also suggest that the Poinae (minus *Poa*) clade and the *Poa* clade may not be sister

taxa; their precise relationship remains unclear due to the equivocal positions of *Phleum* and *Milium*. While major changes in the circumscription of subtribe Poinae will undoubtedly be necessary to align the classification and molecular phylogeny, we feel that increased sampling and stronger support for relationships among major clades are necessary to support further taxonomic change.

Both molecular results and morphology support the inclusion of Cinninae within subtribe Poinae as suggested by Gillespie et al. (2008). All three Cinninae genera (*Cinna, Cyathopus, Limnodea*) have single-flowered spikelets that disarticulate below the glumes, features that previously were used to place them in tribe Aveneae (Clayton and Renvoise 1986). While the majority of Poinae genera are characterized by multiple-flowered spikelets that disarticulate above the glumes, *Apera, Aniselytron*, *Arctagrostis,* and a few species of *Poa* (*Tovarochloa* and some New Guinea species) also have single-flowered spikelets, and *Ventenata* has spikelets that disarticulate below the glumes. Alopecurinae genera are also characterized by single-flowered spikelets that disarticulate below the glumes. When all of these characteristics are considered, Cinninae appears little differentiated morphologically from Poinae. Soreng et al. (2007) discussed homoplasy in these characteristics across tribe Poeae.

The precise affinities of *Milium* and *Phleum* remain unclear. While well supported as members of the PAM clade in combined plastid and nrDNA trees, they appear as two separate lineages distinct from the *Poa* and the Poinae (minus *Poa*) clades in all analyses focusing on the PPAM clade (Figs. 1-3; Gillespie et al. 2007, 2008). In the majority of analyses *Milium* and *Phleum* resolve as a clade (except in the ITS analysis), but this relationship has low support. Likewise, their positions within the PPAM clade have been poorly supported in most analyses. The combined plastid and nrDNA tree (Fig. 2) suggests a sister relationship with *Poa* with moderate bootstrap support and high posterior probability. *Milium*, in particular, is characterized by a high rate of nucleotide substitution in all three DNA regions analyzed, which may be a contributing factor in its equivocal position, possibly obscuring its true affinities. Our previous finding based on ITS data that *Milium* may be part of a separate Miliinae s.l. clade including *Colpodium* and *Zingeria* (Gillespie et al. 2008) needs to be tested with additional samples and data from other DNA regions.

Poinae (minus *Poa*) clade—All Poinae genera analyzed, with the exception of *Poa*, are included within the strongly supported Poinae (minus *Poa*) clade. Also included here are two of three Alopecurinae examined and *Cinna*. In previous plastid analyses relationships within this clade were largely unresolved or poorly supported (Gillespie et al. 2008: Fig. 1); only *Apera-Bellardiochloa* and *Arctophila-Dupontia* clades were strongly supported. ITS analyses were somewhat more resolved, having, in addition, a very well supported *Arctagrostis-Hookerochloa* (incl. *Festucella*)-*Nicoraepoa* clade (Gillespie et al. 2008: Fig. 2a). Combining ETS with ITS resulted in a nrDNA tree with increased support for relationships in the Poinae (minus *Poa*) clade, and analysis of combined plastid and nrDNA

provided even greater support. Structure within this clade was congruent and mostly strongly supported in trees resulting from both sets of analyses, suggesting that the hypothesis of relationships presented here is robust.

Both ITS/ETS and TLF/ITS/ETS analyses resolved two major sister clades in the Poinae (minus *Poa*) clade. *Arctagrostis-Hookerochloa-Nicoraepoa-Cinna* (clade 1) and *Alopecurus-Apera-Arctophila-Beckmannia-Bellardiochloa-Dupontia* (clade 2) each have a high posterior probability in the Bayesian analysis, but are only poorly to well supported in the MP analysis. Both clades contained several strongly supported subclades. In clade 1, the following subclades are strongly supported and of biogeographically interest: *Nicoraepoa* (southern South American) and *Arctagrostis* (arctic), and these sister to *Hookerochloa* (Australia). Clade 2 included subclades: *Apera-Bellardiochloa* (both Eurasia), *Arctophila-Dupontia* (both arctic), and the latter sister to *Alopecurus* (distribution widespread) (the first two subclades strongly supported, the third with moderate to very good support).

Subtribe Alopecurinae: Monophyletic, Paraphyletic or Polyphyletic?—The three genera analyzed of subtribe Alopecurinae, *Alopecurus*, *Beckmannia*, and *Phleum*, did not resolve as monophyletic in separate TLF and ITS analyses or in combined analyses (Gillespie et al. 2007, 2008; results presented here). *Alopecurus* and *Beckmannia* did resolve as a clade in the ETS analysis; in other analyses nodes separating these two genera have at most poor to moderate support (e.g., Figs. 2-3). Constraining *Alopecurus* and *Beckmannia* as monophyletic resulted in trees not considerably longer than the unconstrained analysis (Table 2, 1 –4 steps). Thus, there appears to be no strong evidence contradicting the monophyly of *Alopecurus* and *Beckmannia*. In contrast, *Phleum* resolved as a distinct lineage distant from *Alopecurus* and *Beckmannia* in all analyses. Including *Phleum* in the monophyly constrained analyses substantially increased tree length (Table 2, 6-15 steps). Our results suggest that Alopecurinae, as currently treated, is polyphyletic and support splitting the subtribe into two or three lineages following Tzvelev (1976). Tzvelev recognized three subtribes in his tribe Phleeae: Alopecurinae, Beckmanniinae, and Phleinae. Our data strongly support recognizing *Phleum* as a distinct subtribe Phleinae (Table 3 or Appendix 2). In contrast, we tentatively leave *Beckmannia* within Alopecurinae since we have no strong evidence contradicting this relationship.

The constrained Alopecurinae clade resolved as sister to the *Arctophila-Dupontia* clade in the Poinae (minus *Poa*) clade in all monophyly constrained analyses performed. Analyzing different data partitions, constraining only *Alopecurus* and *Beckmannia* versus all three genera (i.e., including *Phleum*), and including additional species (nine in total) in the ITS analysis did not change its position. The position of the constrained Alopecurinae clade is the same as that occupied by only *Alopecurus* in ITS, ITS/ETS, and TLF/ITS/ETS analyses, and by both *Alopecurus* and *Beckmannia* in the ETS analysis.

Reticulate Evolution – Cases of Intergeneric Hybridization?—Combined plastid and nrDNA analyses presented here strongly support the hypothesis that

hybridization has played a role in the origin and evolution of genera in Poinae (Gillespie et al. 2008). ILD tests confirmed that plastid and nrDNA data for *Arctopoa* and *Nicoraepoa pugionifolia* were highly incongruent, whereas *Aniselytron* was determined to have two highly divergent ITS paralogues. Including the putative hybrids as separate plastid and nrDNA OTUs (in the case of *Aniselytron*: separate TLF/ETS and ITS OTUs, and one combined TLF/ITS/ETS OTU) reduced resolution and support in the trees, particularly in the *Arctagrostis-Hookerochloa-Nicoraepoa-Cinna* clade where relationships among genera were all unresolved. Different levels of plastid versus nrDNA variation and the presence of missing data in the plastid only and nrDNA only OTUs likely contribute to this lower resolution and support. Recombination of two or more divergent repeats to form chimeric ITS and/or ETS sequences may be an additional contribution factor (Alvarez and Wendel 2003), although levels of recombination would presumably be low here if this has occurred. Considerable incongruence between plastid and nrDNA data was also detected within *Poa*, and will be discussed in Soreng et al. (2010) and future papers focusing on hybridization in *Poa*.

ARCTOPOA —*Arctopoa* species are tetraploid or hexaploid and occur in riparian and littoral habitats of central to far eastern Asian mountains, steppes and coasts, and along seashores in boreal North America. Eight collections representing three of five species have now been examined for both plastid and nrDNA data (Gillespie et al. 2008; data presented here). All collections consistently resolve in two divergent positions: plastid OTUs in the *Poa* subg. *Sylvestres* clade, nrDNA OTUs in the Poinae (minus *Poa*) clade (Fig. 3). Additional species and collections previously examined for only plastid or nrDNA resolve in positions consistent with our results, including *A. eminens* (plastid restriction site data, Soreng 1990) and *A. schischkinii* (Tzvelev) Prob. Tzvelev (ITS sequence data, Rodionov et al. 2005). These results provide strong evidence that *Arctopoa* is likely of intergeneric, and presumably ancient, hybrid origin. Our evidence suggests a member of the *Poa* subg. *Sylvestres* clade as a maternal parent (i.e., which donated a plastid genome), and a member of the Poinae *Arctagrostis-Hookerochloa-Nicoraepoa-Cinna* clade as a paternal parent. In both cases, no extant species group or genus can be readily identified as possible parents; further sampling of Asian *Poa* and Poinae may identify putative parents, although these may now be long extinct. Data from additional nuclear linkage groups could potentially provide further insight. *Poa* subg. *Sylvestres*, as currently understood, is endemic to temperate mesic woodlands in North America. The wetland genus *Arctopoa* bears little resemblance morphologically and shares no morphological synapomorphies with this subgenus. The possibility of a sister relationship with *Cinna* as inferred by the nrDNA trees and the Bayesian combined plastid and nrDNA tree needs to be further explored, but again we know of no morphological synapomorphies for this set.

Neither plastid nor nrDNA data support the current subdivision of *Arctopoa* into two sections, as currently circumscribed: sects. *Arctopoa* (*A. eminens*) and

Aphydris (*A. schischkinii*, *A. subfastigiata*, *A. tibetica*, and *A. trautvetteri* (Tzvelev) Prob.) (Probatova 1974; Tzvelev 1976). Both datasets suggest that *A. subfastigiata* and *A. eminens*, the two most salt tolerant species, are most closely related to each other (e.g., identical nucleotide characters in TLF), than either is to *A. tibetica*. This suggests that the distinctive features of *A. eminens* may be derived in this genus.

One of the three collections examined of *Arctopoa tibetica* is suggested to be an infrageneric hybrid (also suggested in Gillespie et al. 2007, where it was included as a single polymorphic sequence). The sample contained two variant ITS sequences, one identical to the other two *A. tibetica* collections sampled, the other closer to *A. eminens* and *A. subfastigiata*. An alternative explanation might be incomplete lineage sorting and retention of two ancestral variants.

ANISELYTRON—*Aniselytron* includes two species, one widespread in south-east Asia (one hexaploid count), the other apparently endemic to Taiwan and the Philippines (ploidy unknown) (Lu and Phillips 2006). The genus was traditionally placed in Aveneae, near, or even included within, *Calamagrostis* Adans. (Clayton and Renvoize 1986), but recent molecular data on the widespread species placed it in the PAM clade (Soreng et al. 2007, with Poinae and allied subtribes in the 'Poeae type' plastid group; Gillespie et al. 2008). While the majority of sequence data (plastid, ETS, and one ITS sequence) place *A. treutleri* with the Poinae genera *Arctagrostis*, *Hookerochloa*, *Nicoraepoa*, and *Cinna*, a divergent ITS paralogue (two different collections) resolves in the *P.* subg. *Sylvestres* clade. This suggests that either hybridization was involved in the origin of *Aniselytron* or there has been more recent hybridization with *Poa*. As with *Arctopoa*, no extant genus or species group, respectively, have yet been identified as putative parents, but further sampling of co-occurring south-east Asian *Poa* and the Cinninae genus *Cyathopus* (the latter sampled only for plastid *matK*; Döring et al. 2007) would be appropriate. *Aniselytron* shares with *Cyathopus* the character of single flowered spikelets, distinct from the typical multi-flowered spiketlets of *Poa*. The floret of *Aniselytron* is very similar to a typical *Poa* floret, but differs in its short stiff hairs on the front of the callus and on lower lemma margins (as occurs in *Agrostis* L.).

Curiously, the positions occupied by *Arctopoa* and *Aniselytron* in both the *Poa* and Poinae (minus *Poa*) clades are very similar, but with plastid and ITS (except *S5229* variant 2) OTUs reversed. Previous sampling also detected a more divergent ITS paralogue in *Aniselytron* that was not readily alignable (Gillespie et al. 2008).

NICORAEPOA —*N. pugionifolia*, of the southern South American genus *Nicoraepoa* (ploidy unknown), was previously suggested to be of intergeneric hybrid origin based on previous incongruent TLF and ITS data (Gillespie 2008). A second sample from a different location and our new ETS results confirm these two incongruent positions in the *Poa* clade (plastid OTUs) and the Poinae (minus Poa) clade (nrDNA OTUs). The species resolves in the *Arctagrostis-Hookerochloa-*

Nicoraepoa clade based on its plastid data, close to, but not in a clade with, other *Nicoraepoa* members (Fig. 3). The lower resolution and levels of support in this clade, including *Nicoraepoa* not resolving as monophyletic in the MP strict consensus tree, compared to the analysis without hybrid taxa (Fig. 2) are likely due, at least in part, to the low levels of plastid variation in this clade along with the absence of ITS/ETS data in these *N. pugionifolia* OTUs. nrDNA data provides strong evidence for a member of *Poa* sect. *Parodiochloa* as the paternal parent of *N. pugionifolia*. *Poa flabellata* is the only geographically-likely parental candidate in *Poa,* as it is the only member of this section occurring in South America (all other species are found on sub-Antarctic islands).

Evolution involving hybridization is also suggested for *Aniselytron* and *N. pugionifolia* by the relatively high levels of infra-sample indel and base variation in sequences of all three DNA regions analyzed. Indel variants in the TLF dataset were detected only in these two species, and in both collections of each sequenced. Divergent ITS sequences were detected in *Aniselytron*, the only case of divergent paralogues found in this study by direct sequencing of PCR amplified products, whereas *N. pugionifolia* had multiple indel variants in ITS, together accounting for the most complex patterns of within sample indel variation in ITS sequences so far detected in the PAM clade. Incomplete concerted evolution in nrDNA and the presence of indel variants in plastid sequences may possibly suggest a relatively recent origin for *A. treutleri* and *N. pugionifolia*.

Conclusions

This study advances our understanding of the phylogeny of the large subtribe Poinae and of the relationships of the smaller subtribes Alopecurinae, Cinninae, and Miliinae. Based primarily on our molecular results we recognize Cinninae within Poinae and subtribe Phleinae as separate from Alopecurinae. Among Poinae genera, *Arctopoa* is an old intergeneric hybrid, and *Aniselytron* and *Nicoraepoa pugionifolia* are interpreted as putative intergeneric hybrids or as resulting from more recent hybridization, with *Poa* involved in all three cases. Poinae as currently circumscribed is paraphyletic as *Poa* is not part of the main Poinae clade whereas *Alopecurinae* is nested within. Additional research is needed prior to taking further decisions on taxonomic changes in the circumscription of Poinae and the status of Alopecurinae. We plan to increase taxon sampling, particularly in Alopecurinae, Miliinae and Phleae, and sample additional DNA regions, along with an in-depth re-examination of morphological characters in a phylogenetic context, to advance our understanding of evolution in this large, taxonomically complex clade.

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Appendix 1

Species and collections of tribe Poeae subtribes Alopecurinae, Cinninae, Miliinae, Poinae, and Puccinelliinae (PPAM clade) and outgroup taxa sampled for plastid *trnT-trnL-trnF* and nrDNA ITS and ETS sequence data. Ploidy of species (where known), voucher information, and GenBank accession numbers of TLF, ITS, and ETS sequences, respectively, are provided. Sequences downloaded from GenBank are indicated by "GB", and literature reference where first published and place of origin, where known, are given. New sequences are indicated by * or, for those also included in Gillespie et al. 2009 (submitted after this paper), by ^; all other sequences are from Gillespie et al. (2007, 2008). Herbarium acronyms follow Holmgren and Holmgren (1998).

* * *

SUBTRIBE ALOPECURINAE

Alopecurus borealis Trin., 2n = 56: Canada, Nunavut, *Gillespie et al. 6576* (CAN), DQ353966, EU792345, GQ324237^. ***Alopecurus geniculatus*** L.: GB: Quintanar et al. 2007, Spain, —, DQ539571, —. ***Alopecurus gerardii*** Vill., 2n = 14: Greece, *Soreng et al. 7494* (US), EU792432, EU792344, GQ324238^. ***Alopecurus vaginatus*** (Willd.) Pall. ex Kunth: GB: Grebenstein et al. 1998, Caucasus, —, Z96920/Z96921, —; GB: Grebenstein et al. 1998, Caucasus, —, Z96922/Z96923, —. ***Beckmannia eruciformis*** (L.) Host: GB: Grebenstein, unpubl., —, AJ389163, —. ***Beckmannia syzigachne*** (Steud.) Fernald, 2n = 14, 28: USA, Wyoming, *Soreng 3513* (US), DQ353965, EU792342, GQ324255^. ***Phleum phleoides*** (L.) Karsten., 2n = 14, 28+: GB: Subbotin et al. 2004, —, AF498396, —. ***Phleum pratense*** L., 2n = (14) 42, 56, 63, 70, 84: USA, New York, cultivated, no voucher (contaminant in USDA 20228 seed acc.), DQ353964, EU792341, GQ324284^.

SUBTRIBE CINNINAE

Cinna arundinacea L., 2n = 28: USA, West Virginia, *Soreng & Olonova 7462* (US), EU792436, EU792343, GQ324260^. ***Cinna latifolia*** (Trevir. ex Göpp.) Griseb.: USA, California, *Peterson et al. 19769* (US), —, —, GQ324261^; GB: Quintanar et al. 2007, Finland, DQ631498/DQ631432, DQ539569, —.

SUBTRIBE MILIINAE

Milium effusum L., 2n = 14, 28: Canada, Quebec, *Gillespie 7422* (CAN), —, GQ324477^, GQ324273^; GB: Quintanar et al. 2007, Finland, DQ631501/DQ631435, —, —. ***Milium vernale*** M. Bieb., 2n = 14, 28: Greece, *Soreng et al. 3748* (US), DQ353963, EU792340, GQ324274^.

Aniselytron treutleri (Kunze) Soják, 2n = 42: China, *Ma 140* (KUN), GQ324394*, —, GQ324274**; China, *Soreng et al. 5229* (US), GQ324395^, EU792441, GQ324469^(1) GQ324468*(2), GQ324240^; China, *Soreng et al. 5264* (US), —, EU792373, —. ***Apera interrupta*** (L.) P. Beauv., 2n = 14: Argentina, *Peterson et al. 19173* (US), EU792439, EU792364, GQ324242^. ***Arctagrostis latifolia*** (R. Br.) Griseb., 2n = 56: Canada, Nunavut, *Archambault 145* (CAN), —, EU792352, GQ324243*; Canada, Nunavut, *Archambault 157* (CAN), EU792434, EU792353, GQ324244^; Canada, Nunavut, *Gillespie et al. 6586* (CAN), DQ353969, EU792351, GQ324245^. ***Arctophila fulva*** (Trin.) Rupr., 2n = 42: Canada, Northwest Territories, *Aiken 99-230* (CAN), DQ354058, EU792347, GQ324246^. ***Arctopoa eminens*** (J. Presl) Prob., 2n = 28, 42, 62: Canada, Labrador, *Gillespie 7010-2* (CAN), EU792446, GQ324470*, GQ324247*; Canada, Labrador, *Gillespie 7010-3* (CAN), EU792447, EU792367, GQ324248*; USA, Alaska, *Soreng & Soreng 6022* (US), DQ353977, EU792366, GQ324249*. ***Arctopoa subfastigiata*** (Trin.) Prob., 2n = 28, 42, 91, 97, Mongolia, W6 18199 W94096 (GRIN ID#), EU792448, EU792372, GQ324251*; Mongolia, W6 18244 W94096 (GRIN ID#), EU792449, EU792371, GQ324250*. ***Arctopoa tibetica*** (Stapf) Prob., 2n = 42, Russia, *Olonova 2003-07* (CAN), EU792444, GQ324471*(1) GQ324472*(2), GQ324252*; China, Tibet, *Soreng & Peterson 5481* (US), DQ353976, EU792368, GQ324253*; Kyrgyz Republic, *Soreng et al. 7626* (US), EU792445, EU792370, GQ324254*. ***Bellardiochloa polychroa*** (Trautv.) Roshev.: Turkey, *Soreng & Guney 4191* (US), —, EU792363, GQ324256^. ***Bellardiochloa variegata*** (Lam.) Kerguelen, 2n = 14: Greece, *Soreng et al. 7519-1* (US), EU792438, EU792361, GQ324257^. ***Dupontia fisheri*** R. Br., 2n = 44, 88: Canada, Nunavut, *Gillespie et al. 6589* (CAN), DQ353967, EU792346, GQ324266^; Canada, Nunavut, *Gillespie et al. 6699* (CAN), DQ353968, GQ324475^, GQ324267^. ***Hookerochloa eriopoda*** (Vickery) S.W.L. Jacobs: Australia, *Jacobs 9128* (NSW), EU792433, EU792349, GQ324270^; Australia, *Jacobs 9129* (NSW), GQ324397^, EU792350, GQ324271^. ***Hookerochloa hookeriana*** (F. Muell. ex Hook.f.) E.B. Alexeev: Australia, *Jacobs 9127* (NSW), EU792435, EU792348, GQ324272^. ***Nicoraepoa andina*** (Trin.) Soreng & L.J. Gillespie: Chile, *Soreng & Soreng 7182* (US), DQ353971, EU792354, GQ324275^. ***Nicoraepoa chonotica*** (Phil.) Soreng & L.J. Gillespie: Chile, *Soreng & Soreng 7309* (US), DQ353974, EU792355, GQ324276^. ***Nicoraepoa pugionifolia*** (Speg.) Soreng & L.J. Gillespie: Argentina, *Peterson et al. 17128* (US), GQ324398*, GQ324478*, GQ324277*; Chile, *Soreng & Soreng 7336* (US), DQ353973, EU792360, GQ324278*. ***Nicoraepoa robusta*** (Steud.) Soreng & L.J. Gillespie: Chile, *Soreng & Soreng 7358* (US), DQ353975, EU792357, GQ324279^; Chile, *Soreng & Soreng 7359 (US),* —, EU792356, GQ324280*. ***Nicoraepoa subenervis*** (Hack.) Soreng & L.J. Gillespie **subsp**. ***subenervis***: Chile, *Soreng & Soreng 7334* (US), DQ353972, EU792359, GQ324283^. ***Nicoraepoa subenervis*** **subsp**. ***spegazziniana*** (Nicora) Soreng & L.J. Gillespie: Argentina, *Peterson et al. 19186* (US), EU792443, EU792358, GQ324281^; Chile, *Soreng 7155* (US), EU792442, GQ324479^, GQ324282^. ***Poa alsodes*** A. Gray: Canada, Quebec, *Gillespie 6467* (CAN), DQ353981, EU792374, GQ324288^. ***Poa autumnalis*** Elliott, 2n = 28: USA, Maryland, *Soreng 4680* (US),

DQ353979, EU792379, GQ324294^. ***Poa badensis*** Haenke ex Willd., 2n = 14, 18—21, 28: Bulgaria, *Hajkova 2004-12* (US), GQ324402^**,** GQ324490^, GQ324295^. ***Poa chaixii*** Vill., 2n = 14: Russia, *Soreng 4677* (US), EU854590, EU792404, GQ324299^. ***Poa diaphora*** Trin., 2n = 14, 28, 42: Turkey, *Soreng & Güney 4165* (US), DQ353988, EU792400, GQ324311^. ***Poa dolosa*** Boiss. & Heldr., 2n = 14: Greece, *Soreng et al. 7495-1* (US), GQ324402^, GQ324502^, GQ324312^. ***Poa flabellata*** (Lam.) Raspail, 2n = 28: Falkland Islands, Wright 4NCD (not vouchered), DQ353982, EU792380, GQ324320^. ***Poa infirma*** Kunth, 2n = 14: Spain, *Catalan 3-2000* (UZ), GQ324427^, GQ324516^, GQ324334^. ***Poa kerguelensis*** (Hook. f.) Steud: Subantarctic Islands, Kerguelen Islands, *Hennion Gen5* (P), EU792457, EU792385, GQ324336^. ***Poa lettermanii*** Vasey, 2n = 14, USA, Colorado, *Soreng & Soreng 7434* (US), GQ324431^, GQ324521^, GQ324345^. ***Poa ligulata*** Boiss., 2n = 14, Spain, (JACA 166095), GQ324432^, GQ324522^, GQ324346^. ***Poa media*** (L.) Cav., 2n = 14, Europe, *Stoneberg SH17* (US), GQ324437^, GQ324527^, GQ324352^. ***Poa molinerii*** Balb., 2n = 14, 28: Slovakia, *Stoneberg SH13* (CAN), DQ354036/DQ354037, EU792389, GQ324354^. ***Poa remota*** Forselles, 2n = 14: Kyrgyz Republic, *Soreng et al. 7540* (US), GQ324452^, GQ324545^, GQ324372^**.** ***Poa saltuensis*** Fernald & Wiegand, 2n = 28: Canada, Ontario, *Gillespie 7043* (CAN), EU792451, EU792378, GQ324374^. ***Poa sibirica*** Roshev. **subsp.** ***sibirica***, 2n = 14: Russia, Khakasia, *Olonova 2002-1* (CAN), DQ354044/DQ354045, EU792401, GQ324376^. ***Poa supina*** Schrad., 2n = 14: USA, cultivated, *Soreng & Cayouette 5950-2* (US), DQ353984, EU792387, GQ324383^. ***Poa sylvestris*** A. Gray, 2n = 28: USA, Maryland, *Soreng 4678-3* (US), DQ353980, EU792375, GQ324384^. ***Poa trichophylla*** Heldr. & Sart. ex Boiss., 2n = 14: Greece, *Soreng et al. 7508* (US), GQ324461^, GQ324554^, GQ324386^. ***Poa wolfii*** Scribn., 2n = 28: USA, Missouri, *Soreng 5800* (US), DQ354032/DQ354033, EU792377, GQ324389^.

SUBTRIBE PUCCINELLIINAE

Catabrosa werdermannii (Pilg.) Nicora & Rúgolo: Argentina, *Peterson et al. 19371* (US), EU792431, EU792333, GQ324259^**.** ***Puccinellia vahliana*** (Leibm.) Scribn., 2n = 14: *Gillespie 5808* (CAN), Canada, Nunavut, EU854591, EU792336, GQ324285^**.**

Outgroups:

AVENEAE SUBTRIBE AVENINAE

Helictotrichon sempervirens (Vill.) Pilg., 2n = 42: *Soreng 4622* (US), USA, New York, cultivated, DQ353955, EU792325, GQ324269^**.**

POEAE SUBTRIBE LOLIINAE

Festuca baffinensis Polunin, 2n = 28: *Gillespie & Consaul 6920* (CAN), Canada, Northwest Territories, DQ353951/DQ353952, GQ324476^**,** GQ324268^**.**

POEAE SUBTRIBE AIRINAE

Deschampsia brevifolia R. Br., 2n = 26, 27, 28, 50, 52: *Gillespie & Consaul 6810b* (CAN), Canada, Northwest Territories, DQ353962, EU792328, GQ324262^