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THE UNIVERSITY OF OTTAWA

Molecular Phylogeny of North American *Festuca* Linnaeus (Poaceae)

by

Stephen James Darbyshire

Thesis submitted to
the School of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Master of Science in Biology

Université d'Ottawa/University of Ottawa
OTTAWA, ONTARIO

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ABSTRACT

Current hypotheses of the phyletic relationships in the genus *Festuca* and generic segregates, as expressed in classification systems, were tested using molecular data (DNA restriction endonuclease site variation of the chloroplast and nuclear genomes). Taxa native or introduced to North America were used as exemplars for seven subgenera: *Drymanthele*, *Subulatae*, *Subuliflorae*, *Obtusae*, *Schedonorus*, *Leucopoa* sensu lato (sections *Leucopoa* and *Breviaristatae*) and *Festuca*; and four generic segregates: *Leucopoa* sensu stricto (section *Leucopoa*), *Argillochloa*, *Vulpia* and *Lolium*. Cladistic analysis of 67 shared, polymorphic chloroplast DNA restriction sites (11 endonucleases) indicated that *Festuca* and subgeneric taxa, as circumscribed in morphologically based classifications, are polyphyletic. Phenetic analysis of 108 polymorphic chloroplast DNA restriction sites (11 endonucleases) and nuclear ribosomal DNA restriction fragment patterns (12 endonucleases) supported the results of the cladistic analysis.

Two main evolutionary lines were indicated within the genus *Festuca* as presently constructed. One contained the vast majority of the genus *Festuca* exemplars, including the subgenera *Drymanthele*, *Subulatae*, *Subuliflorae*, *Obtusae* and *Festuca*, as well as *Vulpia*, *Argillochloa* and subgenus *Leucopoa* section *Breviaristatae*. The other lineage included subgenus *Schedonorus*,

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subgenus *Leucopoa* section *Leucopoa* and the genus *Lolium*.

Analyses support the recognition of four related genera in the two lineages, *Vulpia* and *Festuca* (including subgenus *Leucopoa* section *Breviaristatae*, and the subgenera *Drymanthele*, *Subulatae*, *Subuliflorae*, *Obtusae*, and *Festuca*) in one and *Leucopoa* sensu stricto (including only section *Leucopoa*) and *Lolium* (including *Festuca* subgenus *Schedonorus*) in the other, respectively. The recognition of the monotypic generic segregate *Argillochloa* (= *Festuca dasyclada*) is not supported by the analyses.

RESUMÉ

Les hypothèses récentes sur les relations phylogéniques chez *Festuca* et les genres apparentés, telles qu'exprimées par les systèmes de classification, ont été testées au moyen de données moléculaires (variation des génomes d'ADN chloroplastique et nucléaire aux sites d'endonucléases de restriction). Des taxons indigènes et introduits en Amérique du Nord ont été utilisés comme représentants de sept sous-genres, *Drymanthele*, *Subulatae*, *Subuliflorae*, *Obtusae*, *Schedonorus*, *Leucopoa* au sens large (les sections *Leucopoa* et *Breviaristatae*) et *Festuca*, et de quatre genres apparentés, *Leucopoa* au sens strict (section *Leucopoa*), *Argillochloa*, *Vulpia* et *Lolium*. Une analyse cladistique basée sur 67 sites partagés et polymorphes de restriction d'ADN chloroplastique (11 endonucléases) a révélé que *Festuca* et les taxons infragénériques, tels que délimités par les classifications basées sur la morphologie, sont polyphylétiques. Des analyses phénétiques basées sur 108 sites polymorphes de restriction d'ADN chloroplastique (11 endonucléases) et sur des patrons de fragments de restriction d'ADN nucléaire ribosomal (12 endonucléases) ont supporté les résultats de l'analyse cladistique.

Deux directions évolutives principales sont présentées chez le genre *Festuca* tel qu'on le reconnaît présentement. L'une comprend la grande majorité des représentants du genre *Festuca*, incluant les sous-genres *Drymanthele*, *Subulatae*, *Subuliflorae*,

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Obtusae et *Festuca*, ainsi que *Vulpia*, *Argillochloa* et la section *Breviaristatae* du sous-genre *Leucopoa*. L'autre lignée comprend le sous-genre *Schedonorus*, la section *Leucopoa* du sous-genre *Leucopoa* et le genre *Lolium*. Nos analyses soutiennent la reconnaissance de quatre genres dans les deux lignées: *Vulpia* et *Festuca* (incluant la section *Breviaristatae* du sous-genre *Leucopoa*, et les sous-genres *Drymanthele*, *Subulatae*, *Subuliflorae*, *Obtusae*, et *Festuca*) dans l'une; *Leucopoa* au sens strict (la section *Leucopoa* seulement) et *Lolium* (incluant *Festuca* sous-genre *Schedonorus*) dans l'autre. La reconnaissance du genre monotypique apparenté *Argillochloa* n'est pas soutenue par nos analyses.

**Nubes et inania captant,
qui generibus solum student,
nec speciebus simul cunctis.**

F. J. Ruprecht
ex Q. Horatius Flaccus

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ABBREVIATIONS

Ci	Curie(s) (i.e. 3.7×10^{10} radioactive disintegrations per second)
cpDNA	DNA of the chloroplast genome
dH ₂ O	distilled water
DNA	deoxyribonucleic acid
ETS	external transcribed spacer region at the 5' end of the 18S rDNA subunit
g, mg, μ g	gram(s), milligram(s), microgram(s)
IGS	intergenic spacer region between nuclear genes for subunits of 18S-26S rRNA
IR	inverted repeat region of the chloroplast genome
ITS	internal transcribed spacer region between nuclear genes for rRNA subunits 18S and 5.8S, and, 5.8S and 26S
kb	kilo-base pairs (i.e. 1000 nucleotide pairs)
l, ml, μ l	litre(s), millilitre(s), microlitre(s)
LSC	large single copy region of the chloroplast genome
M, mM	molar, millimolar
OTU	operational taxonomic unit
rDNA	tandem arrayed multigene family for the ribosomal RNA subunits 18S, 5.8S and 26S
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SSC	small single copy region of the chloroplast genome
§	taxonomic rank of section (Greuter et al. 1988)
°C	degrees Centigrade
2n =	somatic chromosome number

1. INTRODUCTION

1.1. The genus *Festuca*

The genus *Festuca* L. of the grass tribe *Poeae* (= *Festuceae* Dumortier) inhabits arctic, temperate and alpine regions of all continents, except Antarctica, with the vast majority of species occurring in the holarctic zone. Species estimates vary greatly depending on generic and specific concepts, but anywhere from 170 (Watson and Dallwitz 1988) to 500 (Tzvelev 1989) species are considered by different authors, making it one of the largest genera in the grass family. Species of local or widespread economic importance, mostly as forage, occur throughout much of the genus range.

Classification of *Festuca* began in *Species Plantarum* where Linnaeus (1753) used the name to refer to a rather nondescript group of grasses with paniculate inflorescences and multi-flowered spikelets. Some of Linnaeus' *Festuca* species have since been placed in separate genera and even tribes; for example: *Danthonia* DC. (*Arundineae* Dumortier), *Glyceria* R. Br. (*Meliceae* Reichenbach), *Kengia* Packer (*Eragrostideae* Stapf) and *Rostraria* Trinius (*Aveneae* Dumortier). One species currently placed in *Festuca*, *Festuca gigantea* (L.) Villars, was originally placed by Linnaeus in *Bromus* L. For the most part the modern taxonomic history of *Festuca* has been of constriction with various groups

being recognized as distinct and given generic status. Taxa such as *Nardurus* Reichenbach, *Scleropoa* Beauv., *Catapodium* Link, *Micropyrum* (Gaud.) Link, *Puccinellia* Parl. and *Scolochloa* Link are now uncontested as separate genera. Others, such as *Vulpia* C. C. Gmelin (*Festuca* subgenus *Vulpia* (C. C. Gmelin) Hackel), *Drymochloa* Holub (*Festuca* subgenus *Drymanthele* Krecz. & Bobrov), *Hellerochloa* Rauschert (*Festuca* subgenus *Helleria* Alexeev), *Schedonorus* Beauv. (*Festuca* subgenus *Schedonorus* (Beauv.) Peterm.), *Argillochloa* W. A. Weber (*Festuca* (subgenus *Festuca*) *dasyclada* (Hackel) W. A. Weber) and *Leucopoa* Grisebach (*Festuca* subgenus *Leucopoa* (Grisebach) Hackel section *Leucopoa*), are sometimes recognized as genera and sometimes as infrageneric taxa. Table 1 shows the major classification systems proposed by various authors with similar systems combined in columns. The treatment used here is given in the left column. The comment of Beal (1896) that *Festuca* is a genus "as to whose limits botanists are the least agreed" is still largely appropriate given the varying treatments of recent authors (cf. Alexeev 1972-1990, Clayton and Renvoize 1986, Kerguelen and Plonka 1989, MacFarlane 1986, Markgraf-Dannenberg 1985, Tzvelev 1989, Watson and Dallwitz 1988). Parallel and convergent evolution of morphology have been major features in the evolution of the genus and tribe so that the traditional criteria of the alpha taxonomist have tended to obscure natural relationships. In spite of the considerable effort made in identifying parallelism in morphological traits and classifying these economically important grasses, the genus

Table 1. Comparison of the subgeneric classification of *Festuca* and allies, and the tribal placement of *Lolium* (in parentheses) by various authors. Only taxa represented in this study with a species exemplar (Tables 2 and 3) are included. Genera are in large bold type; subgenera are in small bold type; sections (§) are in small type.

A dash indicates that the taxon was not included in the cited classification(s).

A blank indicates the taxon was not ranked within *Festuca*, an unrecognized taxon (includes those to which note b applies), or that subtaxonomic divisions were divided in rank.

? - Position of taxon not explicitly ranked by author(s).

a - These authors use the earlier name, subgenus *Montanae* (Hackel) Nyman, in place of subgenus *Drymanthele*. It is not clear that they include the New World species of sections *Aristulatae*, *Texanae*, *Banksia* and *Ruprechtia* in their concepts of this subgenus.

b - Subgeneric taxa are not given.

c - *Festuca gigantea* (of section *Platynia*) was referred to the section *Bovinae*. Hackel later (1889) recognized several subgenera within *Festuca* including *Eufestuca* and *Vulpia*.

d - Other subgenera, now considered as separate genera and not listed here, were recognized as part of *Festuca*. *Festuca kingii* was referred to the genus *Poa*. *Festuca gigantea* was referred to the genus *Bromus*.

e - Hitchcock treated the subgenera listed as sections, except *Hesperochloa* where he followed Rydberg in considering it as a separate genus.

f - The monotypic genus *Argillochloa* is not recognized.

g - Holub did not consider the New World species placed in *Drymanthele* by Alexeev (see note a) but referred only *Festuca altissima* All. and *Festuca drymeja* Mert. & Koch to the genus *Drymochloa*.

h - Subgenus *Schedonorus* is further divided into series *Aphanoneuræ* V. Krecz. & Bobr. (*Festuca arundinacea*) and *Phanoneuræ* V. Krecz. & Bobr. (*Festuca pratensis*).

i - If *Festuca kingii* is considered distinct from the Asian dioecious *Leucopoa* the name *Hesperochloa* may be used. Most authors consider it to be monophyletic and congeneric with Old World species and thus the name *Leucopoa* has priority.

	Alexeev (1977-1987) Clayton & Renvoize (1986) Kerguelen & Plonka (1989) ^a Aiken & Darbyshire (1990)	Roemer & Schultes (1877) ^b	Hackel (1882) ^c Beal (1896) ^d	Piper (1906) Saint Yves (1925) Hitchcock (1950) ^e	Rydberg (1932) ^b	Kreczetowicz & Bobrov (1934) Bar (1970)	Tzelev (1971, 1976)	Gould & Shaw ^{h,i} (1983) Weber (1988, 1990) ^b	Holub (1984) ^{b,o}
Festuca			Festuca	Festuca			Festuca	Festuca	
Drymanthele		Schedonorus	Bovinae		-	Drymanthele	Drymanthele	-	Drymochloa
Ruprechtia		-	-	Eufestuca	-	-	Schedonorus & Subulatae	-	
Subulatae		-	Bovinae	Eufestuca	-	Drymonaetes	Schedonorus & Subulatae	-	
Subuliflorae		-	Bovinae	Eufestuca	-	-	Schedonorus & Subulatae (?)	-	
Obtusae		Schedonorus	Bovinae	Eufestuca	-	-	Schedonorus & Subulatae (?)	-	
Schedonorus		Schedonorus	Bovinae	Eufestuca			Schedonorus		Schedonorus
Schedonorus		Schedonorus	Bovinae			Schedonorus ^h	Bovinae		
Platynkia		Bromus	-		-	Drymonaetes	Platynkia		
Leucopoa		-					Leucopoa		
Leucopoa		-	Bovinae	Hesperochloa ⁱ	Hesperochloa	Leucopoa	Leucopoa	Leucopoa	
Breviaristatae		-	Ovinae	Eufestuca	Festuca	Festuca Phaeochloa	Breviaristatae	Festuca	
Festuca		Festuca	Bovinae & Ovinae	Festuca	Festuca	Eufestuca	Festuca	Festuca & Argillochloa	Festuca
Vulpia		Festuca	Vulpia	Vulpia	Festuca	Vulpia	Vulpia	Vulpia	Vulpia
Lolium (Poae)		Lolium (-)	Lolium (Triticeae)	Lolium (Triticeae)	Lolium (Triticeae)	Lolium (Poae)	Lolium (Poae)	Lolium (Poae)	Lolium (Poae)

remains a heterogenous assemblage of poorly known phylogeny.

Present day circumscriptions of *Festuca* and its allies are largely based on the phylogenetic concepts of E. Hackel (1882), summarized for the Old World taxa in Figure 1. The genus *Festuca* was seen as intersecting with *Poa* and six sections were recognized. The sections *Montanae* Hackel (corresponding to subgenus *Drymanthele* of the authors in the left column of Table 1), *Bovinae* Fr. (corresponding to subgenus *Schedonorus*) and *Ovinae* Fr. (corresponding to subgenus *Festuca*) are present in North America and included in this study. Sections *Scariosae* Hackel (corresponding to subgenus *Schedonorus* section *Scariosae*), *Variae* Hackel (corresponding to subgenus *Festuca* section *Variae*), and *Subbulbosae* Nyman (corresponding to subgenus *Festuca* section *Subbulbosae*) are exclusively Palaearctic in distribution. Subsections named *Intravaginales* and *Extravaginales* were recognized in both subgenus *Festuca* (§ *Ovinae*) and § *Variae*.

Hackel (1882) interpreted section *Montanae* (subgenus *Drymanthele*) as most ancient, sharing more primitive morphological characters with species of the genus *Poa* L. than the more recently derived section *Ovinae* Fr. (subgenus *Festuca*) and having none of the latter's derived characters. Primitive to derived transitions of wide and flat leaves to narrow and folded leaves, rhizomatous habit to caespitose habit and extravaginal to intravaginal shoot production in his various groups were seen as adaptive trends to

Figure 1. Diagrammatic representation of *Festuca* phylogeny in the Hackelian classification system (after Levitsky and Kuzmina 1927) considering only the Old World taxa. The range of reported ploidy levels of each taxon is given.

Poa
Festuca

Montanae Scariosae Bovinae Varias Subbulbosae

14-42

14-28

14-70

14

14



Ovinae

Extravaginales

14-70



Intravaginales

14-70

increasing xerophily. These modifications were perceived as adaptive in reducing and sheltering laminar transpirational surfaces and protecting emergent shoots from desiccation.

Section *Ovinae* (subgenus *Festuca*) was considered to have been derived from section *Bovinae* (subgenus *Schedonorus*). *Festuca rubra* L. (subgenus *Festuca*), with its generally flat leaves, rhizomatous habit and mixed shoot production, was considered to be an intermediate form between sections *Bovinae* (subgenus *Schedonorus*) and section *Ovinae* (subgenus *Festuca*). Some of the major groups (i.e. sections *Montanae* and *Scariosae* Hackel) were interpreted as possibly paraphyletically derived from the genus *Poa*.

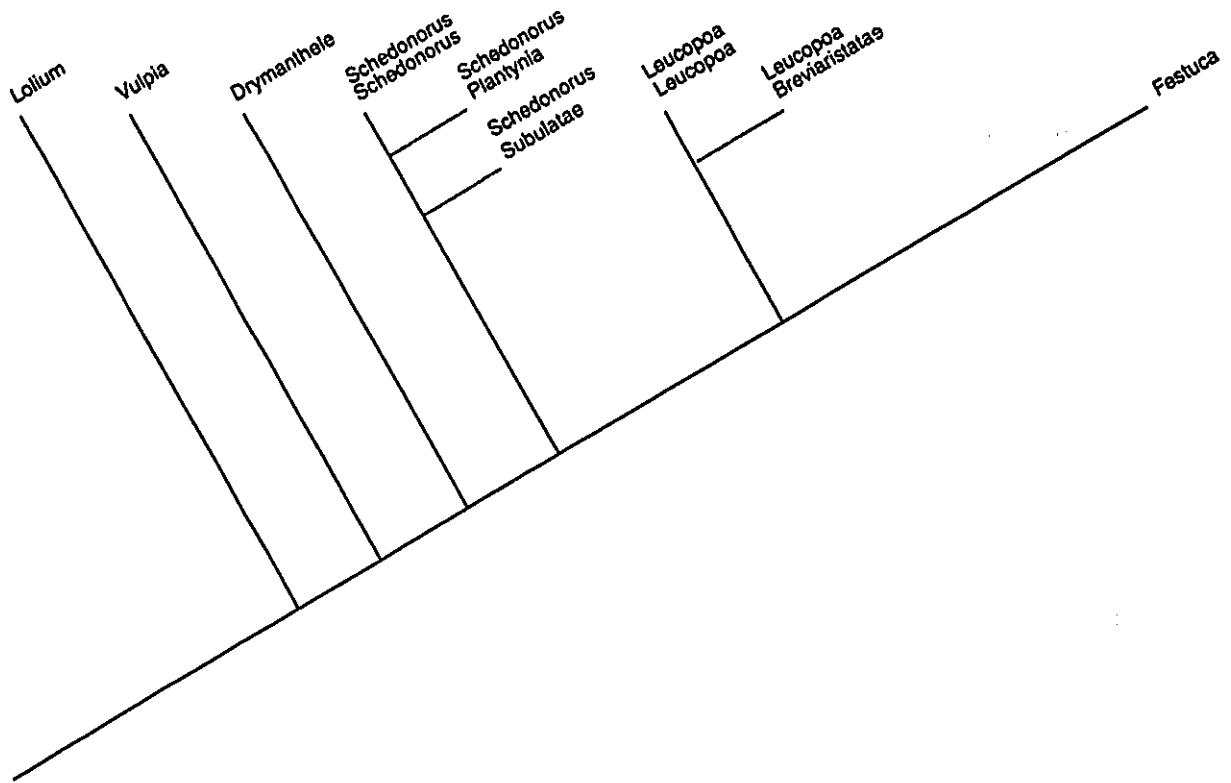
The chromosome studies of Levitsky and Kuzmina (1927) added diploidy to the list of primitive characters and showed that several of the Hackelian sections contained polyploid series of taxa (Figure 1) based on the diploid number of $2n = 14$.

Aneuploids are not reported in *Festuca* with the exception of the non-sexual species *Festuca prolifera* Fernald (Bowden 1960, Löve and Löve 1975). Polyploidization has been an important evolutionary trend in *Festuca* and about 75% of species are estimated to be polyploid (Malik and Thomas 1966). Chromosome numbers in ploidy levels ranging from diploid to decaploid have been detected within subgenus *Schedonorus* and within series *Intravaginales* Hackel and series *Extravaginales* Hackel of

subgenus *Festuca* (see compilations of Alexeev et al. 1987a,b, 1988, 1990). Hackel (1882) and others placed a great many entities, now recognized as species, in a complex network of subspecific taxa within *Festuca ovina* L. These species, referred to here as the 'ovina complex', having only intravaginal shoot production, long prophylls, narrow, tightly folded leaves with adaxial ribs and (mostly) glabrous ovaries, supposedly represent the most advanced species in *Festuca*. A similar group of poorly defined species referred to the *Extravaginales*, the 'rubra complex', is distinguished by its mixed shoot production and short prophylls.

Many recent authors have tended to follow the reputedly phylogenetic classifications of the genus by N. N. Tzvelev and E. B. Alexeev. Working mainly with material from the U.S.S.R., Tzvelev (1971) developed the phylogenetic hypotheses for the genus which led to his later treatment of the species in the Soviet Union (Tzvelev 1976). Developed as a synthesis of morphological, cytological and palaeogeographical data, the view of Tzvelev was of a strictly monophyletic genus and, unlike Hackel, he saw the major groups (subgenera to Tzvelev, sections to Hackel) as parallel lineages rather than deriving one from another (i.e. section *Ovinae* from section *Bovinae*). His interpretation of the genus is represented in Figure 2. Applying his concepts to the North American species four subgenera are recognized, *Drymanthele*, *Schedonorus*, *Leucopoa* and *Festuca*, along

Figure 2. The phylogenetic classification of North American subgenera and sections of *Festuca* after Tzvelev (1971, 1976). Four subgenera are recognized: *Drymanthele*, *Schedonorus*, *Leucopoa* and *Festuca*. Three sections, *Schedonorus*, *Platynia* and *Subulatae*, are recognized in subgenus *Schedonorus*, and two sections, *Leucopoa* and *Breviaristatae*, are recognized in subgenus *Leucopoa*.

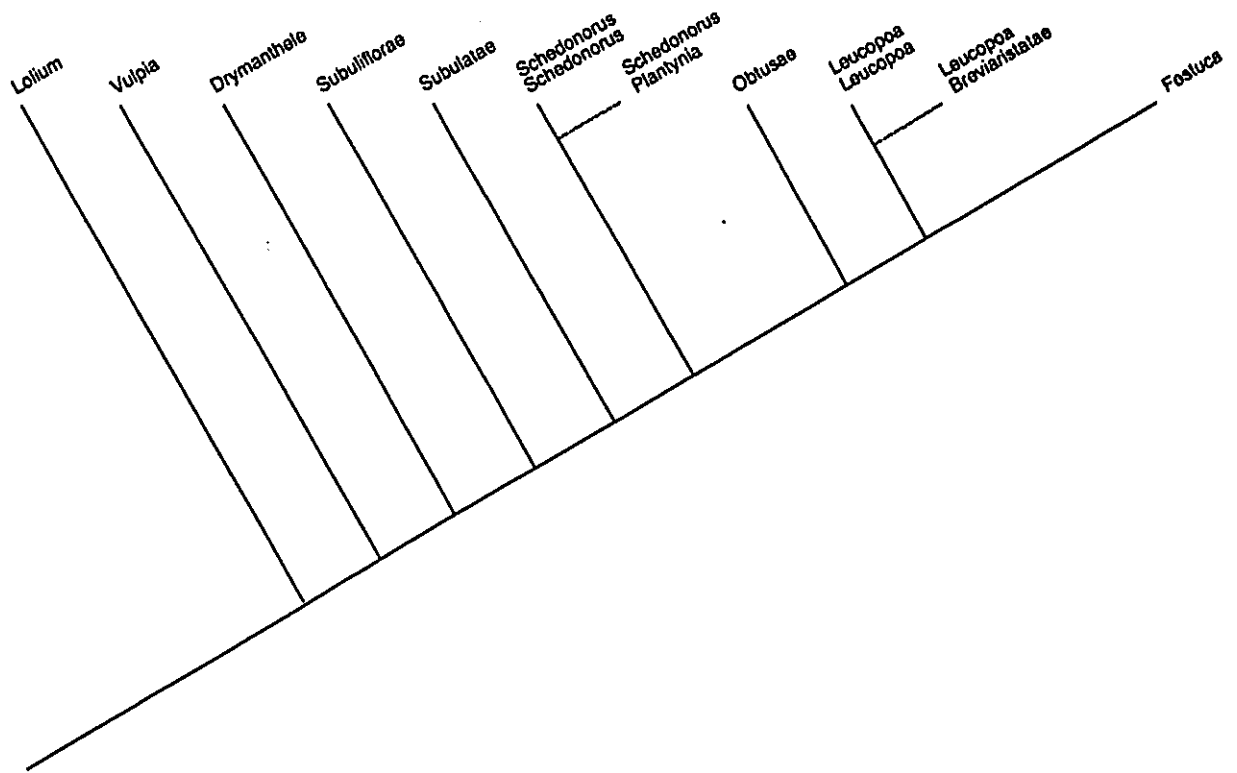


with three sections in *Schedonorus* (*Schedonorus*, *Plantynia* (Dumortier) Tzvelev and *Subulatae* Tzvelev) and two sections in *Leucopoa* (*Leucopoa* and *Breviaristatae* Krivot.).

In an extensive series of papers Alexeev (1972-1990) (summarized in Appendix A) largely followed Tzvelev's concepts but recognized 12 subgenera and 18 sections on an almost global basis, based on a comprehensive analysis of morphology and chromosome numbers. His phylogenetic hypotheses and classification of *Festuca* were similar to those of Tzvelev, although he considered subgenus *Schedonorus* section *Subulatae* Tzvelev a polyphyletic assemblage including (among the North American taxa) subgenera *Schedonorus*, *Subulatae* (Tzvelev) Alexeev, *Obtusae* Alexeev and *Subuliflorae* Alexeev (Figure 3).

The wide distribution of a large, non-tropical genus with many scattered endemics or narrow-ranged taxa suggested to Tzvelev (1971) that generic prototype(s) of *Festuca* must have been present during the mid to upper Cretaceous, prior to the beginning of continental breakup. He suggested that a splitting of weakly differentiated groups had occurred by the beginning of the Palaeocene and that the isolated groups followed separate but parallel radiation on the different continental masses. This would place the beginning of the fescue lineage near the origins of the grass tribe as presently known from the fossil record (Crepet and Feldman 1991). The hypothesis is, however,

Figure 3. The phylogenetic classification of North American subgenera and sections of *Festuca* after Alexeev (1972-1990). Seven subgenera are recognized: *Drymanthele*, *Subuliflorae*, *Subulatae*, *Schedonorus*, *Obtusae*, *Leucopoa* and *Festuca*. Two sections are recognized in each of the two subgenera *Schedonorus* (sections *Schedonorus* and *Platynia*) and *Leucopoa* (sections *Leucopoa* and *Breviaristatae*).



consistent with a postulated rapid and extensive adaptive radiation of the grasses early in their evolution (Stebbins 1982). The restricted subgenera (and generic segregates) of Alexeev would seem to have arisen after continental breakup with the possible exceptions of the subgenera *Festuca* (present throughout the range of the genus), *Drymanthele* (montane areas of central Eurasia and Central and South America) and *Leucopoa* (almost holarctic and southern Africa).

Prototype(s) of *Festuca*, now extinct, are thought to have arisen as montane grasses (Saint Yves 1930) of mesic meadows or forest edges (Tzvelev 1971) from bamboo-like ancestors (Tzvelev 1971, Alexeev 1977a). Primitive forms are not generally found in open habitats or plains. The presence of diploids and polyploid series in many infrageneric taxa suggests that considerable morphological radiation occurred among prototypes prior to parallel polyploidy events (Levitsky and Kuzmina 1927). Subsequent adaptation in infrageneric and species groups has followed environmental trends of increasing xerophily and cryophily. Mountain building events at the end of the Cretaceous combined with continental breakup diversified the climate and habitats of the new land masses. More habitats were generated and climatic shifts increased to form extensive alpine, rain-shadow and arctic regions where the genus is now most abundantly represented.

The present ecogeography of North America was largely shaped by these events and supports six native subgenera and an additional introduced one. Table 1 shows the main proposed infrageneric arrangements and segregation of these taxa. Their arrangement is, to a greater or lesser extent, a synthesis of compatible treatments by authors under which they are listed. The subgenera and sections have been variously aligned based mostly on similarities of leaf and shoot structure, ovary pubescence and/or glume texture.

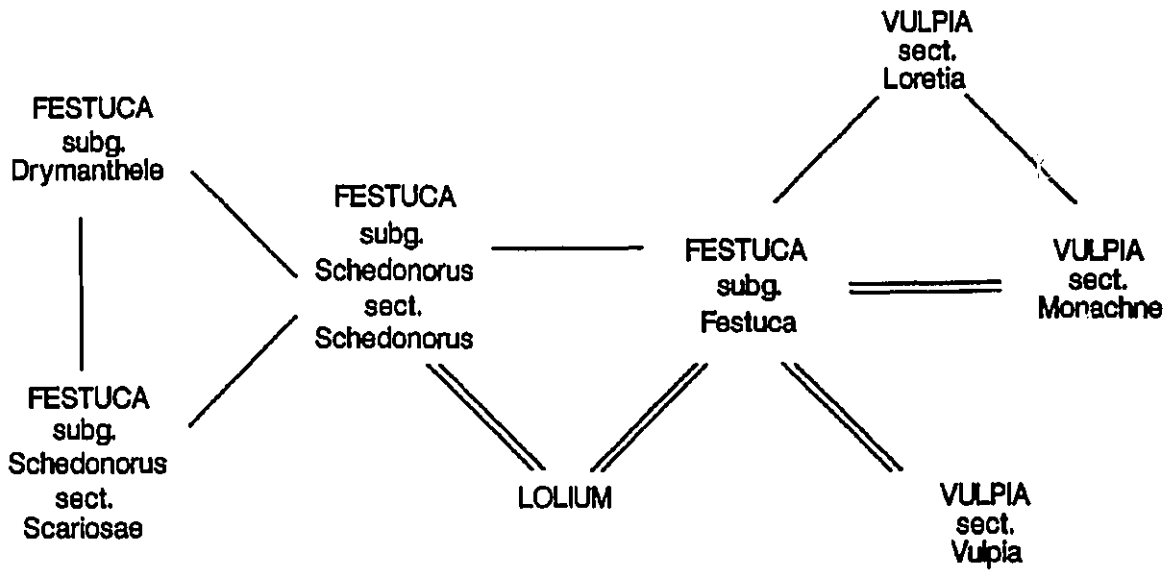
1.2. Generic segregates of *Festuca*

1.2.1. *Lolium* - The relationship between the genera *Festuca* and *Lolium* L. has long been an interesting classification problem. Described in 1753 by Linnaeus, *Lolium* is readily distinguished from *Festuca* by its racemic inflorescence and lateral spikelets with a single glume. This outward appearance suggests the spike-like inflorescences of the tribe *Triticeae* Dumortier, where *Lolium* had, in earlier treatments, often been placed (Table 1). Nevski (1934) was the first to place *Lolium* in the *Poeae* rather than with the *Triticeae*. Information from other diverse non-morphological sources, such as hybridization and cytology (e.g. Avdulov 1931, Peto 1933, Jenkin 1933, 1959) oligosaccharides and water-soluble polysaccharides in caryopses (MacLeod and McCorquodale 1958), fructosan structure (reviewed by Smith 1968, 1973), seed protein electrophoresis (Bulińska-Rodomska and Lester

1988, Butkute and Konarev 1982) and serology (Butkute and Konarev 1980, 1982, Smith 1969, Watson and Knox 1976), chloroplast DNA (cpDNA) restriction endonuclease site patterns (Lehv  slaiho et al. 1987, Soreng et al. 1990), cpDNA reassociation (Hilu and Johnson 1990) and thermal denaturation of genomic DNA (King and Ingrouille 1987), has supported the placement of *Lolium* in *Poeae*.

Although *×Festulolium loliaceum* (Hudson) P. Fournier (the hybrid between *Festuca pratensis* Hudson and *Lolium perenne* L.) was suspected as a hybrid as early as 1796 (see Jenkin 1933), the significance of recurrent spontaneous hybridization between all species of *Festuca* subgenus *Schedonorus* and some species of *Lolium* was not appreciated or incorporated into grass classification until the present century. Extensive studies of hybridization (summarized in Figure 4) and chromosome behaviour (summarized by Jenkin 1933, 1955, 1959) have led many taxonomists and cytogeneticists to suggest that the genera *Festuca* and *Lolium* should be combined (e.g. Buli  nska-Rodomska and Lester 1988, Jauhar 1975, 1976, Knobloch 1963, L  ve 1963, Stebbins 1956, Terrell 1966, Tutin 1956, Ullmann 1936). Differences in chromosome structure between *Festuca* (including subgenus *Schedonorus*) and the more homogeneous structure of *Lolium* has been argued as reason for not amalgamating the genera (Jenkin 1933, Malik and Thomas 1966).

Figure 4. The occurrence of hybrids between *Festuca*, *Vulpia* and *Lolium*. Double lines connecting taxa indicate spontaneous hybridization and single lines indicate artificial hybridization. After Barker and Stace (1986).



1.2.2. *Vulpia* - The genus *Vulpia* is a small, holarctic genus of about 22 species (Clayton and Renvoize 1986). Since its description and separation from the Linnaean genus *Festuca* in 1805 (Gmelin 1805) the taxon has been recognized as very close to *Festuca* and frequently lumped with it (Table 1). Hackel at first (e.g. Hackel 1882) considered *Vulpia* as a separate genus, as do most authors at the present time, but later (e.g. Hackel 1889) gave it the rank of subgenus within *Festuca*. Early in this century *Vulpia* was frequently treated as a subgenus (e.g. Saint Yves 1922-1931) or section (e.g. Piper 1906, Hitchcock 1950) of *Festuca*. Six main characters are used to separate the two genera (longevity, extent of panicle branching, cleistogamy/chasmogamy, relative length of glumes, attenuate/acuminate lemmas and width of caryopsis), but there is overlap in all these characters. The development of the annual habit, attenuate lemmas with long awns and trend to cleistogamy in this polyploid group has clearly marked *Vulpia* as a distinct entity (Cotton and Stace 1976, 1977, Stace and Cotton 1977). Recognized as genera by some authors (e.g. Tzvelev 1989), the monotypic and diploid *Nardurus* (*Vulpia* section *Apalochloa* (Dumortier) Stace) and *Ctenopsis* De Not. (*Vulpia* section *Ctenopsis* (De Not.) Boissier) are included as sections of the genus *Vulpia* by Clayton and Renvoize (1986) along with section *Vulpia* (containing diploid, tetraploid and hexaploid species), section *Monachne* Dumortier (containing a diploid and a tetraploid species), section *Loretia* (Duval-Jouve) Boissier (containing diploids and several species of unknown ploidy) and

the monotypic section *Spirachne* (Hackel) Boissier (diploid). Section *Loretia* most closely approaches *Festuca* in perennial habit, chasmogamy and seed protein profiles (Bulińska-Rodomska and Lester 1988) and all species are diploid (Cotton and Stace 1976).

As well as artificially produced intergeneric hybrids (Barker and Stace 1982, 1984, 1986), naturally occurring hybrids between several species of *Vulpia* and hexaploid and octoploid taxa of *Festuca rubra* L. (sensu lato) are known and described as nothospecies of *xFestulpia* Melderis ex Stace & Cotton (Stace and Cotton 1974, Ainscough et al. 1986) (Figure 4). Hybridization between genera within tribes of the grass family is common (e.g. Dewey 1984, Knobloch 1968, Watson 1990) and 15-18% of genera in the Poaceae are known to hybridize (Darbyshire et al. 1992, Watson 1990).

1.2.3. Other genera - Other generic segregates which have been proposed, but not accepted by Alexeev (1972-1990), include *Leucopoa*, *Drymochloa* (= *Festuca* subgenus *Drymanthele*) and *Argillochloa*. The genus *Leucopoa* includes only the section *Leucopoa* and, unlike the rest of the *Festuca* allies, is a largely dioecious group. Most species are from central Asia, but one, *Festuca kingii* (S. Watson) Cassidy, occurs in central North America. Holub (1984) advocated splitting up *Festuca* and coined the generic name *Drymochloa* for Old World species of subgenus

Drymanthele (section *Drymanthele*), although he did not consider the New World species of the subgenus that Alexeev put in the sections *Aristulatae* Alexeev, *Banksia* Alexeev, *Ruprechtia* Alexeev and *Texanae* Alexeev. He advocated the separation of this group from *Festuca* based on the primitive nature of the membranous, unawned lemmas; pubescent ovary; and broad, flat, more or less ribless, leaves with convolute vernation and limited sclerenchyma. Another segregate is the monotypic genus *Argillochloa* W. A. Weber which is principally distinguished by the stiff, divaricate panicle branching and reduced number of florets per spikelet (Weber 1984). The only species, *Festuca dasyclada* Hackel (*Argillochloa dasyclada* (Hackel) W. A. Weber), is a highly localized endemic of oil shales in southern Utah and western Colorado and placed by most authors in subgenus *Festuca*.

1.3. DNA systematics

1.3.1. Chloroplast DNA - Restriction endonuclease site variation in the chloroplast DNA molecule has proved useful in the examination of plant phylogenetic relationships, particularly at rankings above the species level (Palmer et al. 1988, Clegg and Zurawski 1992). The conservative nature of the structure and sequence of this functionally important genome permits comparison of structural and regulatory sequences of relatively distant relatives (Palmer 1987, Palmer et al. 1988, Zurawski and Clegg 1987). Work on the grasses has focused mostly on systematic

problems at the generic level (reviewed by Hilu 1987) where reassociation studies have shown an 80% similarity in sequences among related taxa (Hilu and Johnson 1990).

In mature leaf tissue there is a high density of identical cpDNA molecules, 20-200 per chloroplast and 20-200 chloroplasts per cell (Palmer 1987, Palmer et al. 1988). The circular molecule is divided into four structural regions (Figure 5) with a large single copy region (LSC) separated from a small single copy region (SSC) by repeated regions that are inverted with respect to each other (IR). Size varies among photosynthetic vascular plants from 120 to 217 kilobases (kb) with most of the length variation occurring in the IR regions (Palmer 1987, Palmer et al. 1988). Estimates of cpDNA size in grasses related to *Festuca* are all close to 135 kb; 135 kb \pm 1 in *Leucopoa*, *Festuca* and other *Poaceae* (Soreng et al. 1990), 135 kb in *Triticum* (Bowman et al. 1981), 135 kb in *Pennisetum americanum* (L.) Leeke (Thomas et al. 1984) and 133 kb in *Hordeum* (Poulsen 1983). About 200 densely packed genes (Palmer et al. 1988) code chloroplast products mainly for photosynthetic and transcription-translation functions, including about 50 polypeptides involved in photosynthesis, all stable rRNA's and tRNA's, about 20 ribosomal proteins, 4 genes for RNA polymerase subunits and initiation factor 1 (Palmer 1987, Palmer et al. 1988).

Using appropriate enzymes large numbers of independent characters

Figure 5. Chloroplast DNA molecule indicating positions of the large single copy region (LSC), small single copy region (SSC), inverted repeat regions (IR), ribulose-1,5-bisphosphate carboxylase (rbcL) and chloroplast rRNA genes (rRNA), and *Petunia* cpDNA clones used in this study. Shaded clones (S6, P18) were unavailable. After Sytsma and Gottlieb (1986).

can be detected which are easily scored (e.g. Bremer 1991), are largely unaffected by the phenotype and are selectively neutral. Homoplasy (i.e. convergence and parallelism) is considered to be very low at specific and generic levels (Palmer et al. 1988, Jansen et al. 1990), although significant levels have been detected among tribes of the subfamily *Pooideae* in the cladograms of Soreng et al. (1990) where the consistency indexes was about 0.65.

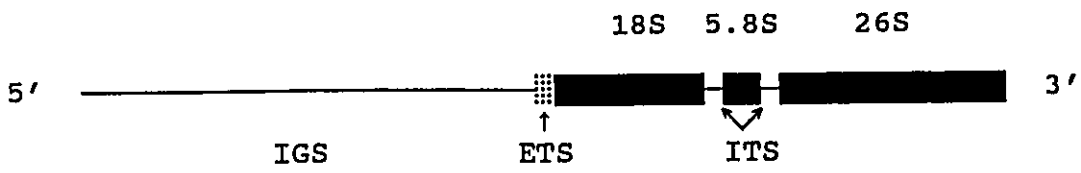
1.3.2. Nuclear Ribosomal DNA - The genes for the ribosomal RNA subunits 18S, 5.8S and 26S are transcribed as a single unit and are arranged in tandem array multigene families localized in nuclear organizer regions (NOR) at one or more chromosomal loci (reviewed by Appels and Baum 1992, Appels and Honeycutt 1986, Dvořák 1990, Hamby and Zimmer 1992, Jorgensen and Cluster 1988). Figure 6 shows the generalized structure of the repeated unit. Higher plants contain 2,000 to 20,000 copies of the rDNA repeat unit per cell. Cell copy number may vary as much as four fold between individuals within a species. Separating each 26S and 18S subunit in the tandem arrays is a non-transcribed intergenic spacer (IGS) varying in length from about one to eight kb, which itself contains 100-200 base pair sequence tandem repeats. Greater sequence and length variation is found in the non-transcribed IGS, rather than the highly conserved ribosomal subunit regions of the rDNA repeat unit. Some differences in copy length within individuals occur, but molecular drive

Figure 6. Nuclear ribosomal DNA repeat unit showing 18S, 5.8S and 26S subunits, intergenic spacer (IGS), external transcribed spacer (ETS) and internal transcribed spacers (ITS). After Jorgensen and Cluster (1988). a: tandem array. b: single unit. After Hamby and Zimmer (1992).

a



b



mechanisms exist which effect a concerted evolution tending to produce repeat unit uniformity. The latter mechanisms are still poorly understood. When more than one chromosomal locus is present the units are generally quite homogenous within each locus. Each ribosomal subunit is separated by a transcribed region of several hundred base pairs called the internal transcribed spacers (ITS). A similar sized external transcribed spacer (ETS) occurs at the 5' end of the 18S subunit. These transcribed regions are removed in the maturation of rRNA.

All classes of rDNA variation, length, nucleotide sequence, nucleotide modification (i.e. methylation), and copy number, may occur within an individual's rDNA array (Jorgensen and Cluster 1988). Nucleotide sequence divergence, at least in the conserved rRNA subunit regions, is minimal among closely related species (Jorgensen and Cluster 1988) and is more useful than other types of variation in the comparison of distant relationships.

1.4. Phylogeny and classification

In organisms of reduced structural organization such as grasses, morphological characters are few in number and highly susceptible to convergence or parallelism. Additionally, relatively simple but gross structural rearrangements (e.g. reduction of a paniculate inflorescence in *Festuca* and *Vulpia* to a raceme in *Lolium* and *Nardurus*) can influence a suite of obvious characters

strongly biasing analysis (Bulińska-Rodomska and Lester 1988, Hilu and Wright 1982). The grass family has proved one of the most difficult for determining directional trends in evolution (Stebbins 1982). Phylogenetic reconstruction of *Festuca* is therefore of interest for developing a 'natural' classification of the genus with its allies and in the understanding of its evolutionary relationships. Because of the extensive morphological and cytogenetic studies, phylogenies based on molecular data will provide an independent data set useful in determining evolutionary relationships and allowing an assessment of morphological and cytological evolutionary trends.

The purpose of this study was to use DNA restriction site/fragment data to test the phylogenetic relationships of North American *Festuca* and related genera as proposed in taxonomic classifications in general (Table 1) and in particular the most recent and most comprehensive phylogenetic hypotheses of E. B. Alexeev (Figure 3; Appendix A). Subsequent use of names follows the taxonomy of Alexeev, unless indicated otherwise.

2. MATERIALS AND METHODS

2.1. Plant material

Species selected to represent genera and infrageneric taxa are given in Table 2 with their code and somatic chromosome number. Chromosome numbers used are taken from Alexeev (1972-1990), Cotton and Stace (1976), Frederiksen (1982) and Tzvelev (1976). Nomenclatural details of their classification are given in Table 3. Selection of exemplars was made to include as many species of low ploidy level (preferably diploids if known) and nomenclatural types. Plant material was collected on a major collecting trip to western North America and several minor local trips. Seed was obtained from herbarium specimens, Plant Gene Resources of Canada and colleagues. Plant material was grown from a single seed or ramet of field collected material. One individual from each of two populations of *Festuca hallii* (Vasey) Piper, *Festuca subverticillata* (Persoon) Alexeev, and *Vulpia myuros* (L.) C. C. Gmelin and three individuals from two populations of *Festuca kingii* were examined (Appendix B). Plants were grown in a greenhouse in Ottawa with daily watering and ambient light supplemented with artificial lighting to give 16:8 L:D cycle. Identification of exemplars was confirmed using keys and descriptions by Aiken and Darbyshire (1990), Alexeev (1980d, 1982a, 1984e, 1985d) and Hitchcock (1951). Specimens for preservation in herbaria were prepared at the time of field

Table 2. List of exemplar taxa in phylogenetic order of Alexeev, with codes and reported chromosome numbers (see text for references). a: Species of the *Festuca ovina* complex; b: Other cytotypes reported, but rare; c: Aiken et al. (1988); d: Populations used are $2n = 28$ (Frederiksen 1982).

TAXON	EXEMPLAR	CODE	$2n =$
FESTUCA			
subgenus <i>Drymanthele</i> section <i>Ruprechtia</i>	<i>F. amplissima</i>	fm	28
subgenus <i>Subulatae</i>	<i>F. subulata</i>	sl	28
subgenus <i>Subuliflorae</i>	<i>F. subuliflorae</i>	sf	28
subgenus <i>Schedonorus</i> section <i>Schedonorus</i>	<i>F. pratensis</i> <i>F. arundinacea</i>	fp fa	14 ^b 42 ^b
section <i>Plantynia</i>	<i>F. gigantea</i>	fg	28, 42
subgenus <i>Obtusae</i>	<i>F. subverticillata</i>	sv	42
subgenus <i>Leucopoa</i> section <i>Leucopoa</i>	<i>F. kingii</i>	fk	56
section <i>Breviaristatae</i>	<i>F. hallii</i> <i>F. thurberi</i>	fh ft	28 28, 42
subgenus <i>Festuca</i> section <i>Festuca</i>	<i>F. toluensis</i> <i>F. viridula</i> <i>F. dasyclada</i> <i>F. rubra</i> <i>F. roemeri</i> ^a <i>F. idahoensis</i> ^a <i>F. minutiflora</i> ^a <i>F. brachyphylla</i> ^a <i>F. saximontana</i> ^a <i>F. trachyphylla</i> ^a <i>F. canadensis</i> ^a <i>F. filiformis</i> ^a	fl fv fd fr fo fn fi fb fs fy fc ff	42 28 ? 42 ^c ? 28 28 28 ^d , 42 42 42 ^b ? 14
VULPIA	<i>V. myuros</i>	vm	42
LOLIUM	<i>L. perenne</i>	lp	14 ^b
POA	<i>P. pratensis</i>	pp	various
PUCCINELLIA	<i>P. distans</i>	pd	42

Table 3. Infrageneric classification and nomenclature of *Festuca* in North America after E. B. Alexeev. Exemplar species (shown in bold) used to represent taxa in this study are given with important synonyms.

Subgenus *Drymanthele* V. Krecz. & Bobrov in Komarov, 1934, Fl. URSS 2: 532. *Drymochloa* Holub, 1984, Folia Geobot. Phytotax. (Praha) 19: 96. (Type: *F. altissima* All., 1789, Auct. ad Fl. Pedem.: 43). Section *Montanae* Hackel, 1882, Monographia Festucarum Europaerum: 195. Subgenus *Montanae* (Hackel) Nyman, 1882, Conspectus florae europaeae: 825. (Type: *F. montana* M. Bieb., non Savi)

Section *Texanae* E. B. Alexeev, 1980, Novosti. Sist. Vyssh. Rast. 17: 44 (Type: *F. versuta* Beal, 1896, Grass. North. Amer. 2: 589. *F. texana* Vasey, 1886, Bull. Torrey Club, 13: 119, non Steudel 1854).

Section *Ruprechtia* E. B. Alexeev, 1980, Novosti. Sist. Vyssh. Rast. 17: 45 (Type: *F. amplissima*).
F. amplissima Rupr. ex E. Fourn., 1886, Mex. Pl. 2: 125.

Subgenus *Subulatae* (Tzvelev) E. B. Alexeev, 1977, Byull. Mosk. obshch. isp. prir. otd. biol. 82(3): 96. *Festuca* subgenus *Schedonorus* section *Subulatae* Tzvelev, 1971, Bot. Zhurn. 56: 1253. (Type: *F. subulata*).

Section *Subulatae* Tzvelev, 1971, Bot. Zhurn. 56: 1253.
F. subulata Trinius in Bongard, 1832, Mém. Acad. Sci. Pétersb. Phis. Math., sér. 6, 2: 173.

Section *Elmera* E. B. Alexeev, 1980, Novosti. Sist. Vyssh. Rast. 17: 47. (Type: *F. elmeri* Scribner & Merrill, 1902, Bull. Torrey Bot. Club 29: 468.

Subgenus *Subuliflorae* E. B. Alexeev, 1980, Novosti. Sist. Vyssh. Rast. 7: 47. (Type: *F. subuliflora*).
F. subuliflora Scribner in Macoun, 1890, Cat. Canad. Pl. 4: 396. *F. ambigua* Vasey, 1893, Contr. US Natl. Herb. 1: 277, non LeGal. 1852.

Subgenus *Schedonorus* (Beauv.) Peterm., 1849, Deutschl. Fl.: 643. *Schedonorus* Beauv., 1812, Ess. Agrost.: 99, sensu stricto. (Lectotype: *F. arundinacea*).

Section *Schedonorus* (Beauv.) Koch, 1837, Syn. Fl. Germ. & Helv.: 813.
F. pratensis Hudson, 1762, Fl. Angl.: 37.
F. arundinacea Schreber, 1771, Spicil. Fl. Lips.: 57.

Section *Plantynia* (Dumortier) Tzvelev, 1976, Grasses
 USSR: 394. *Bromoides* Rouy, 1913, Fl. Fr. 14: 225
 (Type: *F. gigantea*).
F. gigantea (L.) Vill., 1787, Hist. Pl. Dauph.
 2: 110. *Bromus giganteus* L., 1753, Sp. Pl.: 77.

Subgenus *Obtusae* E. B. Alexeev, 1980, Novosti. Sist. Vyssh.
 Rast. 17: 45 (Type: *F. paradoxa* Desv., 1831, Opusc.:
 105. *F. nutans* Biehler, 1807, Pl. Nov. Herb. Spreng.
 Cent.: 10, non Moench 1807).
F. subverticillata (Persoon) E. B. Alexeev,
 1980, Novosti. Sist. Vyssh. Rast. 17: 52. *F.*
obtusae Biehler, 1807, Pl. Nov. Herb. Spreng.:
 11.

Subgenus *Leucopoa* (Grisebach in Ledebour) Hackel, 1.III.1906,
 Feddes Repert. 2(18): 70. *Leucopoa* Grisebach in
 Ledebour, 1852, Fl. Ross. 4: 383. (Type: *F. sibirica*).
Festuca subgenus *Hesperochloa* Piper, 30.III.1906, Contr.
 US Natl. Herb. 10: 40. *Hesperochloa* (Piper) Rydberg,
 1912, Bull. Torrey Bot. Club 39: 106. (Type: *F. kingii*).

Section *Leucopoa* (Grisebach in Ledebour) Krivot., 1960,
 Bot. Mat. (Leningrad) 20: 48.
F. kingii (S. Watson) Cassidy, 1890, Col. Agr.
 Exp. St. Bull. 12: 36. *F. confinis* Vasey,
 Contr. US Natl. Herb. 1: 126, 1884.

Section *Breviaristatae* Krivot, 1960, Bot. Zhurn. 20: 48
 (Type: *F. altaica* Trinius, 1829, in Ledebour Fl.
 Alt. 1: 108. *F. scabrella* Torrey ex Hooker, 1840,
 Fl. Bor. Amer. 2: 252.
F. hallii (Vasey) Piper, 1906, Contr. US Natl.
 Herb. 10: 31.
F. thurberi Vasey, 1874, in Rothrock. Cat. Pl.
 Survey W. 100 Merid.: 56.

Subgenus *Festuca* (Type: *F. ovina* L., 1753, Spec. Plant.: 73).

Section *Festuca*
F. toluensis Kunth, 1815, Nov. Gen. et Sp. 1:
 153.
F. viridula Vasey, 1893, US Dep. Agr. Div. Bot.
 Bull. 13: 93. *F. howellii* Hackel ex Beal, 1896,
 Grass. North. Amer. 2: 591.
F. dasyclada Hackel ex Beal, 1896, Grass.
 North. Amer. 2: 602. *Argillochloa dasyclada*
 (Hackel ex Beal) W.A. Weber, 1984, Phytologia
 55: 1.
F. rubra L., 1753, Spec. Plant.: 74.
F. roemeri (Pavlick) E. B. Alexeev, 1985, Nov.
 sist. vyssh. rast. 22: 23. *F. idahoensis* var.

roemeri Pavlick, 1983, Can. J. Bot. 61: 350.
F. idahoensis Elmer, 1903, Bot. Gaz. 36: 53. *F.*
ovina var. *ingrata* Hackel ex Beal, 1896, Grass.
 North. Amer. 2: 598. *F. ingrata* (Hackel ex
 Beal) Rydberg, 1905, Bull. Torrey Bot. Club,
 32: 608.
F. minutiflora Rydberg, 1905, Bull. Torrey Bot.
 Club 32: 608.
F. brachyphylla Schultes & Schultes f., 1827,
 Add. ad Mant. 3: 646. *F. brevifolia* R. Br.,
 1824, Suppl. to App. Parry's First Voy., Bot.:
 289, non Muhl. 1817).
F. saximontana Rydberg, 1909, Bull. Torrey Bot.
 Club 36: 536.
F. trachyphylla (Hackel) Krajina, 1930, Acta
 Bot. Bohem. 9: 191. *F. stricta* subsp.
trachyphylla (Hackel) Patzke, 1961, Österr.
 Bot. Zeitschr. 108: 506. *F. brevipila* Tracey,
 1977, Pl. Syst. Evol. 128: 287. *F. duriuscula*
 auct., non L.
F. canadensis E. B. Alexeev, 1983, Byull. Mosk.
 obshch. isp. prir. otd. biol. 88(5): 194.
F. filiformis Pourret, 1788, Mém. Acad. Roy.
 Sci. Toulouse 3: 319. *F. tenuifolia* Sibth.,
 1794, Fl. Oxon.: 44. *F. capillata* Lam., 1778,
 Fl. Fr. 3: 597, nom. illeg.

Subgenus *Helleria* E. B. Alexeev, 1980, Novosti. Sist. Vyssh.
 Rast. 17: 51. *Helleria* Fourn., 1886, Mex. Pl. 2: 128 non
 Nees & Mart., 1824. *Hellerochloa* Rauschert, Taxon 31:
 561, 1982. (Type: *F. livida* (Kunth) Willd. ex Sprengel,
 1825, Syst. 1: 150. *Schedonorus lividus* (Kunth) Roemer &
 Schultes, 1817, Syst. Nat. 2: 707. *Helleria livida*
 (Kunth) Fourn. ex Hemsl., 1885, Biol. Centr. Am. Bot. 3:
 582, in syn.; Fourn., 1886, Mex. Pl. 2: 129).

collection and/or after greenhouse cultivation and deposited at the National Museum of Nature, Ottawa (CAN¹) and the Biological Resources Division, Agriculture Canada, Ottawa (DAO).

Collections used are listed in Appendix B.

2.2. DNA preparation and visualization

Extraction, preparation and visualization of DNA samples is described in detail in Appendix C. Total genomic DNA was extracted using the CTAB method of Doyle and Doyle (1987) followed by extraction in phenol-chloroform-isoamyl alcohol (24:24:1) and chloroform-isoamyl alcohol (24:1) with further purification by centrifugation in a cesium chloride-ethidium bromide density gradient. Solutions with 3-5 μ g of sample DNA were single-digested with each of 11 (cpDNA) or 12 (rDNA) restriction endonucleases (Table 4) according to manufacturer's specifications. Enzymes with six base pair recognition sites were used. Digested samples were size fractionated electrophoretically on 0.8% agarose gels at 20 volts. Following electrophoresis, DNA was denatured with 0.4M NaOH and Southern (1975) transfers were made to nylon membrane. Twelve restriction fragments cloned from pUC 19 (Amp^r) inserts of the *Petunia hybrida* Vilm. (*Solanaceae*) chloroplast DNA (Sytsma and Gottlieb 1986), as well as a 9 kb rDNA clone, pTA 71, from wheat (*Triticum*

¹Acronyms according to Holmgren et al. (1990)

Table 4. Restriction endonucleases used and their recognition sequences and cleavage (arrows) sites. G = guanine, C = cytidine, A = adenine, T = thiamine, N = variable, m = those enzymes sensitive to DNA methylation at or near restriction sites (according to supplier, Appendix C).

<i>Bam</i> HI	5'...G↓G A T C C...3' 3'...C C T A G↑G...5'	m
<i>Bcl</i> I	5'...T↓G A T C A...3' 3'...A C T A G↑T...5'	m
<i>Bgl</i> II	5'...A↓G A T C T...3' 3'...T C T A G↑A...5'	
<i>Cla</i> I	5'...A T↓C G A T...3' 3'...T A G C↑T A...5'	m
<i>Dra</i> I	5'...T T T↓A A A...3' 3'...A A A↑T T T...5'	
<i>Eco</i> RI	5'...G↓A A T T C...3' 3'...C T T A A↑G...5'	m
<i>Eco</i> RV	5'...G A T↓A T C...3' 3'...C T A↑T A G...5'	
<i>Hind</i> III	5'...A↓A G C T T...3' 3'...T T C G A↑A...5'	
<i>Pvu</i> II	5'...C A G↓C T G...3' 3'...G T C↑G A C...5'	m
<i>Sca</i> I	5'...A G T↓A C T...3' 3'...T C A↑T G A...5'	
<i>Xba</i> I	5'...T↓C T A G A...3' 3'...A G A T C↑T...5'	m
<i>Xmn</i> I	5'...G A A N N↓N N T T C...3' 3'...C T T N N↑N N A A G...5'	

aestivum L. CV Chinese Spring) (Gerlach and Bedbrook 1979), were labelled with deoxycytidine 5'-(³²P) triphosphate (6000 Ci/mmol) by nick translation (Maniatis et al. 1982) and hybridized to membrane-bound DNA samples at 0.7M salt concentration and 65°C (Warwick et al. 1989). *Petunia* Juss. chloroplast clones used as probes represented about 90% of the genome (un-shaded regions in Figure 5) and were of the following sizes: P1, 23 kb; P3, 21 kb; P4, 19 kb; P6, 15.3 kb, P8, 9.2 kb; P10, 9 kb; P12, 7.6 kb; P14, 4.6 kb; P16, 4.1 kb; P19, 1.5 kb; P20, 1.4 kb; S8, 11.4 kb. After autoradiography using Kodak XAR-2 film, membranes were stripped for re-hybridization.

The two outside lanes on each 17 lane gel were reserved for size markers made from digests of lamda DNA (*Escherichia coli* bacteriophage) with *Hind*III and pBR322 (*Escherichia coli* plasmid cloning vehicle) with *Hinc*II and *Hinf*I. Generated lamda fragments of 23130, 9416, 6557, 4361, 2322 and 2027 base pairs and pBR322 fragments of 3256, 1632, 1107 and 574 base pairs were used for sizing unknown fragments.

2.3. Data interpretation and scoring

Digest fragments of the chloroplast genome were linearly ordered and homologous restriction sites identified on the basis of: 1) implied homologies as indicated by hybridization to adjacent probes *Petunia*, 2) changes in fragment pattern between various

taxa corresponding to, or consistent with, presence or absence of a site, and 3) by comparison with published restriction site maps for *Triticum* L. and *Aegilops* L. (Ogihara and Tsunewaki 1988: *Bam*HI, *Bgl*III, *Hind*III, *Pvu*II, *Sca*I, and *Xba*I), *Hordeum* L. (Poulsen 1983: *Pvu*II) and those of various genera in the tribe *Poeae* including Eurasian species of *Leucopoa* and *Lolium* (Soreng et al. 1990: *Hind*III, *Pvu*II) and *Festuca* (Soreng et al. 1990: *Hind*III, *Pvu*II). Ribosomal DNA data were compared with various published maps for *Triticum* (Jorgensen et al. 1987) and *Hordeum* (Molnar and Fedak 1989, Molnar et al. 1989). Restriction sites were scored as present (1) or absent (0) for the cpDNA sites (Appendix D, Tables 1-3) and given a score of 0 to 9 based on rDNA fragment pattern (Appendix D, Table 3).

Three main factors limiting the confidence with which some homologies could be determined. These were: 1) the large size of cloned chloroplast DNA fragments used as probes; 2) the overlap of SSC region probes with IR regions; and 3) the rearrangement of the grass chloroplast genome compared with the dicot probes in the LSC region. The latter is the result of 3 partially nested inversions occurring in all grasses relative to dicots (Soreng et al. 1990). Only those sites of certain identity were used in the analysis.

2.4. Data analysis

2.4.1. Cladistic analysis - Shared polymorphisms in cpDNA sites (Table 5; Appendix D, Table 1) were used in cladistic analysis. Software included PAUP (Phylogenetic Analysis Using Parsimony) Version 2.4 (Swofford 1985) (Wagner parsimony) and HENNIG86 1.5 (Ferris 1988). Selected options for PAUP were: swap = alt (branch-swapping alternates between nearest neighbour interchanges and subtree re-insertion); and mulpars (saves all equally parsimonious trees found for further branch-swapping). Selected options for HENNIG86 were: mhennig* (applies branch-swapping to initial trees keeping only the shortest); bb* (applies extended branch-swapping to trees keeping only the shortest); and nelsen (for generating Nelsen consensus trees). In that some cladistic programs are sensitive to the order of taxon input (Felsenstein 1991), several configurations of the data were analyzed.

Character state polarity was determined by out group analysis (Stevens 1980, Watrous and Wheeler 1981) using *Poa* (*Poa pratensis* L.) and *Fuccinellia* Parl. (*Puccinellia distans* (Jacq.) Parl) as the designated outgroups. The genera *Poa* (\pm 500 species) and *Puccinellia* (\pm 80 species) along with *Festuca* (\pm 450 species) are the largest in the *Poeae* and represent more than 90% of the species in the tribe (Clayton and Renvoize 1986). The close relationship of *Poa* and *Festuca* has been recognized by various

authors (e.g. Bews 1929, Hackel 1882, Parodi 1953) and, as pointed out by Clayton and Renvoize (1986) and Tzvelev (1971), the genera are not always easily separated based on the primary differences of a keeled versus un-keeled lemma and a round versus linear hilum (respectively). The cladistic analysis of cpDNA restriction site data by Soreng et al. (1990) indicate *Poa* in the sister clade to *Festuca* and as part of the ingroup with respect to the sister clade containing *Puccinellia*.

2.4.2. Phenetic analysis - Data were analyzed with NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) Version 1.6 (Rohlf 1990) using standard procedures reviewed by Sneath and Sokal (1973). Chloroplast DNA data were analyzed separately and combined with rDNA data. An association coefficient, measuring the similarity between pairs of operational taxonomic units (OTU's) over the range of characters, was calculated using the simple matching coefficient. Sequential, agglomerative, hierarchic and nested clustering was done with the unweighted pair-group method using arithmetic averages (UPGMA). The arithmetic average of similarity between OTU's in extant clusters is used in iterative pair-wise comparisons with other candidate OTU's (or clusters), linking those of greatest similarity. Cophenetic values were calculated from implied similarities in tree matrices and compared with respective association coefficient matrices to generate cophenetic correlation values as an indication of the goodness of fit of the two matrices.

Consensus trees were generated within and among the cluster analyses using strict (each subset of the consensus tree is found in all trees), majority rule (each subset of the consensus tree is found in more than 50% of all trees) and Stinebrickner (1984) consensus algorithms.

3. RESULTS

3.1. Chloroplast DNA

Using the first 11 enzymes in Table 4, 341 restriction sites were identified with the chloroplast probings, mostly from the LSC and IR regions (Appendix E). The SSC region was poorly resolved and very few sites could be scored from this region with confidence. One hundred and eight of the 341 sites (31.7%) were polymorphic for at least one taxon and used in the phenetic analysis (Table 5; Appendix D, Table 2). Of the 108 polymorphic sites, 67 (19.6% of the 341 total) were phylogenetically informative (i.e. shared by two or more taxa) and used in the cladistic analysis (Appendix D, Table 1). Six pairs of species were identical in the 67 shared sites: *Festuca rubra* - *Festuca trachyphylla* (Hackel) Krajina, *Festuca canadensis* Alexeev - *Festuca filiformis* Pourret, *Festuca idahoensis* Elmer - *Festuca roemerii* (Pavlick) Alexeev, *Festuca brachyphylla* Schultes & Schultes - *Festuca saximontana* Rydberg, *Festuca subulata* Trinius - *Festuca subuliflora* Scribner and *Festuca arundinacea* Schreber (subgenus *Schedonorus*) - *Lolium perenne*. The first four of these species pairs belong to the ovina complex of subgenus *Festuca* (Table 2). The fifth pair are exemplars belonging the two subgenera *Subulatae* and *Subuliflorae*. The sixth pair indicate the remarkably similar chloroplast genomes between *Festuca* subgenus *Schedonorus* and genus *Lolium*.

Table 5. Mutation number, homologous *Petunia* chloroplast clones (overlap indicated in parentheses), fragment length changes and taxa with restriction site present. Fragments in square brackets were not actually seen but invoked to account for size variation from fragments too small for resolution or present in adjacent un-probed regions. Fragment sizes used to define the mutations are representative for the majority of taxa possessing the site. For a given site mutation, however, the actual fragment sizes observed in a particular taxon may differ from that given because of adjacent site changes (see text for examples). UK refers to large fragments of unknown size greater than 23 kb. Taxon codes as given in Table 2.

No.	<i>Petunia</i> clone	Mutation	Taxa with site
BamHI			
1	S8	4.5 = 3.5+1.0	fp fg fa lp
2	S8	4.8 = 4.5+0.3	ft
3	S8	4.5 = 2.3+2.2	pd
4	S6 (S8)	8.9 = 3.4+[5.5]	fa lp
5	S6 (P16)	3.2 = 2.5+[0.7]	vm pp
6	S6 (P16)	3.2 = 2.6+[0.6]	sv
7	S6 (P16)	3.2 = 2.7+[0.5]	fp fg fm fr fd ff ft fv fy fi fb fs fc fo fn fl fa fl
8	P16	6.7 = 2.7+4.0	sf sl sv fp fg fk fm fd ft fi fh fa vm lp pp pd
9	P16	4.0 = 3.6+[0.4]	sf sl ft fh
10	P3	18 = 14+4.0	sf sl sv fm fr fd ff ft fv fy fi fb fs fc fo fn fl fh vm pd
11	P3	3.4 = 2.0+1.4	fp fg fk fa lp pd
12	P8	6.6 = 4.6+2.0	fm fl
13	P6	3.1 = 2.0+1.1	fk pp
14	P6	[9.8] = 6.4+3.4	sf sl sv fm fr fd ff ft fv fy fi fb fs fc fo fn fl fh pp pd
15	P10	8.7 = 6.4+2.3	sf sl sv fp fg fk fm fd ft fv fi fl fa lp vm
16	P10	2.3 = 1.7+0.6	sv fd fi
17	P10	5.0 = 3.5+1.5	sf sl sv fg fk fm fr fd ff ft fv fy fi fb fs fc fn fo fl fh fa vm lp pp pd
18	P12	5.1 = 4.6+0.5	fr fy
19	P14	6.6 = 5.1+1.5	sf sl sv fp fg fk fm fr fd ff ft fv fy fi fb fs fc fo fn fl fh vm lp pp pd
20	P4	3.0 = 2.8+[0.2]	sf sl sv fm fr fd ff ft

Table 5 (cont.)

			fv	fy	fi	fb	fs	fc	fn	fo
			fl	fh	vm					
			<i>BclI</i>							
21	S8	1.15 = 1.1+[0.05]	sf	sl	sv	fm	fr	fd	ff	ft
			fv	fy	fi	fb	fs	fc	fo	fn
			fl	fh	vm	pp	pd			
22	P16	[12.6] = 8.5+4.1	pp	pd						
23	P3	9.3 = 5.5+3.8	sf	sl	sv	fk	fm	fr	fd	ff
			ft	fv	fy	fi	fb	fs	fc	fo
			fn	fl	fh	vm	pd			
24	P8	5.5 = 3.0+2.5	fk							
25	P10	7.2 = 6.6+0.6	sf	sl	sv	fg	fk	fm	fr	fd
			ff	ft	fv	fy	fi	fb	fs	fc
			fo	fn	fl	fh	fa	vm	lp	
26	P10	6.0 = 4.8+1.2	sf	sl	sv	fp	fg	fk	fm	fr
			fd	ff	ft	fv	fy	fi	fb	fs
			fc	fo	fn	fl	fh	fa	vm	lp
			pd							
			<i>BglIII</i>							
27	P3	5.0 = 4.3+0.7	fp	fg	fa	lp				
28	P3	5.0 = 4.6+[0.4]	vm							
29	P3	8.2 = 5.0+3.2	fp	fg	fm	fl	fa	vm	lp	
30	P3	4.75 = 3.5+1.25	sf	sl	sv	fp	fg	fk	fm	fr
			fd	ff	ft	fv	fy	fi	fb	fs
			fc	fo	fn	fl	fh	fa	vm	pp
			pd							
31	P8	1.7 = 1.0+0.7	fp	fg	fk	fa	lp			
32	P8	2.1 = 1.3+0.7	sf	sl	sv	fk	fm	fr	fd	ff
			ft	fv	fy	fi	fb	fs	fc	fo
			fn	fl	fh	vm	pp			
33	P6	16.3 = 9.0+7.3	sv	fm	fr	fd	ff	fy	fi	fb
			fs	fc	fo	fn	fl	vm		
34	P6	16.3 = 12.3+4.0	fp	fg	fa	lp				
35	P10	2.8 = 2.2+0.6	sf	sl	sv	fp	fg	fk	fm	fr
			fd	ff	ft	fv	fy	fi	fb	fs
			fc	fo	fn	fl	fh	fa	lp	pp
			pd							
36	P4	3.2 = 2.6+0.6	fp	fg	fk	fa	lp	pp	pd	
			<i>Clal</i>							
37	S8	13.0 = 9.2+3.8	vm							
			<i>DraI</i>							
38	P6	3.8 = 3.2+0.6	pp	pd						
39	S8	6.7 = 5.5+1.2	sf	sl	sv	fm	fr	fd	ff	ft
			fv	fy	fi	fb	fs	fc	fo	fn
			fl	fh	vm	pp	pd			
40	S8	5.5 = 4.5+1.0	pp							
41	S8	2.5 = 1.6+0.9	sf	sl	sv	fm	fr	fd	ff	ft
			fv	fy	fi	fb	fs	fc	fo	fn

Table 5 (cont.)

42	P3	6.3 = 2.6+3.7	fl fh vm pp
43	P3	6.3 = 3.0+3.3	fl
44	P8	4.8 = 2.9+1.9	pp
			sf sl sv fg fk fm fr fd
			ff ft fv fy fi fb fs fc
			fo fn fl fh fa vm lp pp
			pd
45	P6	2.5 = 1.25+1.25	sv fv
46	P4	4.4 = 3.0+1.4	sf sl sv fp fg fk fm fr
			fd ff ft fv fy fi fb fs
			fc fo fn fl fh fa lp pp
			<i>EcoRI</i>
47	P16	3.0 = 1.5+1.5	sf sl sv fk fm fr fd ff
			ft fv fy fi fb fs fc fo
			fn fl fh vm
48	P3	7.9 = 6.9+1.0	sf sl sv fm fr fd ff fv
			fy fi fb fs fc fo fn fl
			fh vm pp
			pd
49	P3	7.9 = 5.5+2.4	sf sl sv fk fm fr fd ff
50	P10	4.6 = 2.35+2.25	ft fv fy fi fb fs fc fo
			fn fl fh vm pp pd
51	P10	3.95 = 2.35+1.6	sf sl sv fp fg fk fr fd
			ff ft fv fy fi fb fs fc
			fo fn fh fa vm lp pp pd
			pp pd
52	P10	1.6 = 1.0+0.6	fb fs
53	P12	2.3 = 1.5+0.8	fr ff fy fb fs fc fo fn
54	P4	12.6 = 6.3+6.3	sf sl sv fp fg fk fm fr
55	P4	1.7 = 1.1+0.6	fd ff ft fv fy fi fb fs
			fc fo fn fl fh vm pp pd
			<i>EcoRV</i>
56	S8	21.3 = 18.0+[3.3]	sf sl sv fp fg fk fm fd
			ft fv fi fl fh lp pp pd
57	S8	18.0 = 12.0+6.0	vm
58	P3	2.5 = 1.6+0.9	sf sl fp fg fk fm fr ff
			ft fv fy fb fs fc fn fo
			fl fh fa vm lp pp pd
59	P3	5.9 = 2.9+2.8	sf sl sv fp fg fk fm fr
			fd ft fv fy fi fb fs fc
			fo fn fl fh fa vm lp pp
			pd
60	P3	14.0 = 9.0+5.0	ft
61	P3	14.0 = 11.0+3.0	fp fg fk fa lp pp pd
62	P3	3.0 = 1.7+1.3	pd
63	P8	5.3 = 5.0+[0.3]	sf sl sv fm fr fd ff ft
			fv fy fi fb fs fc fo fn
			fl fh vm pp
64	P6	17.0 = 12.0+5.0	sf sl sv fk fm fr fd ff
			ft fv fy fi fb fs fc fo

Table 5 (cont.)

65	P6	12.0 = 5.7+6.3	fn fl fh vm pp pd
66	P14	4.0 = 2.8+1.2	sf sl
			sf sl sv fm fr fd ff ft
			fv fy fi fb fs fc fo fn
			fl fh vm pp pd
67	P12(P20)	5.7 = 5.4+[0.3]	fp fg fk fa lp pp pd
68	P12(P20)	4.3 = 4.1+[0.2]	fp fg fk fa lp pp
69	P12(P20)	5.3 = 4.3+1.0	pd
			<i>HindIII</i>
70	S8	11.0 = 8.3+2.7	sf sl sv fm fr fd ff ft
			fv fy fi fb fs fc fo fn
			fl fh vm pp pd
71	S8	2.7 = 2.3+0.4	pp
72	S8	7.0 = 6.4+0.6	sf sl vm
73	P16	9.4 = 6.0+3.4	pd
74	P3	6.7 = 6.0+0.7	sf sl sv fp fg fk fm fr
			fd ff ft fv fy fi fb fs
			fc fo fn fl fh fa vm lp
			pp
75	P8	2.8 = 2.6+[0.2]	sf sl sv fm fr fd ff ft
			fv fy fi fb fs fc fo fn
			fl fh vm pp pd
76	P8	2.6 = 1.8+0.8	pd
77	P6	12.8 = 11+1.8	pp
78	P6	12.8 = 11.6+1.2	pd
79	P10	6.2 = 5.5+[0.7]	fr ff fy fb fs fc fo fn
			pp
			<i>PvuII</i>
80	P3	6.8 = 5.5+1.3	fp fg fa lp
81	P3	11.2 = 5.5+5.7	sf sl sv fp fk fm fr fd
			ff ft fv fy fi fb fs fc
			fo fn fl fh fa vm lp pp
			pd
82	P8	5.7 = 2.8+2.9	sf sl sv fm fr fd ff ft
			fv fy fi fb fs fc fo fn
			fl fh
83	P20(P19)	4.2 = 2.8+1.4	sf sl sv fp fg fk fm fr
			fd ff ft fy fi fb fs fc
			fo fn fl fh fa vm lp pp
			pd
			<i>ScaI</i>
84	S8	15.5 = 9.3+6.2	fv
85	P3	3.9 = 2.6+1.3	sf sl sv fp fg fk fm fr
			fd ff ft fv fy fi fb fs
			fc fo fn fl fh fa vm lp
			pd
86	P8	3.1 = 2.9+[0.2]	fp fg fk fa fl pp pd
87	P8	2.9 = 2.6+[0.3]	pp
88	P8	[5.7] = 3.1+2.6	sf sl sv fm fr fd ff ft

Table 5 (cont.)

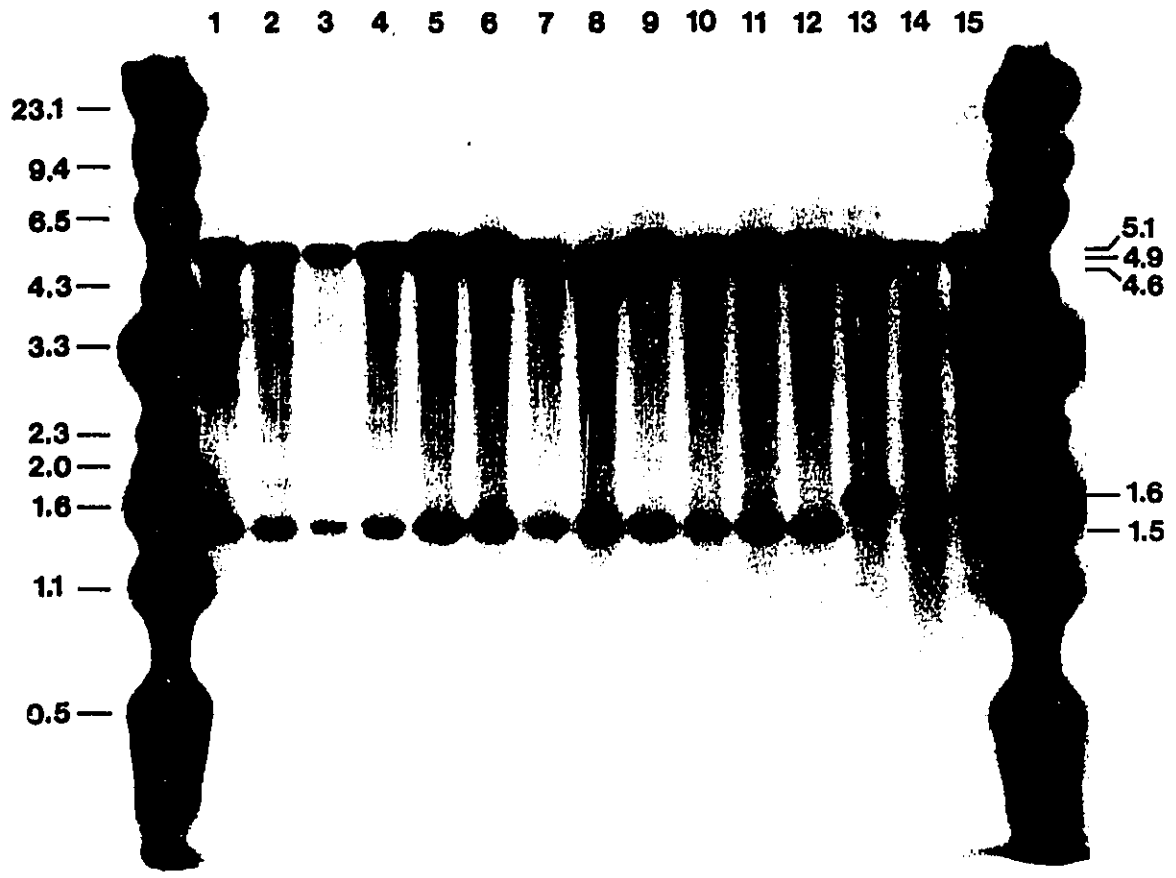
89	P8	[4.5] = 2.4+2.1		fv fy fi fb fs fc fo fn fl fh vm fp fg fk fa lp pp pd
90	P8	[3.9] = 2.6+1.3		sf sl sv fm fr fd ff ft fv fy fi fb fs fc fo fn fl fh vm
91	P6	[8.6] = 3.3+5.3		sf sl sv fp fg fk fm fr fd ff ft fv fy fi fb fs fc fo fn fl fh fa vm lp pp pd
92	P6	[8.6] = 7.1+1.5		fk pp pd
93	P10	3.0 = 2.4+0.6		sf sl fk ft fv fh pp pd
94	P4	18.0 = 14.0+4.0		sf sl sv fp fg fk fm fr fd ff ft fv fy fi fb fs fc fo fn fl fh fa lp pp pd
95	P1	22.5 = 18.0+4.5		
96	S8	7.9 = 5.2+2.7	XbaI	sf sl sv fp fg fk fm fr fd ff ft fv fy fi fb fs fc fo fn fl fh fa lp pp pd fk fb fs fr ff fy fi fb fs fc fo fn fr ff fy fi fb fs fc fo fn pd vm sf sl sv fp fg fk fm fr fd ff ft fv fy fi fb fs fc fo fn fl fh fa vm lp pp fp fg fa lp
97	P3	6.0 = 3.6+2.4		pp
98	P3	3.4 = 2.0+1.4		sf sl sv fm fr fd ff fv fy fi fb fs fc fo fn fl fh vm pp pd
99	P3	[5.9] = 3.4+2.5		sf sl sv fm fr fd ff fv fy fi fb fs fc fo fn fl fh vm pp pd
100	P3	[4.8] = 2.5+2.3		fr ff fy fi fb fs fc fo fn pd vm
101	P3	[7.6] = 4.4+3.2		sf sl sv fm fr fd ff fv fy fi fb fs fc fo fn fl fh vm pp pd
102	P3	15.5 = 9.5+6.0		sf sl sv fm fr fd ff fv fy fi fb fs fc fo fn fl fh vm pp pd
103	P8	18.6 = 15.5+3.1		fr ff fy fi fb fs fc fo fn pd vm
104	P6	3.4 = 1.9+1.5		sf sl sv fm fr fd ff fv fy fi fb fs fc fo fn fl fh vm pp pd
105	P12	8.9 = 7.2+1.7		sf sl sv fm fr fd ff fv fy fi fb fs fc fo fn fl fh vm pp pd
106	P14	[16.9] = 8.9+8.0		fr ff fy fi fb fs fc fo fn pd vm
107	P4(P1)	UK = UK+8.0		fr ff fy fi fb fs fc fo fn pd vm
108	P1(P4)	UK = UK+15.0		fr ff fy fi fb fs fc fo fn pd vm

Restriction fragment sizes given in Table 5 to define particular mutations may differ in the values observed among taxa as a result of adjacent mutations. Examples can be seen in the scoring of the digests illustrated in Figure 7: character 19 should read [6.1] = 4.6+1.5 for *F. rubra* (fr, Figure 7a, lane 8), which is alluded to in character 18, and [6.7] = 5.1+1.6 for *Lolium perenne* (lp, Figure 7a, lane 13) and *Puccinellia distans* (pd, Figure 7a, lane 15) due to adjacent mutations in the 1.5 kb fragment seen in other taxa; character 80 should actually read [12.5] = 11.2+1.3 for *F. gigantea* (fg, Figure 7b, lane 5), because the 5.5 kb fragment present in most exemplars joins with the adjacent 5.7 kb fragment to give the 11.2 kb fragment.

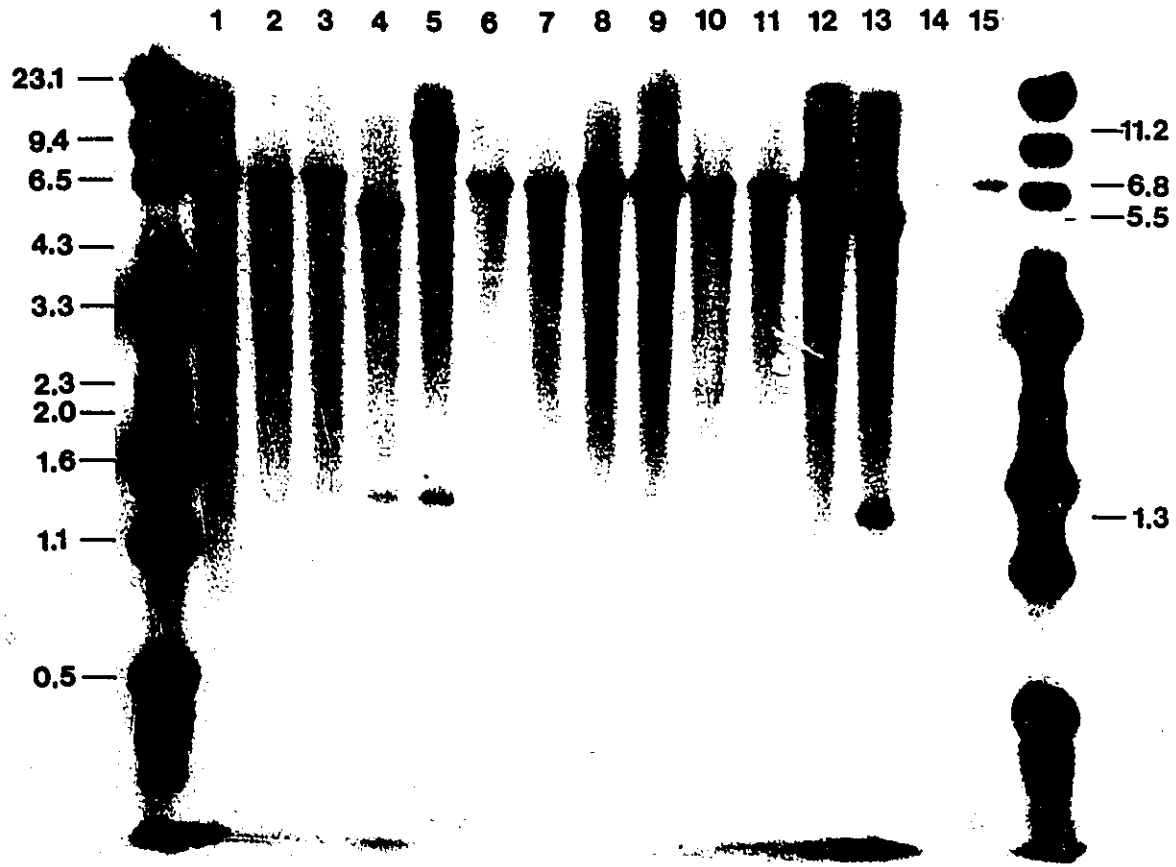
Restriction site maps generated from autoradiographs (e.g. Figure 7) were sufficiently aligned with published maps (Ogihara and Tsunewaki 1988, Poulsen 1983, Soreng et al. 1990) to be confident in the interpretation of *Festuca* cpDNA variation. Complex banding patterns generated by most enzymes cutting in regions homologous to probes P3, P8, P6 and P10 confirmed that at least the two larger of the three inversions detected in other grasses are present in *Festuca* and its allies (Soreng et al. 1990). The most complete maps generated the following size estimates for the chloroplast as a whole: *Hind*III, 140.6 kb; *Pvu*II, 132.1 kb; *Sca*I, 128.1 kb; with an average of 133 kb. Other less complete maps gave smaller estimates. These estimates are within a 10% error range that might be expected in determining fragment lengths and

Figure 7. Autoradiographs of Southern blots. Two outside lanes are marker lanes with fragment sizes indicated to left. Sample fragment length sizes are indicated on the right. Sample lanes: 1, *F. subuliflora* (sf); 2, *F. subulata* (sl); 3, *F. subverticillata* 2593 (sv); 4, *F. pratensis* (fp); 5, *F. gigantea* (fg); 6, *F. kingii* 3821 (pistillate) (fk); 7, *F. amplissima* (fm); 8, *F. rubra* (fr); 9, *F. dasyclada* (fd); 10, *F. filiformis* (ff); 11, *F. therberi* (ft); 12, *Vulpia myuros* 7294 (vm); 13, *Lolium perenne* (lp); 14, *Poa pratensis* (pp); 15, *Puccinellia distans* (pd). a: Digest with BamHI and probed with *Petunia* cpDNA clone P14 (IR region). b: Digest with PvuII and probed with *Petunia* cpDNA clone P3 from the LSC region.

a



b



close to the 134,525 base pairs reported in the *Oryza sativa* L. chloroplast genome (Hiratsuka et al. 1989). Thus the 341 six-base recognition sites studied represent about 1.5% of the entire *Festuca* chloroplast genome, while the 67 shared polymorphic sites represent about 0.3%. The major difference detected between the cpDNA of *Petunia* (154.2 kb) and the grasses used in this study occurred in the IR region. The IR regions were about 4 kb smaller in the grasses, with the difference occurring in the P12 probe region. The IR region was determined to be about 20.5 kb. The LSC and SSC regions could not be sized accurately.

No intraspecific variation in chloroplast restriction sites was detected in the two samples of *Vulpia myuros* (two populations), *Festuca subverticillata* (two populations) or *Festuca hallii* (two populations), or the three samples of *Festuca kingii* (two populations).

3.2. Nuclear rDNA

Variation was detected among taxa in rDNA repeat unit length, restriction site number and amount of base methylation. Overall length of the rDNA repeat unit varied among taxa from about 9.3 kb to 12.2 kb. A difference of about 0.5 kb was detected between two individuals of *Festuca kingii* from the same population in Colorado. Considerable variation in length and number of sites present was noted between even closely related species (e.g.:

Festuca idahoensis and *Festuca roemerii*; *Festuca subulata* and *Festuca subuliflora*). Two repeat unit length variants were detected within plants of subgenus *Drymanthele* (*Festuca amplissima* Fourn.), subgenus *Schedonorus* (*Festuca arundinacea*) and subgenus *Festuca* (*Festuca canadensis*, *Festuca minutiflora* Rydberg and *Festuca roemerii*).

Restriction sites in the rDNA repeat unit for the enzymes for the 12 enzymes varied from no sites up to three sites (Table 6). Fragment patterns of nuclear rDNA were scored phenetically (Table 6; Appendix D, Table 3) and, in spite of the lack of accurate maps, comparisons can be made between the fragment patterns of exemplar taxa in this study and maps available for related grasses (Jorgensen et al. 1987, Gerlach and Bedbrook 1979, Molnar and Fedak 1989, Molnar et al. 1989).

Base modification in rDNA by methylation was detected with certain enzymes that are sensitive to methylation (Table 4). The presence of fragments equal in size to the combined size of two or more smaller fragments indicated modification of the site separating the two fragments (Figure 8a).

*Bam*HI sites present in *Hordeum* (Molnar and Fedak 1989, Molnar et al. 1989) and *Triticum* (Jorgensen et al. 1987) include two conserved sites in the 18S and 26S subunit regions of the rDNA repeat unit. These sites are probably homologous with the two

Table 6. Number of restriction sites seen in nuclear ribosomal DNA repeat unit for 12 restriction endonucleases. Exemplar species codes as given in Table 2. Different phenotypes are indicated by a dash and a phenotype code. Data for *Triticum* (tr) are from Jorgensen et al. (1987), and *Hordeum* spp. (ho) are from Molnar and Fedak (1989) and Molnar et al. (1989). ? = missing data.

Taxa	Restriction Endonucleases											
	<u>Bam</u> HI	<u>Bcl</u> I	<u>Bgl</u> II	<u>Cl</u> aI	<u>Dra</u> I	<u>Eco</u> RI	<u>Eco</u> RV	<u>Hind</u> III	<u>Pvu</u> II	<u>Sca</u> I	<u>Xba</u> I	<u>Xmn</u> I
sf	2	1	3-1	0	1	2	1	1	2	1	1	?
sl	2	0	2	0	1	2	1	1	0	0	1	?
sv	3	0	3-1	0	1	1	1	0	0	0	1	?
fp	2	0	2	0	1	1	1	0	0	0	1	?
fg	2	1	2	0	1	1	1	0	0	0	1	?
fk	2	0	3-1	0	2-1	2	1	1	0	0	1	3-1
fm	3	1	2	0	1	1	2-1	0	0	0	1	2
fr	3	0	3-1	0	1	2	1	0	0	0	1	2
fd	2	0	2	0	1	2	1	0	0	1	1	3-2
ff	2	0	2	0	1	1	2-2	0	0	0	1	2
ft	2	1	2	0	1	2	1	1	0	0	1	2
fv	2	0	2	0	1	2	1	1	0	0	1	2
fy	2	?	2	0	1	2	2-3	0	0	0	1	2
fi	2	1	3-2	0	2-2	2	1	0	0	0	1	3-3
fb	2	0	2	0	1	2	2-4	0	0	0	1	2
fs	2	1	3-2	0	2-2	2	2-5	0	0	0	1	2
fc	2	0	2	0	1	2	2-6	0	0	0	1	3-4
fo	2	1	3-2	0	2-2	2	2-7	0	3	0	1	2
fn	2	1	4	0	2-2	1	1	0	0	0	1	2
fl	3	0	2	0	1	1	2-5	0	0	0	1	2
fh	2	0	3-1	0	1	2	1	1	1	0	2-1	2
fa	2	0	2	0	1	1	1	0	0	0	1	2
vm	3	1	2	0	1	2	2-8	1	0	0	1	2
lp	2	0	2	0	1	1	1	0	0	0	1	2
pp	2	0	2	0	1	2	1	0	1	0	2-2	3-5
pd	2	1	2	0	2-3	1	1	1	0	0	1	3-6
tr	2	?	2	1	?	1	0 or 1	0	1	?	1	?
ho	2 or 3	?	?	?	?	1	?	0 or 1	?	?	?	?

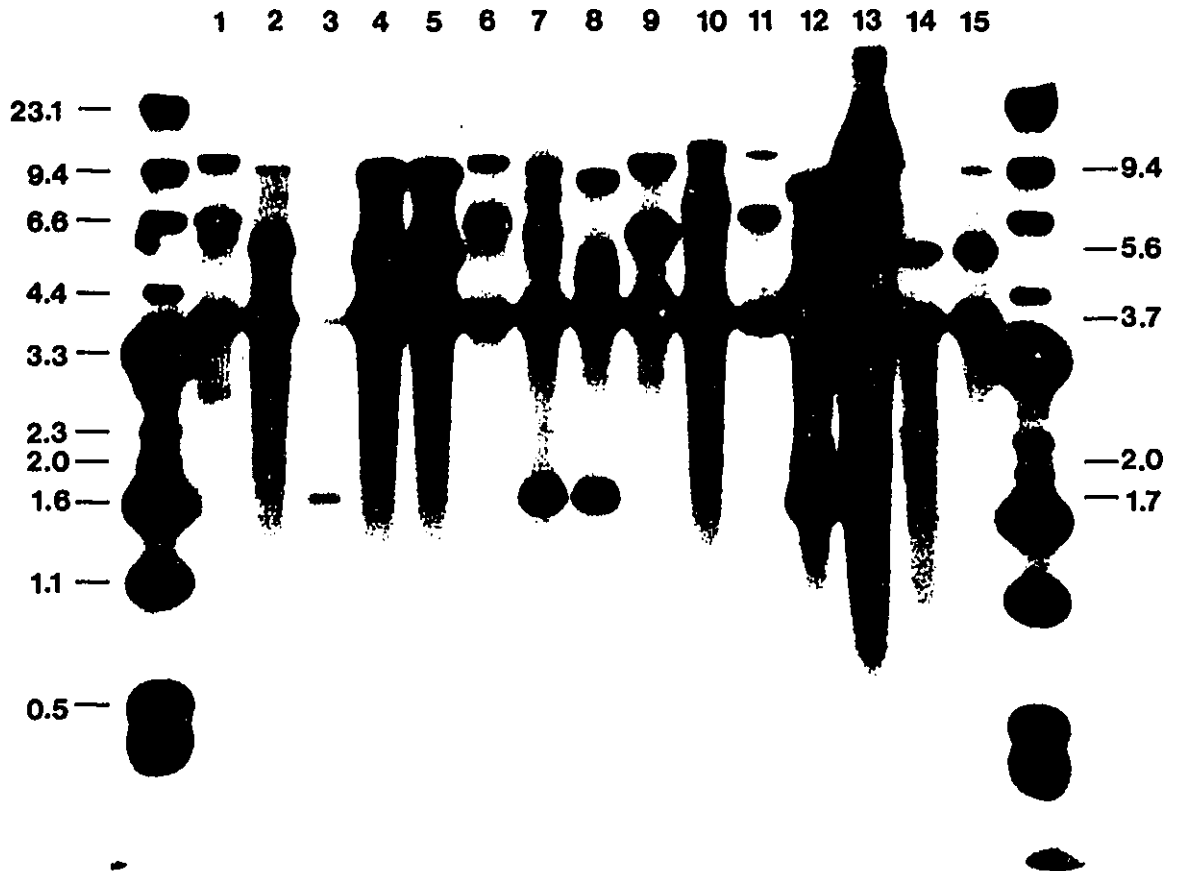
*Bam*HI sites indicated in all taxa, including the outgroup genera *Poa* and *Puccinellia*, by a prominent 3.7 kb fragment (Figure 8). A prominent 1.7 kb fragment (Figure 8) generated by a third site was seen in several taxa (Table 6). If these fragments are homologous then it is likely the third site is at the 3' end of the 26S subunit. No length variation was detected as is seen when a third site occurs in the IGS of *Hordeum*. However, fragments spanning the IGS junction at the 5' end of the 18S subunit, as small as 1.8 kb, are seen in many *Hordeum* species (Molnar et al. 1989). The third site(s) appears in five taxa; exemplars of *Vulpia*, subgenus *Obtusae*, subgenus *Drymanthele* (*Festuca amplissima*) and subgenus *Festuca* (*Festuca rubra*, *Festuca tolucensis* Kunth).

A single *Bcl*I site was seen in some of the taxa (Table 6) but information on this enzyme in *Hordeum* and *Triticum* is not available. The disparate groups that show a single site suggests that non-homologous gains and/or losses have occurred in *Festuca* and related genera.

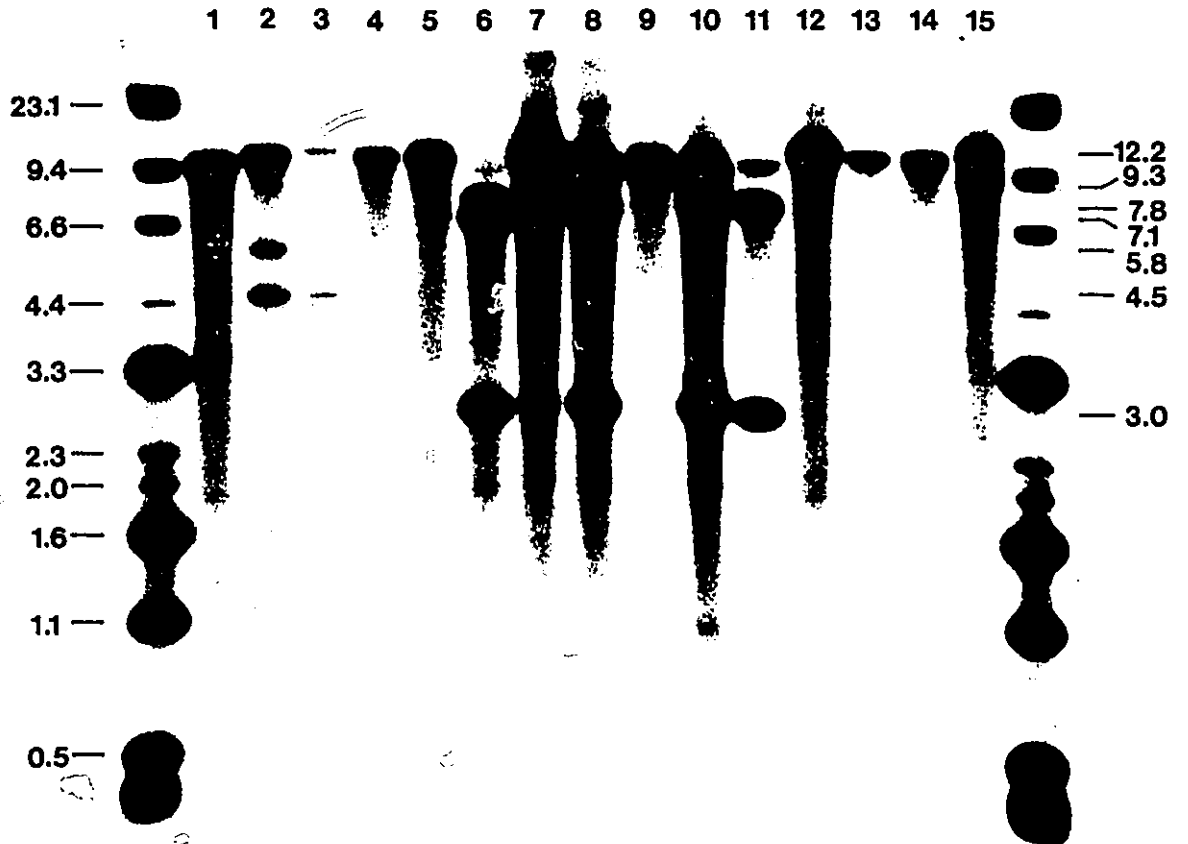
The *Triticum* rDNA repeat has two *Bgl*III sites in the 26S region which produce a fragment of about 1.3 kb (Jorgensen et al. 1987). These two sites seem to be conserved in *Festuca* and related genera as all samples showed a 1.3 kb fragment. A fragment of about 1.1 kb was seen in four North American species of the ovina complex (*Festuca idahoensis*, *Festuca minutiflora*, *Festuca roemeri*

Figure 8. Autoradiographs of Southern blots probed with the rDNA clone pTA71. Two outside lanes are marker lanes with fragment sizes indicated to left. Sample fragment length sizes are indicated on the right; not all shown for Figure a. a: Digest with *Bam*HI. Sample lanes as in Figure 7. b: Digest with *Dra*I. Sample lanes: 1, *Vulpia myuros* 2211 (vm); 2, *F. kingii* 3831(pistillate) (fk); 3, *F. kingii* 3831(staminate) (fk); 4, *F. viridula* (fv); 5, *F. trachyphylla* (ft); 6, *F. minutiflora* (fi); 7, *F. brachyphylla* (fb); 8, *F. saximontana* (fs); 9, *F. canadensis* (fc); 10, *F. roemerii* (fo); 11, *F. idahoensis* (fn); 12, *F. toluensis* (fl); 13, *F. hallii* 3716 (fh); 14, *F. hallii* 32699 (fh); 15, *F. arundinacea* (fa).

a



b



and *Festuca saximontana*) indicating a third site possibly toward the 3' end of the 26S subunit. In addition to the 1.1 kb fragment, *Festuca idahoensis* also had another fragment about 4.0 kb. In subgenera *Subuliflorae*, *Obtusae*, and *Leucopoa* as well as *Festuca rubra* (subgenus *Festuca*), a third site was detected 1.6 kb from the 1.3 kb fragment.

No *Cla*I restriction sites were detected in the repeat unit of any of the samples examined, however a single site is reported from the 5.8S region in *Triticum* (Jorgensen et al. 1987).

All taxa showed at least one *Dra*I restriction site and several showed a second (Figure 8). Three second-site phenotypes were seen: 1) dividing the repeat unit into two fragments of about 4.5 and 5.8 kb, in all three specimens of *Festuca kingii*; 2) dividing the repeat unit into fragments of about 3.0 and 7.8 kb, in the same four ovina complex species seen to contain the 1.1 kb *Bgl*III fragment (*Festuca idahoensis*, *Festuca minutiflora*, *Festuca roemeri* and *Festuca saximontana*); and 3) dividing the repeat unit into fragments of about 2.8 and 6.5 kb, in the outgroup taxon *Puccinellia distans*.

One or two *Eco*RI and *Eco*RV sites were seen in repeat units but the size variation of small and faint fragments generated in those taxa with two sites suggests that the majority of their sequences are within the IGS. These second sites detected may or

may not be homologous among various taxa examined. The primary site of *EcoRI* may be homologous to the primary site toward the 5' end of the 26S subunit in *Triticum* and *Hordeum*. As a first *EcoRV* site is found only sometimes in the 5.8S subunit of *Triticum* (Jorgensen et al. 1987) its homology with that of taxa studied here is less certain.

Very few grasses of the subfamily *Pooideae* examined to date possess any *HindIII* restriction site in their rDNA, although a site is reported in *Hordeum bulbosum* L. (Molnar et al. 1989), *Poa eminens* J. S. Presl, and *P. labradorica* Steudel (Darbyshire et al. 1992). Several taxa showed a single *HindII* site including exemplars of *Vulpia*, *Puccinellia* and subgenera *Subuliflorae*, *Subulatae*, *Leucopoa*, and, among subgenus *Festuca*, *Festuca idahoensis*, *Festuca toluensis* and *Festuca rubra*.

The rDNA of most taxa contained no *PvuII* sites. It is unlikely that the single sites in *Festuca subuliflora* (subgenus *Subuliflorae*), *Festuca hallii* (subgenus *Leucopoa* section *Breviaristatae*), *Poa pratensis* and the 5.8S-26S ITS site in *Triticum* (Jorgensen et al. 1987) are all homologous. A unique 4-banded pattern (fragments of >23, 6.6, 3.3 and 2.5) for *Festuca roemerii* (subgenus *Festuca*, ovina complex) was tentatively considered to have three sites.

Only two samples were found with rDNA sites for *ScaI*, *Festuca*

subuliflora (subgenus *Subuliflorae*) and *Festuca dasyclada* (subgenus *Festuca*).

All exemplar rDNAs contained at least one *Xba*I site which is probably homologous to the single site found at the 3' of the 18S subunit in *Triticum* (Jorgensen et al. 1987). Second sites were indicated for *Festuca hallii* (subgenus *Leucopoa* section *Breviaristatae*) and *Poa pratensis*, however, these appear to correspond to two different sites.

Data on *Xmn*I rDNA sites are unavailable for *Triticum*, *Hordeum* and several of the samples. All taxa examined had two sites generating a 0.5 kb fragment along with one or more larger fragments. Six unique patterns were generated by various additional sites within the remaining portion of the repeat unit in six of the taxa, including the outgroups (*Poa pratensis* and *Puccinellia distans*), subgenus *Leucopoa* section *Leucopoa* (*Festuca kingii*) and subgenus *Festuca* (*Festuca dasyclada*, *Festuca minutiflora* and *Festuca canadensis*).

3.3. Analysis

The data set produced a similar basic tree topology in all cladistic and phenetic analyses showing three main lineages or clusters, respectively. Apart from the outgroup of *Poa* and *Puccinellia* (group III, Figures 9-14), two main ingroup lineages

are apparent in all analyses. A basic split between the group containing *Festuca kingii*, *Festuca arundinacea*, *Festuca pratensis*, *Festuca gigantea*, and *Lolium perenne* (group II) and all other taxa (group I) is indicated. Poor resolution was encountered in two areas in all analyses as indicated by polychotomies in consensus trees (Figures 9-12); within *Festuca rubra* and the ovina complex (subgenus *Festuca* including *Festuca canadensis*, *Festuca brachyphylla*, *Festuca filiformis*, *Festuca idahoensis*, *Festuca minutiflora*, *Festuca roemerii*, *Festuca saximontana* and *Festuca trachyphylla* (*Festuca minutiflora* was consistently placed basally in this group), and within the *Schedonorus-Lolium* group (II).

3.3.1. Cladistic analysis - The most parsimonious trees found by both PAUP and HENNIG86 were 102 steps in length. The PAUP program produced more than one hundred most-parsimonious trees with a consistency index of 0.66. An example is shown in Figure 9. In considering longer trees more consistent with current phylogenetic hypotheses than the most-parsimonious trees, a series (more than 100) of 103-step trees (sub-parsimonious) with a consistency index of 0.65 were found with PAUP. An example is shown in Figure 10. The HENNIG86 program produced the Nelsen consensus tree seen in Figure 11, from a total of 103 most-parsimonious trees (102 steps) found. The consistency index of this tree was 0.65 and the retention index was 0.86.

Figure 9. One of the most-parsimonious trees (102 steps) found by PAUP using cpDNA data (Appendix D, Table 1). Numbers above branches indicate the number of synapomorphies supporting that branch. The subgenera and sections of E. B. Alexeev are indicated to the right of taxa.

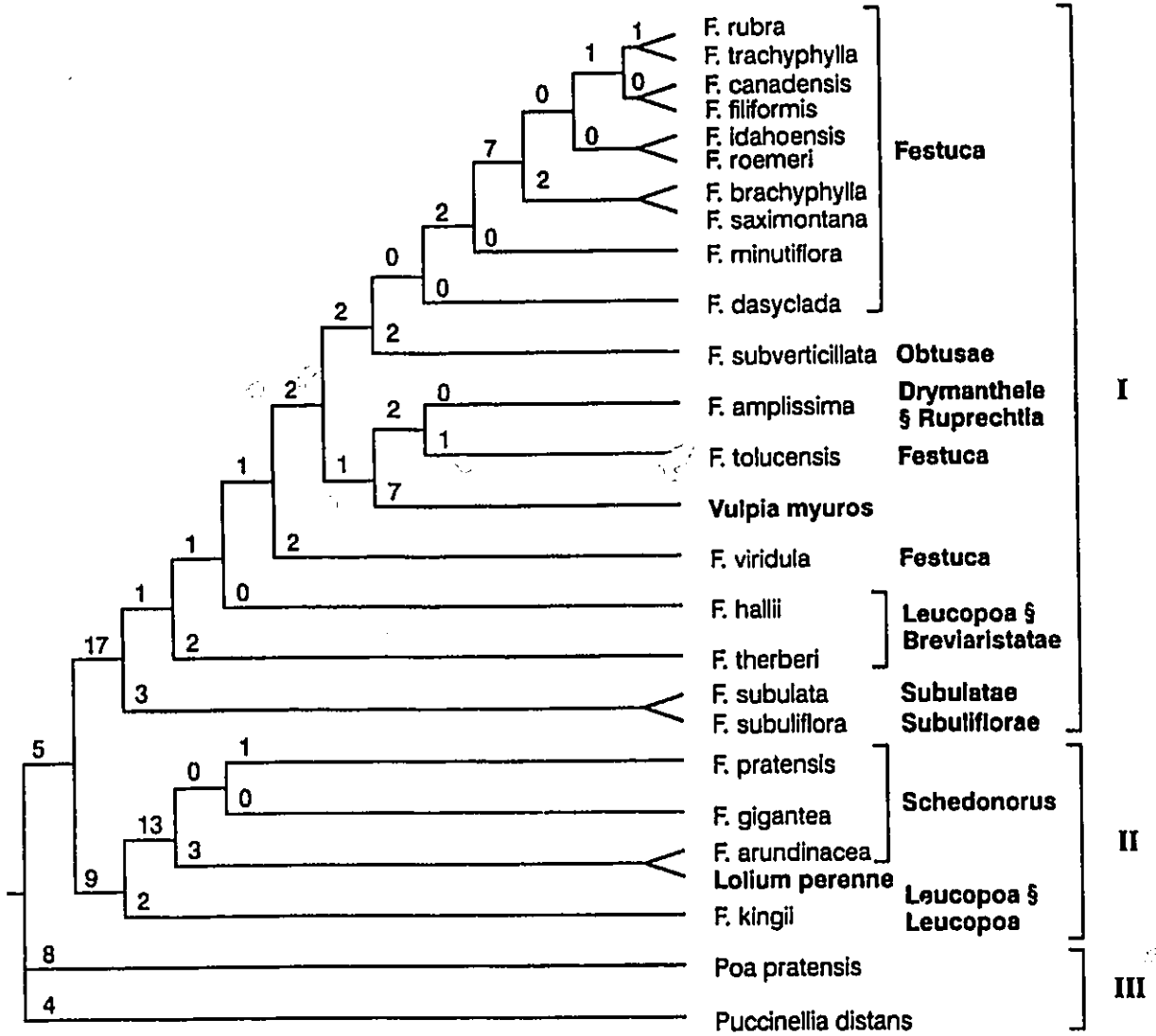
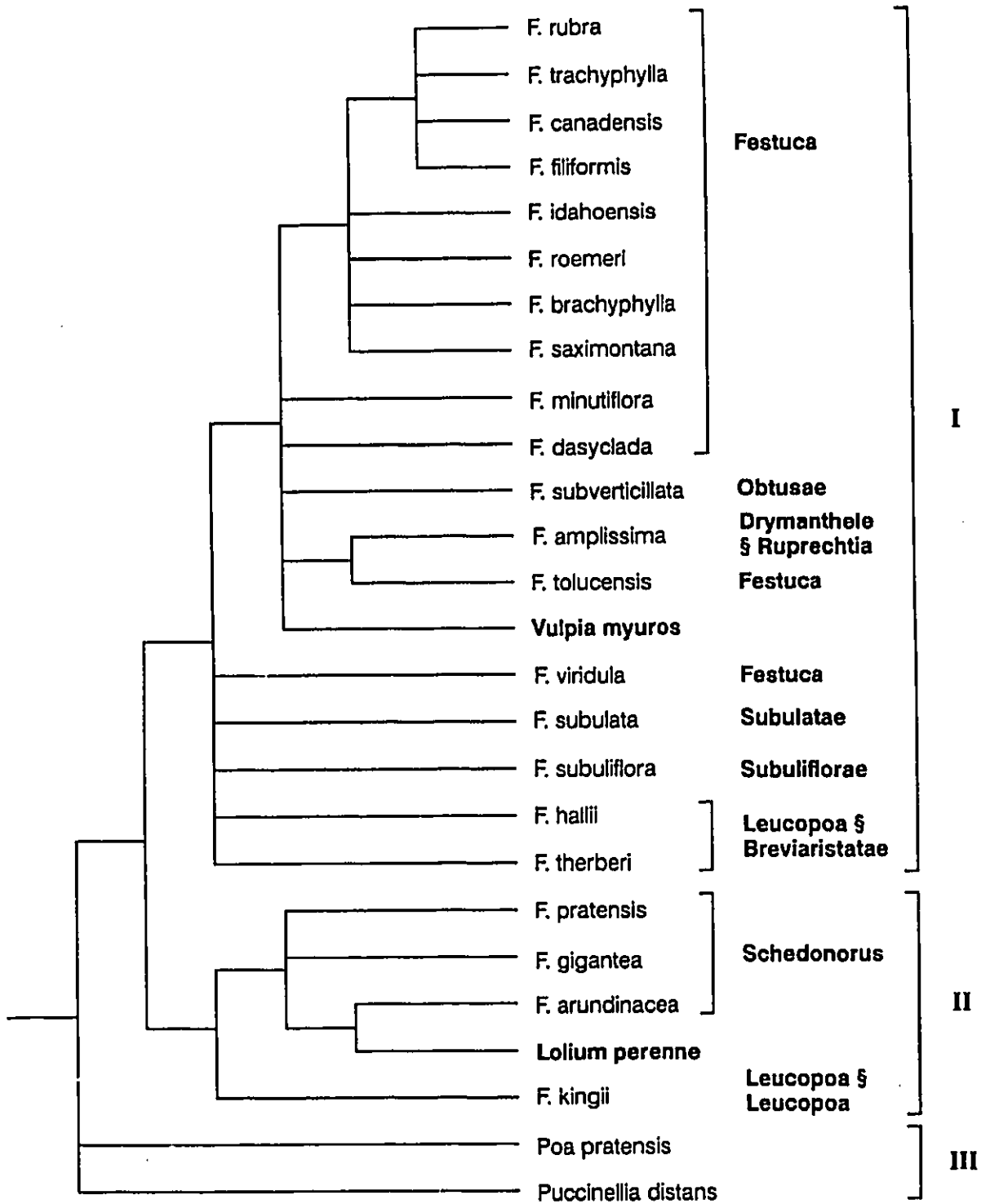


Figure 10. One of the sub-parsimonious trees (103 steps) found by PAUP using cpDNA data (Appendix D, Table 1). Numbers above branches indicate the number of synapomorphies supporting that branch. The subgenera and sections of E. B. Alexeev are indicated to the right of taxa.

Figure 11. Nelsen consensus tree produced from 103 most-parsimonious trees by HENNIG86 using cpDNA data (Appendix D, Table 1). The subgenera and sections of E. B. Alexeev are indicated to the right of taxa.



In all the analyses the ingroup (all samples excluding the outgroup of *Poa* and *Puccinellia*, group III) separated into two distinct clades: group I, containing *Vulpia*, and the subgenera *Drymanthele*, *Festuca*, *Obtusae*, *Subulatae*, *Subuliflorae*, and *Leucopoa* section *Breviaristatae*; and group II, containing subgenus *Schedonorus*, subgenus *Leucopoa* section *Leucopoa* and *Lolium*. This division was supported by 26 synapomorphies in the most-parsimonious trees (17 and 9 for groups I and II, respectively) and 23 in the sub-parsimonious trees (11 and 12, respectively) and represents the most robust dichotomy found.

Within group II subgenus *Leucopoa* section *Leucopoa* was placed basally to the *Lolium-Schedonorus* clade, which included *Lolium perenne* and all three taxa of subgenus *Schedonorus*. In the most-parsimonious trees the *Leucopoa* section *Leucopoa* clade was supported by two synapomorphies (4 in the sub-parsimonious trees) from its sister clade, which is supported by 13 synapomorphies (11 in the sub-parsimonious trees). Poor resolution was seen within the *Lolium-Schedonorus* clade and identical character states were detected in *Lolium perenne* and *Festuca arundinacea*.

Differences between the parsimonious and sub-parsimonious trees were primarily at the base of group I and included the exemplars of *Vulpia*, *Drymanthele*, *Obtusae*, *Subulatae*, *Subuliflorae*, *Leucopoa* section *Breviaristatae* and two species of subgenus *Festuca* (*Festuca viridula* Vasey and *Festuca toluensis*).

Primitive forms within group I are indicated as either the wide-leaved, forest dwelling subgenera *Subulatae* and *Subuliflorae* in most-parsimonious trees or the morphologically and physiologically specialized *Vulpia* in sub-parsimonious trees. The reputedly primitive subgenus *Drymanthele* is relatively advanced in group I in the most-parsimonious trees (Figures 9-11) and in a clade with *Vulpia*. Sub-parsimonious trees show it as basal and in the sister clade to that of *Vulpia*. With the basal placement of *Vulpia* within group I and subgenus *Drymanthele* with the majority of *Festuca*, sub-parsimonious trees match phenograms and established classifications more closely than do the most-parsimonious trees.

In the cladistic analysis (and phenetic analysis involving only chloroplast data) the two Central American species, *Festuca amplissima* (subgenus *Drymanthele*) and *Festuca toluensis* (subgenus *Festuca*), were grouped together and distinguished as a separate clade (cluster). The two members of subgenus *Leucopoa* section *Breviaristatae*, *Festuca thurberi* Vasey and *Festuca hallii*, appeared as monophyletic only in some sub-parsimonious trees (Figure 10). There are no supporting synapomorphies, however, for this clade. The hexaploid exemplar of *Festuca rubra* and the diploid *Festuca filiformis* were indicated as the most derived species in group I, originating from within the ovina complex. The morphologically unusual *Festuca dasyclada* (*Argillochloa dasyclada*) was basal to the group containing

Festuca rubra and the ovina complex. Subgenus *Obtusae* formed the sister clade to the group including *Festuca dasyclada* and most of subgenus *Festuca*. Despite poor resolution within this overall group (subgenus *Obtusae*, *Festuca dasyclada*, *Festuca rubra* and the ovina complex), the clade was formed in both most-parsimonious (Figure 9) and sub-parsimonious (Figure 10) trees.

3.3.2. Phenetic analysis - The three lineages seen in the cladistic analysis appeared as distinct clusters in the phenetic analysis, whether based on cpDNA data or on the combined cpDNA and rDNA data. Using the cpDNA data alone four UPGMA trees were generated, however, the combined cpDNA and rDNA data generated only two trees. Variation in branching occurred only within the poorly resolved ovina complex group. The cophenetic correlation with cluster matrices showed a very good fit at 0.98 for both data sets. Consensus trees for the two data sets are given in Figure 12 (cpDNA) and Figure 13 (cpDNA and rDNA) and were largely resolved with consensus fork indices of 0.92 and 0.83, respectively. Topologies were identical whether strict consensus, majority rule consensus or Steinerbrickner consensus methods were used, except for some minor branching differences among members of the ovina complex.

The fundamental dichotomy between the *Leucopoa* section *Leucopoa-Schedonorus-Lolium* lineage (group II) and the rest of *Festuca* and *Vulpia* (group I) is seen. Groups I and II separate at the first

Figure 12. Consensus UPGMA tree produced from cpDNA data (Appendix D, Table 2) by NTSYS-pc.

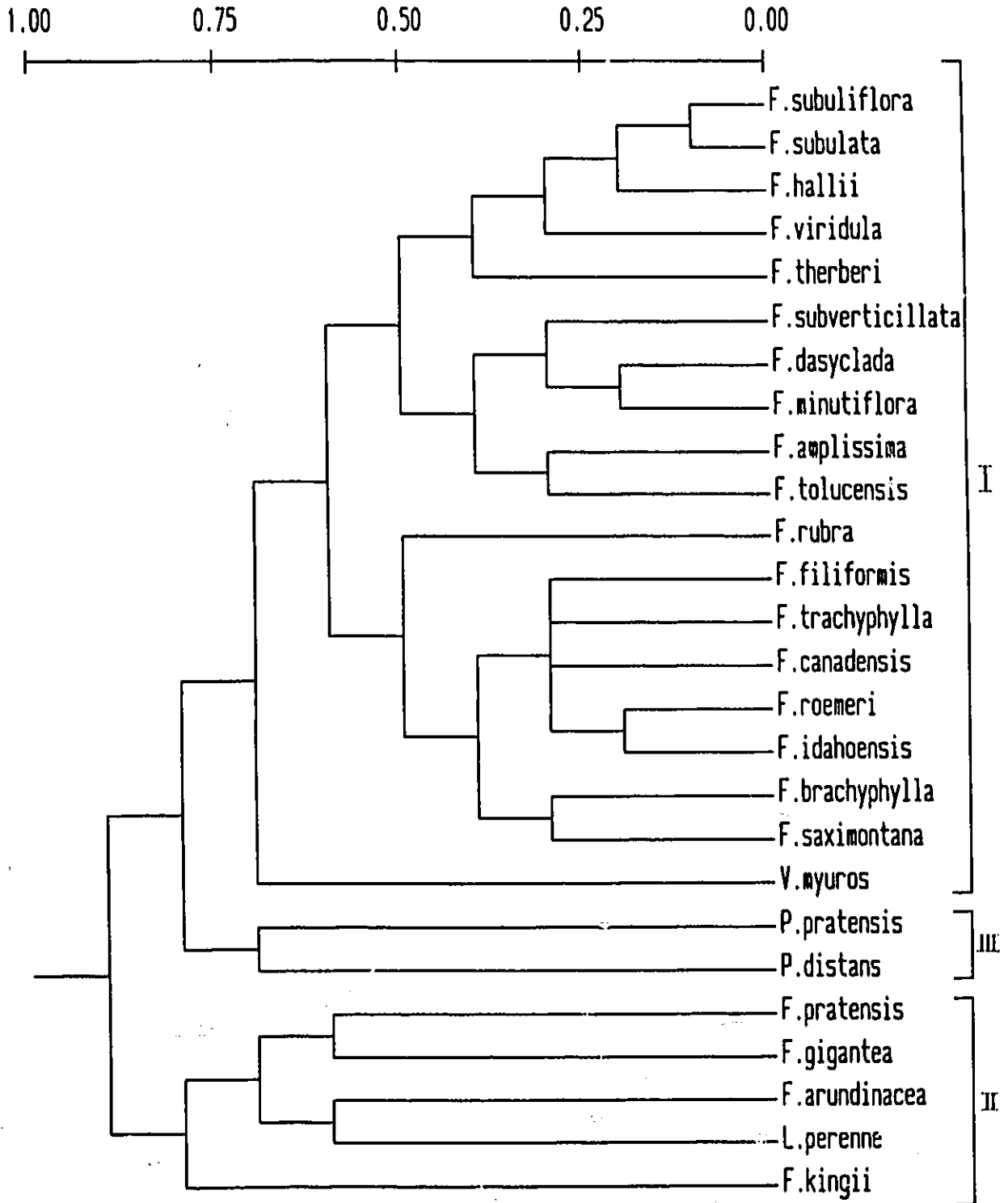
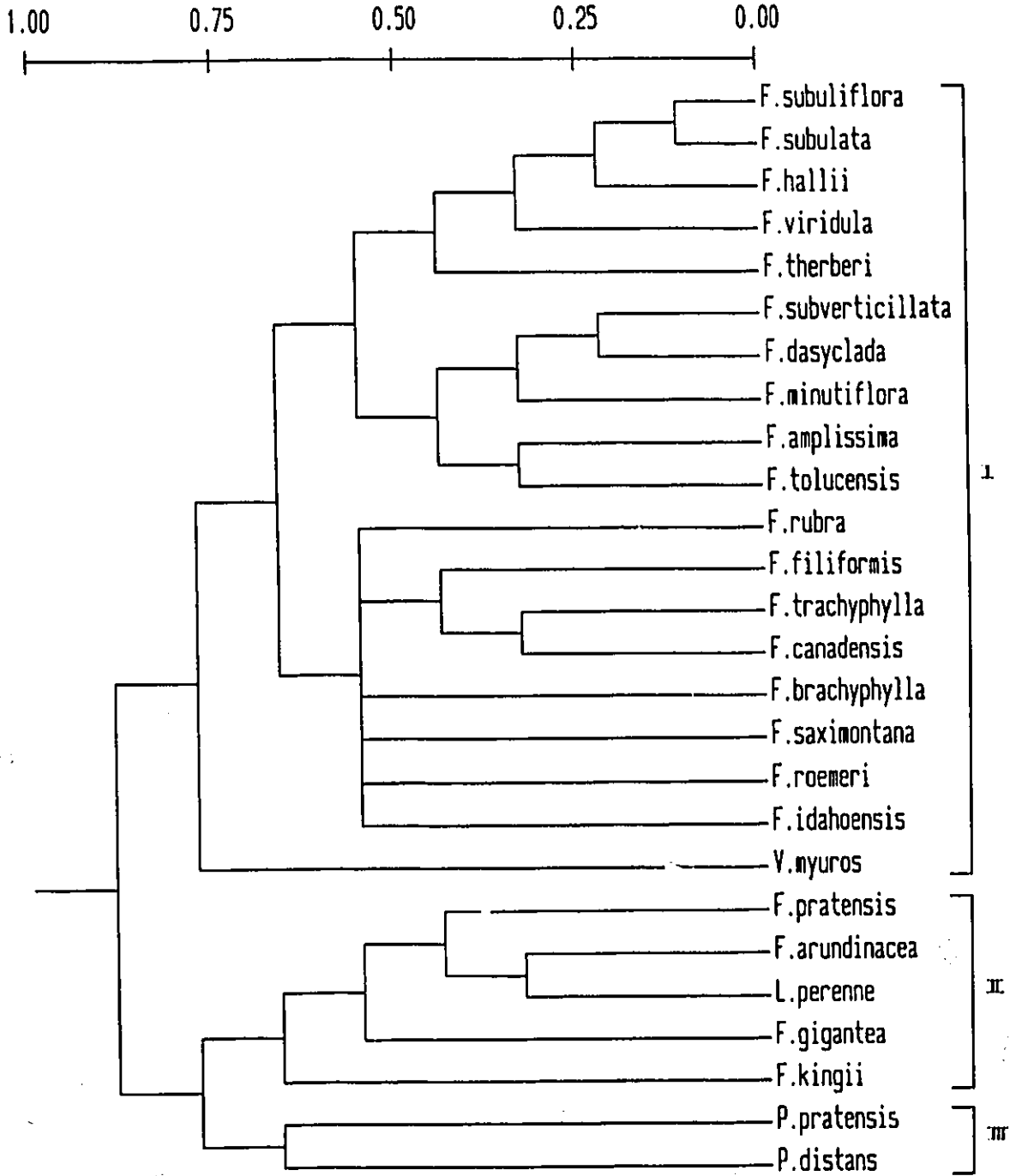


Figure 13. Consensus UPGMA tree produced from cpDNA and rDNA data (Appendix D, Table 3) by NTSYS-pc.



node with a dissimilarity of 0.900 in the analysis of cpDNA data alone and at a dissimilarity value of 0.889 in the analysis of combined data. The major difference in these two dendrograms is the placement of the relatives *Poa* and *Puccinellia* (group III). When only the cpDNA data is considered group III is clustered with group I at a dissimilarity level of 0.800 (Figure 12). With the combined data set the two genera making up group III were clustered with group II at a level of 0.778. In both analyses *Vulpia* was basal in group I, separating at levels 0.700 and 0.788 with respective data sets, and *Leucopoa* section *Leucopoa* was basal in group II, separating at levels 0.800 and 0.667.

The arrangement of taxa within group I varied somewhat between analysis of the cpDNA-only data and the combined data sets. However, the same three main clusters were recognized at distances of 0.600 and 0.667, respectively. In one of these clusters two sub-clusters were also recognized with both data sets at distances of 0.500 and 0.556, respectively. Apart from *Vulpia*, two main clusters were recognized in group I from the cpDNA data. One combined most of the ovina complex, except *Festuca minutiflora*, and the other contained subgenera *Drymanthele*, *Obtusae*, *Subulatae*, *Subuliflorae* and *Leucopoa* section *Breviaristatae*, as well as *Festuca dasyclada*, *Festuca toluensis*, *Festuca viridula* and *Festuca minutiflora* of subgenus *Festuca*. This latter group was further divided into two sub-clusters, one consisting of subgenera *Subulatae*, *Subuliflorae*,

Leucopoa section *Breviaristatae* and *Festuca viridula*, the other of subgenera *Obtusae* and *Drymanthele*, and *Festuca dasyclada*, *Festuca minutiflora* and *Festuca toluensis* of subgenus *Festuca*.

4. DISCUSSION

Over-all size and structure of both the chloroplast genome and rDNA repeat unit of the taxa in this study were found to be similar to those reported for other related grasses. The patterns of restriction site variation detected in this study indicated different relationships among the taxa than have been hypothesized based on morphology. The polyphyletic nature of *Festuca* detected in this study is consistent with the findings of many other biochemical (Aiken and Gardiner 1991, Bulińska-Rodomska and Lester 1988, Butkute and Konarev 1980, 1982, Hilu and Johnson 1990, King and Ingrouille 1987, Lehväslaiho et al. 1987, MacLeod and McCorquodale 1958, Smith 1968, 1973, Smith 1969, Soreng et al. 1990, Watson and Knox 1976), cytological (e.g. Jauhar 1975, Jenkin 1933, 1959, Malik and Thomas 1966) and even certain morphological (Padoux 1971, Bulińska-Rodomska and Lester 1988) data sets.

4.1. The genus

The genus, as pointed out by Estes and Tyrl (1982), "is an artificial construct"; a concept rather than a fact. Most classification systems of any complexity rely on a concept similar to the genus for grouping similar kinds of entities. The individual types can be further identified in the name with a modifier. Gleason (1952) suggests that this concept may

"antedate the human race." Whether incorporated in classification systems of the pre-Linnaean 'folk' taxonomies (e.g. 'oak' and 'maple'), modern manufactured goods or people (through personal names), a generic concept links those types related to each other in a particular way of interest to the taxonomist. Linnaeus, who formalized the generic concepts of Tournefort in binomial nomenclature, saw the primary function of the genus as a memory aid. Admitting that his classifications were not necessarily 'natural', he did insist on the reality of natural genera: "*Genus omne est naturalis, in primordia tale creatum*" (Linnaeus 1751). After the publication of *Species Plantarum*, with its 'sexual system', the concern of taxonomists became the creation of 'natural' genera based on overall similarity. Since the advent of Darwin the further demand of interpreting classification in a phylogenetic hierarchy has been emphasized by most, but not all, taxonomists.

A vast array of organisms displaying continuous and discontinuous variation, coupled with incongruent concepts of isolated specialists, has led to classifications of unequal hierarchical ranks. A desire for equivalency of generic rank across widely related groups of organisms and the objective quantification of 'predictive' generic units has resulted in several proposed systems for detecting 'natural' discontinuities among genera. Examples include a 'phenon-line' measure of overall dissimilarity (e.g. Borgmeier 1957, Sneath and Sokal 1973), adaptive shifts in

ecological 'zone' or 'sphere' (e.g. Inger 1958, Miller 1949, Simpson 1944, 1953), qualitative shifts in 'biological shape' of groups (e.g. Anderson 1937, Lemen and Freeman 1984), and extent of hybridization (e.g. Dubois 1988) including variants such as genome analysis (see Jauhar and Crane 1989, Kellogg 1989, Seberg 1989 and references therein). Whether the 'genus' is read from the inferred phylogeny of a cladogram (Kornet 1988), represented as a discontinuous cluster (Sneath and Sokal 1973), or a combination of both (Estes and Tyrl 1982), or subjected to any standardized identification criteria, the hierarchical entity called a genus is artificial in that it is ultimately defined subjectively.

The genus, however, is of enormous importance in the use intended by Linnaeus, as a hierarchical rank in the overall classification of organisms, and as a predictive model of relationships.

Supraspecific taxa are natural or 'biologically real' only if constructed in an evolutionary framework. Even though unreal, genera may be useful and powerful abstractions (Estes and Tyrl 1982). Utility and reality may be optimized by incorporating as much inferred phylogeny as possible into a classification. Three recent works documenting the grass genera of the world give genus numbers of 651 (Clayton and Renvoize 1986), 765 (Watson 1990) and 905 (Tzvelev 1989), indicating that a consensus on the circumscription of grass genera is a long way off.

Cladistic and phenetic analyses of cpDNA and rDNA restriction sites of taxa are largely congruent and support the recognition of four genera in the two lineages, *Vulpia* and *Festuca* (including subgenus *Leucopoa* section *Breviaristatae*, and the subgenera *Drymanthele*, *Subulatae*, *Subuliflorae*, *Obtusae*, and *Festuca*) in one and *Leucopoa* sensu stricto (including only section *Leucopoa*) and *Lolium* (including *Festuca* subgenus *Schedonorus*) in the other. The recognition of the monotypic generic segregate *Argillochloa* (= *Festuca dasyclada*) is not supported by the analyses. Each genus represents a qualitative shift in reproductive strategy, life history strategy and/or biochemical adaptation as well as substantially different genomes each with a polyploid series. A broader concept of *Festuca* to include *Leucopoa* and *Vulpia* as subgenera would require the lumping of *Lolium* to be monophyletic. This would make the genus very unbalanced with concepts elsewhere in the tribe *Poeae*. Of the works mentioned above, only Watson (1990) recognizes all four of these genera and none lump *Lolium* with *Festuca*.

4.2. Phylogenetic relationships

Phylogenetic history of the maternally inherited fescue chloroplast genome (Corriveau and Coleman 1988) may not represent the relationships of species involved in hybridization. Biases placed on phylogenies of cpDNA by interspecific hybridization and intraspecific polymorphism (Harris and Ingram 1991, Soltis et al.

1992) are considered to diminish with increased evolutionary time due to its simple replication and transmission, and its conserved nature (Clegg and Zurawski 1992). The presence of diploid forms in most of the major taxa and three of the proposed genera in this study, *Festuca*, *Lolium*, and *Vulpia*, suggests that they are not of hybrid origin. Significant differentiation of the cpDNA genome prior to the evolution of polyploidy is also seen in the genera *Triticum* and *Aegilops* (Ogihara and Tsunewaki 1988).

4.2.1. *Festuca* and *Lolium* - Whether phylogeny is inferred from shared derived characters (Hennig 1966) or phenetic similarity (Sneath and Sokal 1973), the DNA restriction site data suggest a close phylogenetic relationship between *Festuca* subgenus *Schedonorus* and *Lolium* (group II) on one hand, and the narrow-leaved forms of subgenus *Festuca* (and others in group I) on the other. Divergence and membership in these two lineages were distinct and consistent in all analyses. They are strongly supported by 17 and 9 synapomorphies (respectively) in the most-parsimonious Nelsen consensus tree (Figure 11) and separated at dissimilarity levels of 0.8 to 0.9 in phenetic networks (Figures 12-13). Cladograms most congruent with morphological and cytological data were only a single step longer than the most-parsimonious trees, where the divergence of groups I and II was also supported strongly with 23 synapomorphies (11 and 12, respectively) (Figure 10).

The presence of two distinct groups is in agreement with almost all other non-morphological studies (see references cited above), although the polyphyletic nature of *Festuca* sensu lato has not always been appreciated because of insufficient sample size. These findings are also in complete agreement with those of Lehv slaiho et al. (1987), whose study of cpDNA restriction fragment patterns in several genera of Poaceae found that *Lolium multiflorum*, *Festuca pratensis* and *Festuca arundinacea* were more similar to each other than any of the three were to *Festuca rubra*. Chloroplast DNA reassociation studies by Hilu and Johnson (1991) showed the highest sequence reciprocal-similarity values for *Festuca arundinacea* and *Lolium multiflorum* L. cpDNA among the genera studied. The realignment of *Festuca* subgenus *Schedonorus* with genus *Lolium* would be consistent with most of the biochemical and cytological evidence, and even some morphological evidence of the evolutionary relationships between these taxa and subgenus *Festuca*.

The genus *Lolium* has been described by some as being derived from the more ancient genus *Festuca* based on its reduced inflorescence, limited polyploid development and distribution, and chromosome symmetry criteria (Jenkin 1933, Essad 1962, Malik and Thomas 1966, 1967). The DNA data are largely congruent and show it to be derived from an ancestor similar to subgenus *Schedonorus*, although the latter is in a lineage distinct from other *Festuca*.

4.2.2. *Leucopoa* (= *Festuca* Subgenus *Leucopoa*) - The cpDNA restriction site cladogram of Soreng et al. (1990) indicated a close relationship between *Leucopoa* section *Leucopoa* and *Schedonorus* by placing the Asiatic *Festuca sclerophylla* Boissier & Hohenacker and Eurasian *Festuca arundinacea* as a monophyletic clade, but the divergence of this clade from subgenus *Festuca* was not detected as appropriate taxa were not used. The congruence of the cladograms presented here (Figures 9-11), with those of Soreng et al. (1990), circumstantially suggests that the Asian taxa of *Leucopoa* section *Leucopoa* and the North American *Festuca kingii* (= *Hesperochloa kingii* (S. Watson) Rydberg) are monophyletic. Non-morphological data are insufficient at this time to assess the placement of *Festuca kingii* in the monotypic genus *Hesperochloa* (Piper) Rydberg.

Among the taxa studied dioecious species are known only in some of the species of *Leucopoa* section *Leucopoa* and some highly specialized species of *Poa*. In crossing experiments between the largely cleistogamous and selfing species of *Lolium*, Naylor (1960) found that the F₁ plants were either staminate or pistillate sterile. A similar process earlier in the evolution of the group II lineage might have provided the mechanism for evolution of dioecy in *Leucopoa* section *Leucopoa*. It is also interesting to note that diploid forms have not been reported in the latter taxon.

Exemplars of the taxon *Leucopoa* section *Breviaristatae*, *Festuca hallii* and *Festuca thurberi*, showed no phyletic or phenetic affinity with section *Leucopoa*. In all analyses these two taxa were consistently related with species of group I and not with section *Leucopoa* in group II (Figures 9-13). Exemplars of the section *Breviaristatae* did not form a distinct cluster or separate clade in group I, instead, all analyses showed them to be closely related to the subgenera *Subulatae* and *Subuliflorae* and *Festuca viridula* (subgenus *Festuca*). Tzvelev (1971) speculated that the morphological similarity between species of section *Breviaristatae* and subgenus *Festuca* is a result of xerophytic convergence. No evidence was found to suggest that section *Breviaristatae* be included in subgenus *Festuca*. Phylogenetic analysis showed that other, reputedly primitive subgenera (e.g. subgenus *Obtusae*), as more closely related to subgenus *Festuca* sensu stricto than section *Breviaristatae*. Phenetic analysis separated section *Breviaristatae* from the majority subgenus *Festuca* exemplars at a dissimilarity level of about 0.6.

4.2.3. *Vulpia* - The small genus *Vulpia* is speculated to have diverged from chasmogamous, long-lived perennial *Festuca*-like ancestors adapting to cleistogamy and ephemeral annual habit (Bulińska-Rodomska and Lester 1988, Hackel 1882, Stace and Cotton 1977). Although represented here by the advanced hexaploid *Vulpia myuros* (type species of the genus), other species in the

primitive section *Loretia* are chasmogamous, perennial and diploid (Bulińska-Rodomska and Lester 1988, Cotton and Stace 1976, 1977). The greater number of generations per unit time in an annual versus perennial plant will allow for more anagenic evolution with greater divergence and increased chance of homoplastic mutations. This may account for the labile position of *Vulpia myuros* in various restriction site analyses and in the cladistic analysis of seed protein data (Bulińska-Rodomska and Lester 1988). The sub-parsimonious cladograms (Figure 10) indicated *Vulpia* as the sister group to all exemplars of *Festuca* in group I (supported by a total of 7 characters). It is also largely congruent with the cladistic analysis of morphological data by Bulińska-Rodomska and Lester (1988). Phenetic analysis of both cpDNA (Figure 12) and combined (Figure 13) data sets also produced trees suggesting a similar relationship to that of the sub-parsimonious trees. The level of dissimilarity and basal divergence from group I *Festuca* species provides support for the recognition of *Vulpia* as a distinct genus. Most-parsimonious cladograms, however, placed *Vulpia myuros* as one of a number of paraphyletic clades between the basal *Subulatae* and *Subuliflorae* and the ovina complex. In a classification following this phylogenetic hypothesis, *Vulpia* could only be recognized as a genus concurrent with the recognition of various *Festuca* subgenera and species of subgenus *Festuca* as distinct genera. Further analysis with more primitive species in *Vulpia*, particularly section *Loretia*, may resolve the discrepancies seen

in this study between most-parsimonious and sub-parsimonious phylogenies.

4.2.4. *Festuca* - The sample species belonging to genus *Festuca*, as circumscribed here, consists of the following taxa (in the sense of authors in the left column of Table 1): subgenus *Drymanthele* section *Ruprechtia*, subgenus *Subulatae*, subgenus *Subuliflorae*, subgenus *Obtusae*, subgenus *Leucopoa* section *Breviaristatae* and subgenus *Festuca*. There is no indication to warrant the recognition of the genus *Argillochloa* as separate from *Festuca*.

4.2.4.1. Subgenus *Festuca* - The subgenus *Festuca* is, by definition (Greuter et al. 1988), the subgenus which contains the type species of the genus, *Festuca ovina*. Consideration of taxa for inclusion in the subgenus must start with those species closely related to this diploid. All exemplars of the *ovina* complex (except *Festuca minutiflora*), as well as *Festuca rubra*, were placed together in a monophyletic clade or distinct sub-cluster. Other exemplars of subgenus *Festuca* (*Festuca dasyclada*, *Festuca minutiflora*, *Festuca toluensis*, and *Festuca viridula*) were indicated as polyphyletic or placed in a heterogeneous sub-cluster including taxa from five other subgenera.

The phylogenetic position of *Festuca rubra* with respect to the *ovina* complex cannot be confidently determined due to lack of

variation in the data set(s) among these taxa. However, the chloroplast genome of *Festuca rubra* was included in the more derived group I along with the ovina complex (Figures 9-11). The close relationship between these two taxa is emphasized by the identical character states detected in the informative cpDNA sites of *Festuca rubra* and *Festuca trachyphylla*, the latter a hexaploid member of the ovina complex. Phenetic analysis linked *Festuca rubra* with the majority of the ovina complex (except *Festuca minutiflora*) at the basal node in the overall cluster, but found *Festuca trachyphylla* to be more similar to other members of the ovina complex than to *Festuca rubra* (Figures 12, 3). Hackel (1882) saw *Festuca rubra* as a species intermediate between his sections *Bovinae* (subgenus *Schedonorus*) and *Ovinae* (subgenus *Festuca*), based on its extravaginal shoot production, prophyllum structure and frequently broad and flat leaves. These characters were used to separate his section *Ovinae* into two subsections: *Extravaginales vel mixtae*, including *Festuca rubra* and its allies; and *Intravaginales*, including *Festuca ovina* and its many allies.

Cladistic analysis suggests that the ovina complex may contain more than one lineage. Seven derived characters distinguished *Festuca minutiflora* from the clade containing the rest of the ovina complex (including the Eurasian diploid *Festuca filiformis*) in both most-parsimonious (Figure 9) and sub-parsimonious (Figure 10) trees. In the phenetic analysis this species, because of its

marked dissimilarity, clustered with non-ovina complex species (Figures 12, 3). The tetraploid species *Festuca minutiflora* may represent a primitive divergence from the ovina complex where speciation at the diploid level occurred prior to the establishment of independent polyploid lineages. On the other hand, it may be an allopolyploid derived from a cross between a ovina complex species and a non ovina complex species of subgenus *Festuca*.

4.2.4.2. Other subgenera - Subgenus *Subuliflorae* was distinguished from subgenus *Subulatae* by Alexeev (1980) primarily on a single morphological trait, the striking and unique development of a long and pubescent lemma callus in *Festuca subuliflora* (the only member of subgenus *Subuliflorae*). Although differences in number of restriction sites in the rDNA were found, they were identical for all 108 cpDNA sites scored. The lack of divergence and indicated monophyly of cpDNA genome suggests that separate subgeneric status is unwarranted for these two taxa.

The subgenus *Drymanthele* has been taken by most authors as the most primitive in *Festuca* based on vegetative morphology. The very high 2C DNA levels in Eurasian diploids (as high as tetraploids in others groups) suggested to Morgan et al. (1986) that *Drymanthele* may not be a progenitor of other subspecific taxa. Originally based on a group of Old World species with

diploid and hexaploid forms, Alexeev (1980d) was the first author to assign New World species to new sections of the subgenus. Few of these have been examined cytologically, but *Festuca amplissima* is known to be tetraploid and hexaploid (Alexeev 1984e). A close relationship of the cpDNA genome of the subgenus *Drymanthele* exemplar, *Festuca amplissima*, and that of *Festuca toluensis* (subgenus *Festuca*) was seen in all analyses. Both these species are from montane forests of Central America. This suggests that the Central American species of montane fescues are more closely related than Alexeev's classification implies. The relatively primitive placement of the clade within group I and the clustering of these Central American species with species other than those of the ovina complex, suggests possible homoplasy among either the morphological characters used by Alexeev to define the supposedly monophyletic subgenus *Drymanthele* (at least in section *Ruprechtia*) and/or for characters used to assign *Festuca toluensis* to subgenus *Festuca*.

4.3. Comparison with morphological characters

Morphological trends in structures and growth habit, where delicate emerging shoots and leaf transpirational surfaces are increasingly protected from desiccation, are interpreted by most authors (e.g. Hackel 1882, Saint Yves 1930, Tzvelev 1971) as indicative of phylogenetic trends. Primitive conditions include extravaginal shoot production (versus intravaginal production),

loosely tufted or rhizomatous habit (versus densely tufted habit), wide leaf blades which are flat (versus folded or rolled) and have minimal abaxial sclerenchyma (versus extensive or continuous development) and no adaxial ribs. Advanced characters are most highly developed in subgenus *Festuca* and in particular in the ovina complex. The analysis of DNA data presented here supports the proposed phylogenetic directionality of these characters based on other data. Narrow-leaved and densely tufted fescues, adapted to low moisture and temperature of open habitats, are derived from the wide-leaved and loosely tufted ancestors of forest or edge habitats. Morphological convergence (to caespitose habit and narrow leaves with distinct adaxial ribs and heavy sclerenchyma) is interpreted to have occurred between the genera *Leucopoa* (section *Leucopoa*) and *Festuca*, as well as within the genus *Festuca* itself.

Taxa of groups II (including *Lolium*, subgenus *Schedonorus*, *Leucopoa* section *Leucopoa*) and III (outgroup genera *Poa* and *Puccinellia*) possess relatively broad, folded or rolled leaves. Wide-leaved forest forms in group I, *Festuca subverticillata*, *Festuca subuliflora*, *Festuca subulata*, and *Festuca amplissima*, are placed basally to the ovina complex clade (Figures 9-11) or cluster (Figures 12, 3), however, various analyses did not consistently indicate them as the most primitive forms in group I. Most-parsimonious cladograms (Figures 9, 11) placed the wide-leaved western subgenera *Subuliflorae* and *Subulatae* as most

primitive. Subgenus *Obtusae*, however, a distinctive eastern forest taxon, was well advanced as sister clade to that including the ovina complex and *Festuca dasyclada* (*Argillochloa*). The sister clade to the group including subgenus *Obtusae* contained *Festuca amplissima* (subgenus *Drymanthele*), *Festuca toluensis* (subgenus *Festuca*), and *Vulpia myuros*, although the latter three taxa are linked by only one synapomorphy.

The montane and subalpine species *Festuca viridula* (subgenus *Festuca*) has narrow blades (<2 mm wide) with adaxial ribs and sclerenchyma only along the abaxial epidermis. These characters have lead most researchers to place it in subgenus *Festuca*, close to the ovina complex. The analysis of the DNA data, however, places it basally to some of the wide-leaved forest species. In the most-parsimonious cladograms (Figure 9, 11) *Festuca viridula* is more advanced than subgenus *Subulatae* and *Subuliflorae*, but is placed basally to the wide-leaved taxa of subgenus *Drymanthele* and *Obtusae*. Sub-parsimonious cladograms (Figure 10) placed it basally in a clade containing the wide-leaved subgenera *Subulatae* and *Subuliflorae* and in the sister group to the that containing the wide-leaved subgenus *Obtusae*.

In sub-parsimonious cladograms the wide-leaved forms were scattered through three major clades of group I. One of these three clades, containing the wide-leaved subgenus *Drymanthele* and the narrow-leaved *Festuca toluensis* (subgenus *Festuca*), was

placed basally to the other two. The wide-leaved subgenera *Subuliflorae* and *Subulatae* were in a clade which also contained other cordilleran and amphiberian taxa including the narrow-leaved taxa of section *Breviaristatae* and *Festuca viridula*. A third clade containing the narrow-leaved ovina complex had, as its most primitive member, the wide-leaved subgenus *Obtusae*. A close phylogenetic relationship between *Festuca dasyclada* and subgenus *Obtusae* is indicated by the congruent relationship in most-parsimonious (Figures 9, 11) and sub-parsimonious (Figure 10) trees and their phenetic clustering.

Hackel (1882), Tzvelev (1971) and Alexeev (1977a) suggest that the hairy ovary apex is a primitive condition in fescues. *Festuca minutiflora* and the tetraploid *Festuca baffinensis* N. Polunin are the only North American members of the ovina complex possessing hairs on the ovary, although all non-ovina complex species of subgenus *Festuca* have densely pubescent ovaries (Aiken and Darbyshire 1990). Tzvelev (1971) speculated that the pubescent ovary functions in protecting developing ovaries from excessive moisture and is a primitive character present in *Festuca*-like ancestors adapted to more mesic conditions. The presence of apical ovary hairs is suggested by the DNA data to be sympleisiomorphic with parallel loss occurring in all three major groups: most members of the ovina complex (but not other members of subgenus *Festuca*) and *Vulpia* (group I); *Lolium* and subgenus *Schedonorus* (group II); and the two outgroup genera (group III).

5. CONCLUSION

Molecular data have proved useful in revealing phylogenetic relationships among *Festuca* and related genera. The genus *Festuca*, as circumscribed in recent monographic treatments is shown to be polyphyletic. This is consistent with similar DNA studies (Lehväslaiho et al. 1987, Soreng et al. 1990) and closely correlated with other biochemical, cytological, and crossing data (see references above). Divergence and monophyletic structure are used to recognize four genera among the taxa examined. Two main evolutionary lineages were identified. Genera in one lineage included *Vulpia* and *Festuca* (including subgenus *Leucopoa* section *Breviaristatae*, and the subgenera *Drymanthele*, *Subulatae*, *Subuliflorae*, *Obtusae*, and *Festuca*), and in the other included *Leucopoa* sensu stricto (including only section *Leucopoa*) and *Lolium* (including *Festuca* subgenus *Schedonorus*). The taxonomic realignment of *Festuca* subgenus *Schedonorus* with genus *Lolium* and disassociation of section *Breviaristatae* from genus *Leucopoa* is required for classification to reflect natural relationships among these grasses. No evidence was found to support the recognition of the generic segregates *Argillochloa* or *Drymochloa* (= subgenus *Drymanthele*).

Phylogenetic reconstruction using an independent data set, such as molecular data, allows evaluation of evolutionary trends and phylogenetic history of morphological, cytological and life

history characters. Convergence in morphology of leaf and shoot structure, as adaptative trends towards increasing xerophily, is confirmed between the two main lineages and within *Festuca*. Analysis of DNA data indicate the presence of parallel polyploid series among the four genera and in various other subgeneric evolutionary lineages (e.g. the ovina complex). The four recognized genera correspond to divergent trends in life history such as annual habit and cleistogamy in *Vulpia*, dioecy in *Leucopoa*, short-lived perennial mesophiles in *Lolium*, and long-lived perennial xerophiles (or cryophiles) in *Festuca*.

These molecular data were, however, poor at resolving taxonomic relationships below the level of the four genera recognized. Most of the North American subgenera of *Festuca* (sensu Alexeev (1972-1990)) appear as paraphyletic lines basal to the ovina complex in cladistic analysis, or as poorly differentiated clusters in phenetic analysis. A framework for the evolution of these four genera is provided, however, that can be tested and improved by further studies.

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APPENDIX A. Synopsis of the taxonomic work on the genus *Festuca* by E.B. Alexeev. Species recognized are listed in alphabetic order within supraspecific taxa. Taxonomic ranking is indicated as follows: GENUS; Subgenus; Section; species. Type species of supraspecific taxa are indicated with an asterisk.

FESTUCA L.

Spec. Plant. : 73, 1753.

Drymanthele Krecz. & Bobrov in Komarov, Fl. URSS 2: 532, 1934.
 = **Montanae** (Hackel) Nyman, Consp. Fl. Europ. :825, 1882; **Montanae** Hackel, Monogr. Festuc. Europ. :195, 1882.
 = **DRYMOCHLOA** Holub, Folia Geobot. Phytotax. (Praha) 19:96, 1984.

Aristulatae E. B. Alexeev, Bot. Zhurn. 66(10): 1495, 1981.

bidenticulata E. B. Alexeev, Bot. Zhurn. 66(10): 1496, 1981.

= **fratercula** auct. non Ruprecht ex Fournier.

swallenii E. B. Alexeev, Bot. Zhurn. 66(10): 1495, 1981.*

Texanae E. B. Alexeev, Novost. Sist. Vyssh. Rast. 17: 44, 1980.

aguana E. B. Alexeev, Bot. Zhurn. 66(10): 1493, 1981.

panamica E. B. Alexeev, Bot. Zhurn. 67(9): 1290, 1982.

versuta Beal, Grass. N. Amer. 2: 589, 1897.*

= **texana** Vasey, Bull. Torrey Bot. Club 13: 119, 1886, non Steudel 1855.

Ruprechtia E. B. Alexeev, Novost. Sist. Vyssh. Rast. 17: 45, 1980.

amplissima Ruprecht ex E. Fournier, Mex. Pl. 2: 125, 1886.*

= **fratercula** Ruprecht ex Fournier, Mex. Pl. 2: 124, 1886.

jaliscana E. B. Alexeev, Bot. Zhurn. 66(10): 1493.

Drymanthele [E. B. Alexeev, Novost. Sist. Vyssh. Rast. 17: 44, 1980.]

- altissima All., Auct. ad Fl. Pedem.: 43, 1789.
- asthenica Hooker fil., Fl. Brit. Ind. 7: 354, 1897.
- drymeja Mert. & Koch, Deutschl. Fl. 1: 670, 1823.
= montana Bieberstein, Fl. Taur.-Cauc. 3: 75,
1819, non Savi, 1798.*
- handelii (St. Yves) E. B. Alexeev, Byull. Mosk.
obshch. isp. prir. otd. biol. 82(3): 95, 1977.
- leptopogon Stapf in Hooker fil., Fl. Brit. Ind. 7: 354,
1897.
- modesta Nees ex Steudel, Syn. Pl. Glum. 1: 316, 1854.
= ? handelii (St. Yves) E. B. Alexeev, Byull.
Mosk. obshch. isp. prir. otd. biol. 82(3): 95,
1977.

Banksia E. B. Alexeev, Bot. Zhurn. 69(3): 348, 1984.

archeri E. B. Alexeev, Novost. Sist. Vyssh. Rast. 24:
8, 1987.

muelleri Vickery, Contr. NSW Natl. Herb. 1(1): 9, 1939.

purpurascens Banks & Solander ex Hooker fil., Fl.
Antarct.: 383, 1847.*

Mallopetalon (Doell in Martius) E. B. Alexeev, Bot. Zhurn. 69(3):
346, 1984; F. subtaxon *Mallopetalon* Doell in Martius, Fl.
Brasil. 2(3): 115.

fimbriata Nees in Martius, Fl. Brasil. 2(1): 472, 1829.
= ampliflora Doell in Martius, Fl. Brasil. 2(3):
116, 1878.

Subulatae (Tzvelev) E. B. Alexeev, Byull. Mosk. obshch. isp.
prir. otd. biol. 82(3): 97, 1977.

Subulatae Tzvelev, Bot. Zhurn. 56(9): 1253, 1971.

cochabambana E. B. Alexeev, Bot. Zhurn. 70(9): 1241,
1985.

elata Keng ex E. B. Alexeev, Byull. Mosk. obshch. isp.

prir. otd. biol. 82(3): 97, 1977.

extremiorientalis Ohwi, Bot. Mag. (Tokyo) 45: 194, 1931.

= subulata subsp. japonica (Hackel) Koyama & Kawano, Canad. J. Bot. 42: 875, 1964.

flacca Hackel ex E. B. Alexeev, Bot. Zhurn. 69(11): 1543, 1984.

leptopogon Stapf in Hooker fil., Fl. Brit. Ind. 7: 354, 1897.

ohwiana E. B. Alexeev, Byull. Mosk. obshch. isp. prir. otd. biol. 83(5): 94, 1978.

parvigluma Steudel, Syn. Pl. Glum. 1: 305, 1854.

sinensis Keng ex E. B. Alexeev, Byull. Mosk. obshch. isp. prir. otd. biol. 93(1): 112, 1988.

sodiroana Hackel ex E. B. Alexeev, Bot. Zhurn. 69(11): 1545, 1984.

sororia Piper, Contr. US Natl. Herb. 16: 197, 1913.

subulata Trinius in Bongard, Mém. Acad. Sci. Pétersb., sér. 6, 2: 173, 1832.*

= jonesii Vasey, Contr. US Natl. Herb. 1: 278, 1893.

ulochaeta Nees ex Steudel, Syn. Pl. Glum. 1: 305, 1854.

Elmera E. B. Alexeev, Novost. Sist. Vyssh. Rast. 17: 47, 1980.

elmeri Scribner & Merrill, Bull. Torrey Bot. Club 29: 468, 1902.*

Glabricarpae E. B. Alexeev, Bot. Zhurn. 67(9): 1291, 1982.

breviglumis Swallen, Contr. US Natl. Herb. 29(9): 398, 1950.*

caldasii (Kunth) Kunth, Rev. Gram. 1: 132, 1829.

chiriquensis Swallen, Ann. Missouri Bot. Gard. 30(2): 116, 1943.

presliana A.S.Hitchcock, Contr. US Natl. Herb. 24(8):

320, 1927.

reclinata Swallen, Contr. US Natl. Herb. 29(6): 254, 1948.

steinbachii E. B. Alexeev, Bot. Zhurn. 70(9): 1243, 1985.

Subuliflorae E. B. Alexeev, Novost. Sist. Vyssh. Rast. 17: 47, 1980.

subuliflora Scribner in Macoun, Cat. Canad. Pl. Add. a Corr. 1-4: 396, 1890.*

= *ambigua* Vasey, Contr. US Natl. Herb. 1: 277, 1893, non LeGal. 1852.

= *denticulata* Beal, Grass. N. Amer. 2: 589, 1896.

Asperifolia E. B. Alexeev, Bot. Zhurn. 66(10): 1496, 1981.

asperella E. B. Alexeev, Bot. Zhurn. 66(10): 1496, 1981.

lugens (Fournier) A.S.Hitchcock ex Hernandez, Bol. Soc. Bot. Mexico 23: 165, 1958.*

= *mirabilis* Piper, Contr. US Natl. Herb. 10: 47, 1906.

Schedonorus (Beauvois) Peterman, Deutschl. Fl.: 643, 1849, s. str.

= F. section *Schedonorus* (Beauvois) Koch, Syn. Fl. Germ. et Helv.: 813, 1837 s. str.

= F. subtaxon *Bovinae* Fries ex Andersson, Gram. Scand.: 17, 1852; section *Bovinae* (Fries ex Andersson) Hackel, Monogr. Festuc. Europ.: 79, 148, 1882

Plantynia (Dumortier) Tzvelev, Zlaki SSSR: 394, 1976.

= *Schedonorus* sect. *Plantynia* Dumortier, Fl. Belg. Prodr.: 159, 1827; F. section *Bromoides* Rouy, Fl. Fr. 14: 225, 1913.

gigantea (L.) Villars, Hist. Pl. Dauph. 2: 110, 1787*.

mazzettiana E. B. Alexeev, Byull. Mosk. obshch. isp. prir. otd. biol. 82(3): 99, 1977.
 = mairei Hackel ex Hand.-Mazz., Symb. Sin. 7: 1288, 1936, non St. Yves 1922.

Schedonorus

apennia De Not., Prosp. Fl. Ligur. 2: 502, 1844.

arundinacea Schreber, Spicil. Fl. Lips.: 57, 1771.*
 = elatior L., Spec. Plant.: 75, 1753.
 subsp. arundinacea
 subsp. fenas (Lag.) Arcang., Compend. Fl. Ital., ed. 2: 61, 1894.
 subsp. orientalis (Hackel) Tzvelev, Spisok rast. Gerb. fl. SSSR 18: 17, 1970.

pratensis Hudson, Fl. Angl.: 37, 1762.

Scariosae Hackel, Monogr. Festuc. Europ. :80, 193, 1882.

mairei St. Yves, Candollea 1: 45, 1922.

scariosa (Lag.) Aschers. & Grab., Syn 2:502, 1900.

Erosiflorae E. B. Alexeev, Novost. Sist. Vyssh. Rast. 23: 11, 1986.

quadridentata Kunth, Nov. Gen. et Sp. 1: 125, 1815.*

Obtusae E. B. Alexeev, Novost. Sist. Vyssh. Rast. 17: 45, 1980.

Obtusae [E. B. Alexeev, Novost. Sist. Vyssh. Rast. 25: 13, 1988.]

paradoxa Desv., Opusc.: 105, 1831.*

subverticillata (Persoon) E. B. Alexeev, Novost. Sist. Vyssh. Rast. 17: 52, 1980
 = obtusa Biehler, Pl. Nov. Herb. Spreng. Cent.: 10, 1807.

Fauria E. B. Alexeev, Novost. Sist. Vyssh. Rast. 25: 13, 1988.

japonica Makino, Bot. Mag. (Tokyo) 20: 83, 1906.*

Xanthochloa (Krivot.) Tzvelev, Bot. Zhurn. 56(9): 1253, 1971.

karatavica (Bunge) B. Fedtsch., Izv. Peterb. Bot. Sada 14, suppl. 2: 86, 1915.*

= kronenburgii Hackel, Allg. Bot. Zeitschr. 6: 132, 1905.

Leucopoa (Grisebach in Ledebour) Hackel, Feddes Repert. 2, 18: 70, 1.III.1906.

= Hesperochloa Piper, Contr. US Natl. Herb. 10(1): 40, 30.III.1906.

Amphigenes (Janka) Tzvelev, Bot. Zhurn. 56(9): 1253, 1971.

carpatica F. Dietr., Nachtr. Vollst. Lexic. Gaertn. u. Bot. 3: 333, 1817.*

Leucopoa (Grisebach in Ledebour) Krivot., Bot. Mat. (Leningrad) 20: 48, 1960.

blepharogyna (Ohwi) Ohwi, Acta Phytotax. Geobot. (Kyoto) 10(2): 114, 1941.

caucasica (Boisser) Hackel ex Trautv., Tr. Peterb. Bot. Sada 9(1): 401, 1884.

hedgeri (Bor) E. B. Alexeev, Byull. Mosk. obshch. isp. prir. otd. biol. 82(3): 100, 1977.

hubsugulica Krivot., Bot. Mat. (Leningrad) 17: 77, 1955.

kingii (S.Watson) Cassidy, Col. Agric. Expt. Sta. Bull. 12: 36, 1890.

= confinis Vasey, Contr. US Natl. Herb. 1: 126, 1884.

komarovii Krivot., Bot. Mat. (Leningrad) 17: 80, 1955.

krivotulenkoa E. B. Alexeev, Ovsyaitsy Kavkaza: 64,

1980.

lucida Stapf in Hooker fil., Fl. Brit. Ind. 7: 355, 1897.

nepalica E. B. Alexeev, Byull. Mosk. obshch. isp. prir. otd. biol. 82(3): 99, 1977.

olgae (Regel) Krivot., Bot. Mat. (Leningrad) 20: 56, 1960.

schischkinii Krivot., Bot. Mat. (Leningrad) 17: 83, 1955.

sclerophylla Boisser & Hohen. in Boisser, Diagn. Pl. Or. ser. 1, 13: 59, 1853.

sibirica Hackel ex Boisser, Fl. Or. 5: 626, 1884.*

takedana Ohwi, Acta Phytotax. Geobot. (Kyoto) 10(2): 113, 1941.

Breviaristatae Krivot., Bot. Mag. (Leningrad) 20: 48, 1960.

alata (St. Yves) Roshevitz in Komarov, Fl. URSS 2: 528, 1934.

altaica Trinius in Ledebour, Fl. Altaica 1: 109, 1829.*

barqusinensis Malysh., Novost. Sist. Vyssh. Rast. 7: 296, 1971.

californica Vasey, Contr. US Natl. Herb. 1: 277, 1893.

hallii (Vasey) Piper, Contr. US Natl. Herb. 10(1): 40, 1906.

hitchcockiana E. B. Alexeev, Byull. Mosk. obshch. isp. prir. otd. biol. 87(2): 111, 1982.

iranica E. B. Alexeev, Byull. Mosk. obshch. isp. prir. otd. biol. 82(3): 101, 1977.

popovii E. B. Alexeev, Byull. Mosk. obshch. isp. prir. otd. biol. 83(5): 94, 1978.

= *insularis* M. Pop., Bot. Zhurn. 18: 4, 1957, non Steudel 1855.

thurberi Vasey in Rothr., Cat. Pl. Survey W. 100 Merid.: 56, 1874.

tristis Kryl. & Ivanitzk., Sist. Zam. Gerb. Tomsk. Univ. 1: 1, 1928.

Festuca

= *Eufestuca* Grisebach, Spicil. Fl. Rum. 2:432, 1844.
= *Arnochloa* Kirschl., Fl. vogéso-rhén. :285, 1870.

Variae Hackel, Monogr. Festuc. Europ.: 169, 1882.

karabaghensis Musajev, Bot. Zhurn. 72: 94, 1987.

pallidula E. B. Alexeev in E. B. Alexeev, A. P. Sokolovskaya & N. S. Probatova, Byull. Mosk. obshch. ips. prir. otd. biol. 93(2): 97, 1988.

varia Haenke in Jacq., Collect. Bot. 2: 94, 1789.*

versicolor Tausch, Flora 4: 359, 1821.

woronowii Hackel, Vestn. Tifl. Bot. Sada 24: 17, 1912.
subsp. *caucasica* (St. Yves) E. B. Alexeev, Ovsyaitsy Kavkaza: 74, 1980. = subsp. *caucasica* (St. Yves) Markgraf-Dannenberg, Willdenowia 11(2): 208, 1981.
subsp. *woronowii*

Cataphyllophorae E. B. Alexeev, Bot. Zhurn. 69(11): 1546, 1984.

columbiana E. B. Alexeev, Bot. Zhurn. 69(11): 1546, 1984.

dasyantha Kunth, Nov. Gen. et Sp. 1: 125, 1815.

procera Kunth, Nov. Gen. et Sp. 1: 124, 1815.*

Festuca

abyssinica Hochst. ex A. Richter, Tent. Fl. Abyss. 2: 432, 1851.

acamptophylla (St. Yves) E. B. Alexeev, Bot. Zhurn. 71(8): 1113, 1986.

- acanthophylla* E. Desv. in C. Gay, Fl. Chil. 6: 434, 1853.
- afghanica* Bor in Rech., Fl. Iran. 70: 766, 1970.
- alaica* Drobov, Tr. Bot. muz. Akad. nauk SSSR 16: 134, 1916.
- alexeenkoi* E. B. Alexeev, Byull. Mosk. obshch. ips. prir. otd. biol. 78(3): 104, 1973.
- algeriensis* E. B. Alexeev, Novost. Sist. Vyssh. Rast. 11: 55, 1974.
- amblyodes* Krecz. & Bobrov in Komarov, Fl. URSS 2: 529, 771, 1934.
- amethystina* L., Spec. Plant.: 74, 1753.
- amurensis* E. B. Alexeev, Byull. Mosk. obshch. ips. prir. otd. biol. 85(4): 87, 1980.
- ancachsana* E. B. Alexeev, Byull. Mosk. obshch. ips. prir. otd. biol. 89(4): 115, 1984.
- andicola* Kunth, Nov. Gen. et Sp. 1: 124, 1815.
- arizonica* Vasey, Contr. US Natl. Herb. 1: 277, 1893.
- asperula* Vickery, Contr. NSW Natl. Herb. 1(1): 12, 1939.
- asplundii* E. B. Alexeev, Byull. Mosk. obshch. ips. prir. otd. biol. 89(4): 115, 1984.
- auriculata* Drobov, Tr. Bot. Inst. Acad. Nauk SSSR 14: 159, 1915.
- azgarica* E. B. Alexeev, Byull. Mosk. obshch. ips. prir. otd. biol. 78(3): 107, 1973.
- azucaria* E. B. Alexeev, Bot. Zhurn. 69(11): 1546, 1984.
- baffinensis* Polunin, Bull. Natl. Mus. Canada 92, Biol. 24: 91, 1940.
- balansae* E. B. Alexeev, Byull. Mosk. obshch. ips. prir. otd. biol. 83(5): 97, 1978.
- beamani* E. B. Alexeev, Bot. Zhurn. 66(10): 1500, 1981.
- beckeri* (Hackel) Trautv., Acta Horti Petropol. 9(1):

- 325, 1884.
subsp. *beckeri*
subsp. *polesica* (Zapal.) Tzvelev, Spisok rast.
Gerb. fl. SSSR 18: 15, 1970.
subsp. *sabulosa* (Andersson) Tzvelev, Spisok rast.
Gerb. fl. SSSR 18: 14, 1970.
- benthamiana* Vickery, Contr. NSW Natl. Herb. 1(1): 12,
1939.
- bhutanica* E. B. Alexeev, Byull. Mosk. obshch. ips.
prir. otd. biol. 83(4): 117, 1978.
- boliviana* E. B. Alexeev, Bot. Zhurn. 70(9): 1243, 1985.
- boriana* E. B. Alexeev, Byull. Mosk. obshch. ips. prir.
otd. biol. 83(4): 115, 1978.
- borissii* Reverd., Sist. Zam. Gerb. Tomsk. Univ. 83: 8,
1965.
- brachyphylla* Schultes & Schultes fil., Add. ad Mant. 3:
646, 1827.
- brevipaleata* (St. Yves) E. B. Alexeev, Bot. Zhurn.
71(8): 1113, 1986.
- brevissima* Jurtzev, Bot. Zhurn. 57(6): 645, 1972.
- brunnescens* (Tzvelev) Galushko,
= subsp. *brunnescens* (Tzvelev) E. B. Alexeev,
Byull. Mosk. obshch. ips. prir. otd. biol. 78(3):
109, 1973.
- burmanica* E. B. Alexeev, Byull. Mosk. obshch. ips.
prir. otd. biol. 83(4): 116, 1978.
- buschiana* (St. Yves) Tzvelev, Bot. Zhurn. 56(9): 1254,
1971.
- callieri* (Hackel) Doerfl. ex Domin, Acta Bot. Bohem. 8:
61, 1929.
- calligera* (Piper) Rydberg, Bull. Torrey Bot. Club 36:
537, 1909.
- callosa* (Piper) St. Yves, Candollea 2: 291, 1925.
- camerunensis* E. B. Alexeev, Bot. Zhurn. 72(9): 1267,
1987.

- camusiana* St. Yves, Bull. Soc. Bot. Geneve II 18: 156, 1926.
- canadensis* E. B. Alexeev, Byull. Mosk. obshch. ips. prir. otd. biol. 88(5): 104, 1983.
- caprina* Nees, Fl. Afr. Austr. 1: 443, 1841.
- cartagana* E. B. Alexeev, Bot. Zhurn. 67(9): 1291, 1982.
- chalcophaea* V. Krecz. & Bobrov in Komarov, Fl. URSS 2: 513, 768, 1934.
- chiisanensis* (Ohwi) E. B. Alexeev, Byull. Mosk. obshch. ips. prir. otd. biol. 85(4): 86, 1980.
- chimbrazensis* E. B. Alexeev, Bot. Zhurn. 72(9): 1549, 1987.
- chionobia* Egorova & Siplivinsky, Novost. Sist. Vyssh. Rast. 6: 226, 1970.
- chodatiana* (St. Yves) E. B. Alexeev, Bot. Zhurn. 71(8): 1113, 1986.
- chumbiensis* E. B. Alexeev, Byull. Mosk. obshch. ips. prir. otd. biol. 83(4): 118, 1978.
- claytonii* E. B. Alexeev, Bot. Zhurn. 71(8): 1117, 1986.
- cleefiana* E. B. Alexeev, Bot. Zhurn. 72(9): 1548, 1987.
- coelestis* (St. Yves) V. Krecz. & Bobrov in Komarov, 2: 514, 770, 1934.
- cretacea* T. Pop. & Proskor., Bull. Obshch. Estestvoisp. Voronezh. Univer. 2(1): 46, 1927.
- cryptantha* T.A.Cope, Kew Bull. 39(4): 834, 1984.
- cumminsii* Stapf in Hooker fil., Fl. Brit Ind. 7: 349, 1897.
- cundinamarcae* E. B. Alexeev, Bot. Zhurn. 72(9): 1548, 1987.
- daghestanica* (Tzvelev) E. B. Alexeev, Ovsyaitsy Kavkaza: 77, 1980.
- dahurica* (St. Yves) Krecz. & Bobrov in Komarov, Fl. URSS 2: 517, 771, 1934.

dasyclada Hackel in Beal, Grass. N. Amer. 2: 602, 1896.
 = *Argillochloa dasyclada* (Hackel in Beal) W.A.
 Weber, Phytologia 55(1): 1, 1984.

debilis (Stapf in Hooker fil.) E. B. Alexeev, Byull.
 Mosk. obshch. ips. prir. otd. biol. 83(4): 109, 1978.

densipaniculata E. B. Alexeev, Bot. Zhurn. 72(9): 1551,
 1987.

densiuscula (Hackel ex Piper) E. B. Alexeev, Byull.
 Mosk. obshch. ips. prir. otd. biol. 87(2): 113, 1982.

dolichophylla J.S. Presl in C. B. Presl, Rel. Haenk. 1:
 258, 1830.

djimilensis Boissier & Bal., Bull. Soc. Bot. Fr. 21: 18,
 1874.

earlei Rydberg, Bull. Torrey Bot. Club 32: 608., 1905.

elbrusica E. B. Alexeev, Byull. Mosk. obshch. ips.
 prir. otd. biol. 78(3): 107, 1978.

elgonensis E. B. Alexeev, Bot. Zhurn. 72(9): 1266,
 1987.

eriosstoma Hackel, Oesterr. Bot. Zeitschr. 103: 32,
 1903.

exaristata E. B. Alexeev, Bot. Zhurn. 71(8): 1116,
 1986.

fiebrigii Pilger, Bot. Jahrb. 37(5): 510, 1906.

filiformis Pourr., Mém. Acad. Sci. Toulouse 3: 319,
 1788.

= *tenuifolia* Sibth., Fl. Oxon.: 44, 1794.

forrestii St. Yves, Rev. Bret. No. 2: 16, 72, 1927;
 Candollea 3: 383, 1928.

frederikseniae E. B. Alexeev, Novost. Sist. Vyssh.
 Rast. 22: 28, 1985.

georgii E. B. Alexeev, Byull. Mosk. obshch. ips. prir.
 otd. biol. 83(5): 94, 1978.

glaucoidea (Vetter) E. B. Alexeev,

glumosa Hackel ex E. B. Alexeev, Bot. Zhurn. 72(9):
 1549, 1987.

- goloskokovii E. B. Alexeev, Novost. Sist. Vyssh. Rast. 13: 25, 1976.
- guatemalica E. B. Alexeev, Bot. Zhurn. 66(10): 1498, 1981.
- hartmannii (Markgraf-Dannenberg) E. B. Alexeev, Byull. Mosk. obshch. ips. prir. otd. biol. 83(4): 121, 1978.
- hedbergii E. B. Alexeev, Bot. Zhurn. 71(8): 1113, 1986.
- hephaestophila Nees ex Steudel, Syn. Pl. Glum.: 310, 1854.
- heterophylla Lamark, Fl. Fr. 3: 600, 1778.
- hieonymi Hackel, Oesterr. Bot. Zeitschr. 103: 33, 1903.
- hintoniana E. B. Alexeev, Bot. Zhurn. 67(9): 1292, 1982.
- hondae E. B. Alexeev, Byull. Mosk. obshch. ips. prir. otd. biol. 86(1): 70, 1981.
= formosana E. B. Alexeev, Byull. Mosk. obshch. ips. prir. otd. biol. 83(5): 98, 1978, non Honda
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subsp. *nipponica* (Ohwi) E. B. Alexeev, Byull. Mosk. obshch. ips. prir. otd. biol. 89(2): 119, 1984.

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- porcii* Hackel, Monogr. Festuc. Europ.: 147, 1882.
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- simpliciuscula* (Hackel) E. B. Alexeev, Bot. Zhurn. 66(3): 351, 1984.
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- skvortsovii* E. B. Alexeev, Byull. Mosk. obschch. ips. prir. otd. biol. 76(2): 119, 1971.
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- soratana* E. B. Alexeev, Bot. Zhurn. 70(9): 1244, 1985.
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 subsp. *valesiaca*
 var. *asperrima* (Hackel) E. B. Alexeev,
 var. *hirsuta* (Link) E. B. Alexeev, Byull. Mosk. obshch. ips. prir. otd. biol. 78(3): 109, 1973.
 var. *pseudovina* (Wiesb.) Schinz. & R. Keller, Fl. Schweiz, ed 2, Krit. Fl.: 26, 1905.
 var. *valesiaca*
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APPENDIX B. Collection vouchers for species used in this study. Species are listed in alphabetical order.

***Festuca amplissima* Rupr. ex Fourn.**

Mexico, Estado Mexico, 11 km E of 155 jct. (S. of Amecameca) on side ride S of road to Paso del Cortes, Volcanos National Park, west slope of Popocatepetl-Ixtaccihuatl, 2988 m elev., south facing slopes of canyon along roadsides in rich woodlands, *Pinus leiophylla*, *Abies religiosa*, *Cupressus*, *Quercus*, rich deep sandy loam, common, forming massive tussocks 2-3 m tall, 87.X.2, R. & N. Soreng 3305.

***Festuca arundinacea* Schreber**

Canada, British Columbia, New Westminster Dist., Abbotsford, 49°03'N 122°17'W, intersection of highways #1 and #11 along ramp, single clump at edge of *Phalaris arundinacea* stand in moist sandy-clay soil, 88.VI.29, S. J. Darbyshire 3723.

***Festuca brachyphylla* Schultes & Schultes fil.**

U.S.A., Colorado, Larimer Co., Mount Cameron, west side of Blue Lake, 40°37'30"N 105°54'30"W, sandy rocky ridge, alpine tundra, 88.VII.12, S. J. Darbyshire 3908.

***Festuca canadensis* E. B. Alexeev**

Canada, Ontario, Lampton Co., Bosanquet Tp. Mun., Ipperwash, 43°12'20"N 81°59'0"W, open area on wooded dunes with lichens, *Carex eburnea*, *Arctostaphylos uva-ursi*, *Poa compressa*, *Smilacena stellata*, *Pinus spp.*, *Thuja occidentalis*, *Juniperus communis*, *Senecio paupercula*, very common in small tufts, 85.VI.23, S. J. Darbyshire, M. J. Oldham & M. Delisle-Oldham 2588.

***Festuca dasyclada* Hackel ex Beal**

U.S.A., Colorado, Rio Blanco Co., Rio Blanco, Piceance Creek, T.4S R.94W, 39°38'N 107°58'W, rocky shale canyon with *Juniperus scoparius*, *Pseudotsuga menziesii*, *Pinus edulis*, 88.VII.13, S. J. Darbyshire 3916.

***Festuca filiformis* Pourret**

Canada, Ontario, Nipissing Dist., Canisbay Tp., Algonquin Provincial Park, Lake-of-Two-Rivers, airfield, 45°34'N 78°31'W, open sandy outwash plain, rare, 85.X.12, S. J. Darbyshire 2903.

***Festuca gigantea* (L.) Vill.**

Plant Gene Resources of Canada Accession No. 3212, received from Belgium, Antwerp, Botanical Garden, 1977.

Festuca hallii (Vasey) Piper

Canada, Manitoba, Riding Mountain National Park: Audy Lake, 50°45'N 100°15'W, shrubby prairie, 27.VI.88, S. J. Darbyshire 3716; Birdtail Bench, Central Trail, 83.VII.21, W. J. Cody 32699.

Festuca idahoensis Elmer

Canada, Alberta, Twin River Ecological Reserve, 49°01'N 112°21'W, widespread, tending to be associated with *F. hallii*, 82.VII.28, S. G. Aiken & S. J. Darbyshire 2508.

Festuca kingii (S. Watson) Cassidy

U.S.A.: Colorado, Boulder Co., Boulder, lower Boulder Canyon, 40°40'N 105°19'W, grassy hillside, south side of canyon (north aspect) with *Pinus ponderosa*, 88.VII.9, S. J. Darbyshire 3831, staminate and pistillate plants; Wyoming, Albany Co., Medicine Bow National Forest, 15 miles southwest of Laramie, mile 324 on highway #180 at intersection of road south to Tie Siding, 2400 m elev., gravel road side embankment, 88.VII.8, S. J. Darbyshire 3821.

Festuca minutiflora Rydberg

U.S.A., Clear Creek Co., Mount Evans, 39°35'N 105°38'W, 3800-3950 m elev., rocky alpine tundra, 88.VII.10, S. J. Darbyshire 3850.

Festuca pratensis Hudson

Plant Gene Resources of Canada Accession No. 2153, received from Vavilov Institute of Plant Industry (WIR 29779), 77.I.10, cv. Jygeva 47, USSR, Estonia.

Festuca roemerii (Pavlick) E. B. Alexeev

Canada, British Columbia, Vancouver Island, Saanich Peninsula, Mount Douglas Park, 48°29'N 123°21'W, open grass bald, shallow soil over bedrock, with *Quercus garryi*, *Arbutus menziesii*, *Cytisus scoparius*, *Aira praecox* and lichen ground cover, 88.VII.4, S. J. Darbyshire 3790.

Festuca rubra L.

Canada, Newfoundland, St. Barbe South Dist., Bonne Bay, East Arm, Tucker's Head near Lomond Cove, 49°28'N 57°46'W, talus limestone cliffs, 7 m elev, growing adjacent to *F. proliferata* (Piper) Fernald, 84.VII.23, S. G. Aiken & S. J. Darbyshire 2932.

Festuca saximontana Rydberg

U.S.A., Wyoming, Albany Co., Medicine Bow National Forest, 15 miles southwest of Laramie, mile 324 on highway #180 at intersection with road south to Tie Siding, 2400 m elev., gravel roadside embankment, 88.VII.8, S. J. Darbyshire 3822.

Festuca subulata Trinius in Bongard

Canada, British Columbia, Vancouver Island, Port Renfrew, Botany Bay, 48°31'N 124°27'W, along trail through thicket of *Gaultheria shallon*, 88.VII.2, S. J. & J. A. Darbyshire 3760.

Festuca subuliflora Scribner in Macoun

Canada, British Columbia, Vancouver Island, Saanich Peninsula, John Dean Park, just outside park boundary, 48°36'N 123°27'W, a few plants at edge of disturbed clearing in *Pseudotsuga menziesii*, *Thuja plicata* woods, 88.VII.1, S. J. Darbyshire 3751.

Festuca subverticillata (Persoon) E. B. Alexeev

Canada, Ontario: Essex Co., Mersea Tp., 5 km NE of Leamington, 42°05'15"N 82°32'20"W, moist deciduous woods with sandy ridges and moist depressions, 85.VI.24, S. J. Darbyshire 2593; Ottawa Carleton Regional Municipality, Osgoode Tp., 5 km south of Greely, 45°13'15"N 75°33'30"W, in moist woods of *Acer rubrum*, *Tilia americana*, *Fraxinus nigra*, 87.XI.12, S. J. Darbyshire 3629.

Festuca thurberi Vasey

U.S.A., Colorado, Clear Creek Co., Arapahoe National Forest, near Ponder Point Picnic Ground, 39°41'N 105°36'W, 3000 m elev., west aspect, grassy slope in open woods of *Pinus aristata* and *Populus tremuloides*, 88.VII.10, S. J. Darbyshire 3844.

Festuca tolucensis Kunth

Mexico, Estado Mexico, Volcanos National Park, Ixtaccihuatl, along trail above La Joya, 4085 m elev., steep west facing slopes, 1 km north of La Joya, in deep rich volcanic soil, dominated by *Festuca tolucensis* and *Calamagrostis tolucensis*, 87.X.3, R. Soreng & N. Soreng 3324a.

Festuca trachyphylla (Hackel) Krajina

Canada, Ontario, Thunder Bay Dist., Coldwell Tp., Neys Provincial Park, park gate, 48°48'N 86°37'W, growing in gravel at edge of asphalt, 85.VII.28, S. J. Darbyshire 2771.

Festuca viridula Vasey

Canada, British Columbia, Manning Provincial Park, Blackwall Mountain, subalpine meadow, 88.VII.6, S. J. Darbyshire 3793.

Lolium perenne L.

Plant Gene Resources of Canada Accession No. 7768, received from Hankkija Plant Breeding Institute, 1979, cv Valinge, Finland, Hyryla.

Poa pratensis L.

Canada, Ontario, Ottawa Carleton Regional Municipality, Gloucester, just south of Uplands Airport along Rideau River, seeded lawn, 88.XI.6, S. J. Darbyshire s. n.

Puccinellia distans (Jacq.) Parl

Canada, Ontario, Ottawa Carleton Regional Municipality, Ottawa, highway # 417 at St. Laurent Blvd., disturbed saline soil at edge of highway ramp, with *Spergularia* and *Chenopodium*, 88.XI.6, S. J. Darbyshire & P. Uptegrove s. n.

Vulpia myuros (L.) K. C. Gmelin

Canada: British Columbia, Vancouver Island, Trevor Channel, Poett Nook, 48°52'N 125°03'W, between parking lot and forest in disturbed grassy area, 82.VII.6, S. G. Aiken, S. J. Darbyshire & L. E. Pavlick 2211; Ontario, Kent Co., Dover Tp., St. Clair National Wildlife Area, on border dyke with Balmoral Marsh, rare and local on sandy moist ground at edge of marsh, 87.VII.7, M. J. Oldham & M. Delisle-Oldham 7294.

APPENDIX C. DNA extraction, purification, endonuclease digestion and restriction fragment visualization. Suppliers are indicated by superscripts: 1, Sigma Chemical Co.; 2, BDH Chemicals; 3, Fisher Scientific; 4, Pharmacia LKB; 5, Boehringer Mannheim; 6, New England Biolabs; 7, J. T. Baker Inc.; 8, ICN Biotrans Nylon Membranes; 9, Dupont.

EXTRACTION

1. About 3-4 g (fresh weight) apparently healthy, green leaves were pulverized under liquid nitrogen with a mortar and pestle and about 10-15 ml of hot (65°C) CTAB buffer [0.1M trishydroxymethylaminomethane (Tris)¹ (pH 8.0), 1.4M sodium chloride² (NaCl), 20mM ethylenediaminetetraacetic acid (EDTA)¹, 2% mixed hexadecyltrimethylammonium bromide¹, 0.2-0.4% b-mercaptoethanol¹].
2. Sludge transferred to a 50 ml polypropylene tube using another 5 ml of buffer to rinse mortar.
3. Incubated at 65°C for 30-45 minutes.
4. 15 ml (equal volume) phenol³: chloroform²: iso-amyl alcohol³ (25:24:1) added.
6. Sample shaken vigorously for 30 seconds by hand.
7. Centrifuged at 6000 rpm for 5 minutes.
8. Aqueous upper layer transferred into new 50 ml polypropylene tube.
9. About equal volume of chloroform: iso-amyl alcohol (24:1) added.
10. Sample shaken vigorously for about 10 seconds on vortex mixer.
11. Centrifuged at 6000 rpm for 5 minutes.
12. Aqueous upper layer transferred to new 50 ml polypropylene tube.
13. DNA precipitated by adding 0.1× volume of 3.3M sodium acetate³ (pH 5.5).
14. About 2× new volume 95% ethanol at -20°C added and tube inverted repeatedly to mix well.
16. Held for 20 minutes (maximum) at -80°C or 1 hour (minimum) at -20°C.

17. Centrifuged at 6000 rpm for 15 minutes and supernatant poured off.
18. Pellet washed in about 5 ml 70% ethanol (-20°C); centrifuged at 6000 rpm for about 3 minutes and supernatant poured off.
19. Pellet re-dissolved in 5 ml of 50mM NaCl: 50mM Tris: 5mM EDTA (pH 8.0) by placing on shaker at 37°C.
20. 5.0 g cesium chloride¹ added and dissolved.
21. Using a 10 ml syringe and 16G needle, 0.15 ml (10 mg/ml) ethidium bromide¹ added to a 6.3 ml Beckman quick seal polyallomer centrifuge tube then sample added.
22. Tubes balanced in pairs and sealed with heat sealer, and shaken to mix contents.
23. Centrifuged at 55,000 rpm for at least 16 hours at 15°C (Beckman L8-70M ultracentrifuge).
24. After unloading rotor, tubes clamped in front of long-wave UV lamp to visualize fluorescing band.
25. Tubes punctured about 1 mm below band with 20G syringe needle, with tip inserted horizontally to the centre of tube.
26. 11.5 ml polypropylene tube placed under needle to catch drips.
27. Top of tube punctured with 21G needle attached to 10 ml syringe with plunger withdrawn. Using minimum force necessary on plunger, drips caught until all fluorescent material was collected.
28. About equal volume (or less) isopropanol³ taken from upper layer of isopropanol-saturated cesium chloride in dH₂O mixture was added.
29. Tubes capped and rocked 4-5 times, not vigorously, allowed to stand until layers separate, then upper pink layer discarded with transfer pipette.
30. Steps 28 and 29 repeated until no more 'colour' could be extracted.
32. dH₂O added to double volume in tube.
33. Steps 13-17 inclusive were repeated to precipitate DNA.
34. Pellet washed (loosen but not dissolved) once in about 5 ml

70% ethanol (-20°C) and centrifuged 3 minutes. Supernatant poured off.

35. Remaining liquid evaporated from pellet in vacuum desiccator.
36. DNA re-suspended overnight in 400 ml sterile 2:5:1 buffer (2mM Tris: 5mM NaCl: 0.1mM EDTA (pH 7.7)).
37. Sample transferred to 1.5 ml Eppendorf tube.
38. DNA precipitated by adding 0.5× volume (20 ml) of 3.3M sodium acetate (pH 5.5).
39. 1 ml 95% ethanol (-20°C) added. Tubes capped and repeatedly inverted to mix contents well.
40. After at least 1 hours at -20°C, sample microcentrifuged for 5 minutes Supernatant poured away.
41. With minimum disturbance to pellet, 0.5 ml 70% ethanol (-20°C) added. Rocked gently to wash.
42. Microcentrifuged for 3 minutes and supernatant poured away. Swab used to remove droplets from tube walls.
43. Remaining liquid evaporated from pellet in vacuum desiccator.
44. DNA re-suspended by gentle shaking overnight on bench in 100 ml sterile 2:5:1 buffer. Stored at -20°C.

ESTIMATE DNA CONCENTRATION

45. A 3 ml aliquot of sample diluted in 297 ml of 2:5:1 buffer.
46. Optical density of this diluted sample read at 260 nm (Bausch and Lomb spectrophotometer), and DNA concentration of original sample calculated.
47. Sample diluted to 1 mg/ml with sterile 2:5:1 buffer.
48. Samples stored at -20°C.

DNA DIGESTION WITH RESTRICTION ENDONUCLEASES

49. In a 1.5 ml Eppendorf tube add in the following order:
 - 13 ml sterile H₂O
 - 4 ml 5× restriction reaction buffer for chosen enzyme^{4,5,6}
 - 3-5 mg sample DNA in solution
50. 5-10 units of restriction endonuclease enzyme^{4,5} (Table 4)

added.

51. Mixed well, letting all droplets coalesce.
52. Incubated at 37°C for all enzymes (*Bcl*I at 50°C) for at least 4 hours.
53. Reaction stopped by adding 10 ml Stop buffer/tracking dye [Running buffer (89mM Tris; 2.5mM EDTA; 89mM boric acid²) with 10% ficoll¹, 20% Sucrose¹, 0.5% sodium dodecyl sulfate¹ (SDS), 0.1% bromophenol blue¹, 0.1% antifoam "B"²]. Mixed well.

AGAROSE GEL ELECTROPHORESIS

54. 0.8% agarose¹ prepared in running buffer (step 53).
55. Boiled briefly and swirled to mix well.
56. Placed in water bath and allow to cool to 55°C.
57. Poured into gel mould, 17 slot comb inserted to form sample wells, allowed to solidify for 45 minutes minimum.
58. Dams removed from gel mould. Gel submerged in running buffer in electrophoresis apparatus. Comb removed carefully.
59. Each well loaded with 25-30 ml restricted DNA sample from STEP 53.
60. Run at 20 volts for about 16 hours.
61. When bromophenol blue dye reached foot of gel, gel removed from rig.
62. Gel slid off support plate into 500 ml H₂O containing 25 ml 10 mg/ml ethidium bromide.
63. Stained about 30 minutes, and viewed on short wave UV-transilluminator to check digestion.

ALKALINE SOUTHERN TRANSFER TO NYLON MEMBRANE

64. Gel trimmed to minimum size.
65. Gel shaken gently in 500 ml 0.25M hydrochloric acid⁷ (HCl) for 8 minutes.
66. Gel transferred to 500 ml dH₂O for one minute.

67. Gel shaken in 0.4M sodium hydroxide³ (NaOH) for at least 30 minutes.
68. 2 pieces of Biotrans nylon membrane⁸ trimmed to fit gel.
69. 4 pieces of Whatman 3MM and 4 pieces of Whatman 17MM filter paper trimmed to the same size as the membrane.
70. Membranes soaked in dH₂O for 0.5-2 hours and filter paper soaked in dH₂O for 10-15 minutes prior to use.
71. "Gel Sandwich" assembled as follows:
 - 1½ inch paper serviettes (as flat as possible)
 - 2 pieces of Whatman 17MM paper
 - 2 pieces of Whatman 3 MM paper
 - 1 piece of nylon membrane
 - gel
 - 1 piece of nylon membrane
 - 2 pieces of Whatman 3 MM paper
 - 2 pieces of Whatman 17 MM paper
 - 1½ inch paper serviettes
 - plexiglass plate
 - weight, balanced evenly

Level checked periodically and transfer allowed to proceed overnight.
72. Wet serviettes replaced with dry ones and weight re-balanced after several hours.
73. "Gel sandwich" disassembled. Membranes shaken in 3M NaCl/0.5 M Tris (pH 7.0-8.0) for 15 minutes.
74. Membranes shaken in 2× SSC (1× SSC = 3M NaCl, 0.3M citric acid trisodium salt) for 10 minutes.
75. Membranes air dried 30 minutes to 2 hours.
76. DNA covalently bonded to membrane with shortwave UV exposure for 3 minutes.
77. Membranes dried in vacuum oven at 80°C for 1-2 hours.
78. Membranes stored in filter paper sleeves in air-tight zip-lock bag at 4°C.

MEMBRANE PREHYBRIDIZATION

79. Membranes unwrapped (if new membranes, go to step 81).

80. Membranes wetted by covering with 2× SSC solution for 5 minutes.
81. FOR NEW MEMBRANES: Membranes washed in 500 ml of 0.1× SSC, 0.5% SDS at 65°C for 1 hour.
82. Membranes to be probed stacked 10-12 at a time and heat sealed in polyethylene bag.
83. One corner of bag cut off.
84. 100 ml of prehybridization solution (0.5% nonfat dry milk (skim milk powder); 2% SDS; 4× SSC) added.
85. Air bubbles removed by rolling a pipette across the bag on an angled surface.
86. Bag heat sealed across cut corner of bag.
87. Incubated with rotary shaking (200 rpm) at 65°C overnight.

DNA NICK TRANSLATION - MAKING THE RADIOACTIVE PROBE

88. Into a 2 ml screw-top reaction tube or Eppendorf tube add:
4 μ l nick translation buffer (Eppendorf Nick Translation Kit)
0.2 μ l DNA clone probe (1 mg/ml solution)
5.8 μ l sterilized H₂O
89. 2-3 μ l [α^{32} P]dCTP⁹ added.
90. 2 μ l DNase I/DNA polymerase I solution added, contents mixed well.
91. Incubated at 16°C for 90 minutes.
92. A 5 cm Sephadex G50 column⁴ prepared and equilibrated with about 30 ml of TE buffer (10mM Tris; 1mM EDTA (pH 8.0)).
93. Nick translation reaction stopped by adding 80 ml TE buffer and 100 ml phenol: chloroform: iso-amyl alcohol (25:24:1) with a few grains of bromophenol dye.
94. Vortexed briefly to emulsify.
95. Centrifuged for 2 minutes.
96. Nick translation solution loaded into Sephadex column.
97. Translations eluted with 100 μ l aliquots of TE buffer and collected in 3 drop fractions.

98. 4 most active fractions in the first peak of activity to be eluted from the column kept.
99. Heated in boiling water bath for 5 minutes to denature and then plunged into ice.

PROBING MEMBRANES/HYBRIDIZATION

100. Corner of prehybridizing bag cut off and excess liquid drained leaving about 10-15 ml.
101. Radioactive fractions added to bag.
102. Air excluded as much as possible.
103. Bag secured flat in radio-opaque box container and incubated at 65°C overnight on rotary shaker.

MEMBRANE WASHING & AUTORADIOGRAPHY

104. Corner of bag cut off and as much liquid as possible drained.
105. Bag cut open and filters transferred to 500 ml membrane wash buffer (2× SSC; 0.5% SDS) at room temperature with gentle shaking.
106. Membranes shaken for five minutes. Room temperature wash repeated once more.
107. Membranes washed 2 more times for 30-40 minutes at 65°C.
108. Membranes set out to dry on paper towels.
109. Membranes assembled in X-RAY cassettes for autoradiography.
110. Cassette loaded with Kodak X-Omat AR film and left at room temperature or in -80°C freezer for desired exposure time.
111. Membranes rinsed with 500 ml boiling strip/rinse solution (0.1× SSC). Shaken gently for 5 minutes at 65°C. Repeated 3 additional times.
112. Membranes stored in air-tight plastic bags at 4°C until reused.

APPENDIX D. Data matrices used in the various analyses. Taxon codes are given in Table 2 of main text.

Table 1. Matrix of cpDNA restriction site (67 polymorphic shared) characters used in analysis with PAUP 2.4. Site present, 1; site absent, 0 (Table 5, main text). Taxa by row.

```

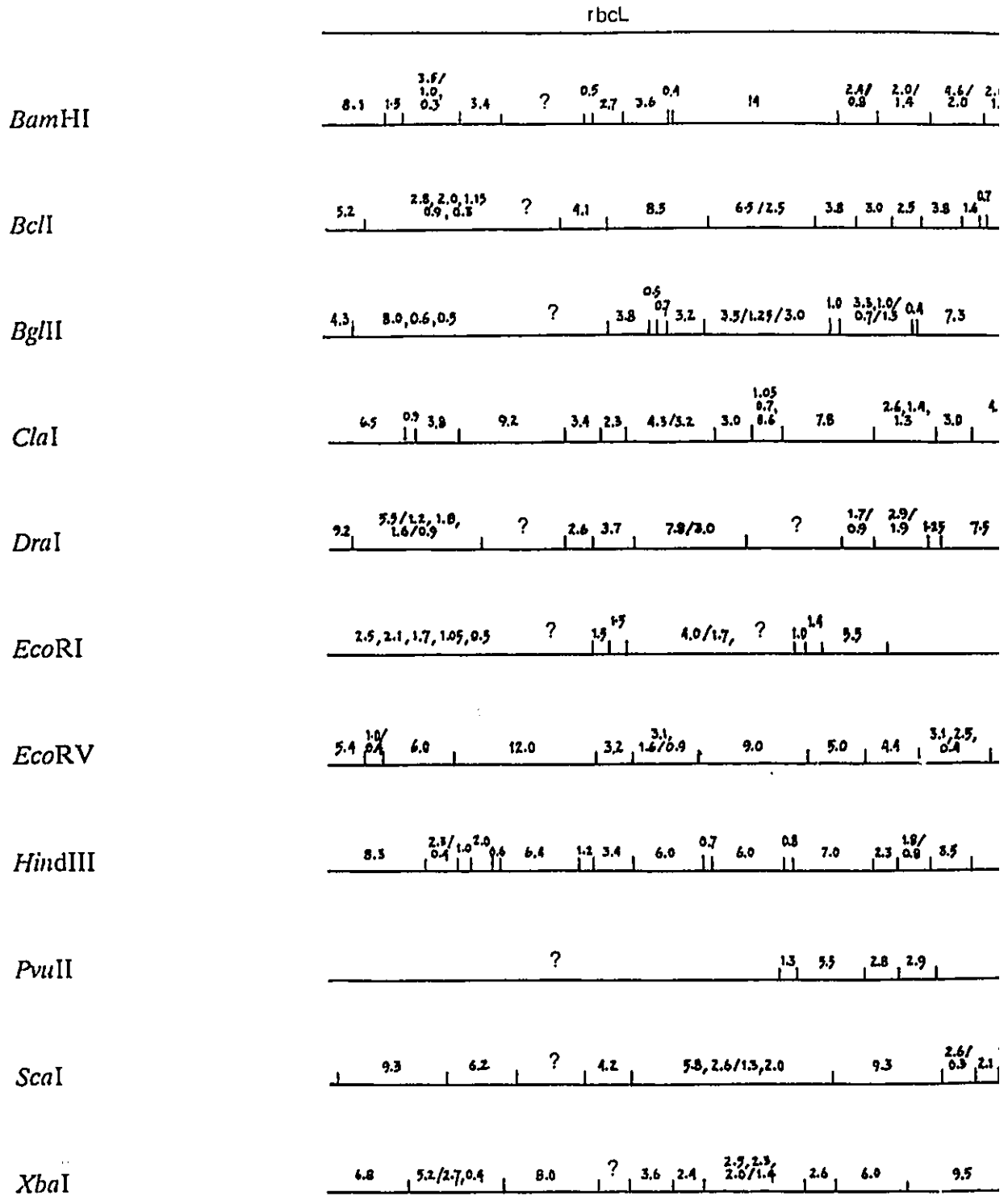
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fp 1001100100010000000111001100001000100011110000110000101010000001000
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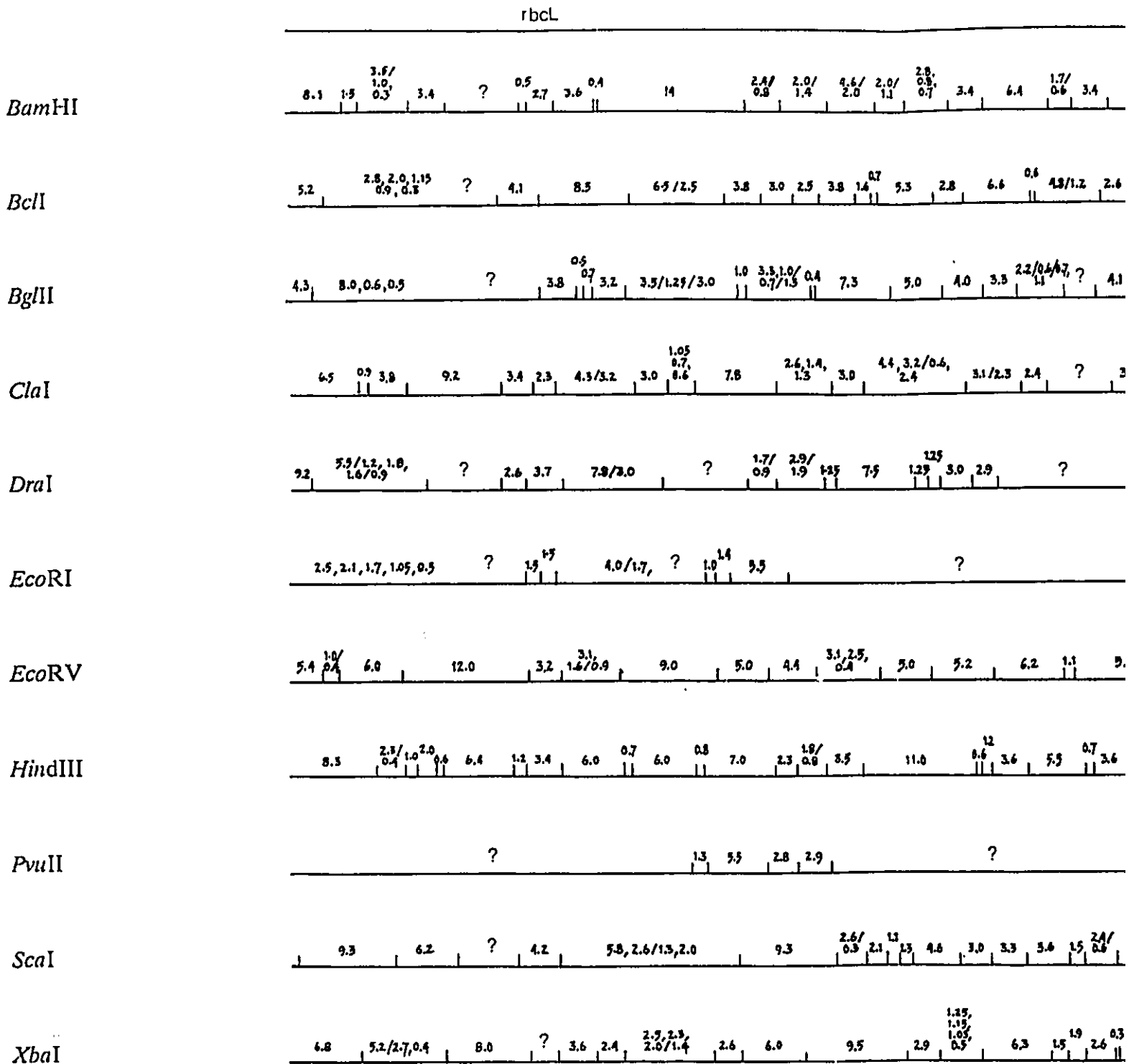
Table 2. Matrix of cpDNA restriction site (108 polymorphic) characters used in analysis with NTSYS-pc 1.6. Coding as in Table 1 (Appendix D). Taxa by columns.

sf	sl	sv	fp	fg	fk	fm	fr	fd	ff	ft	fv	fy	fi	fb	fs	fc	fo	fn	fl	fh	fa	vm	lp	pp	pd
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0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
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1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1

APPENDIX E. Generalized maps of the restriction endonuclease s exemplars. Map at top shows position of ribulose-1,5-bisphosph (IR). Fragments of unknown order are separated by commas or a



APPENDIX E. Generalized maps of the restriction endonuclease sites detected in t exemplars. Map at top shows position of ribulose-1,5-bisphosphate carboxylase (r bcl) (IR). Fragments of unknown order are separated by commas or a slash if they adjc



detected in the chloroplast genome combined for 26
 carboxylase (rbcL) and the inverted repeat regions
 if they adjoin. ? = unknown region.

