Evolutionary history of the canary grasses (*Phalaris*, Poaceae)

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ABSTRACT

Canary grasses (*Phalaris*, Poaceae) include 21 species widely distributed throughout temperate and subtropical regions of the world with centers of diversity in the Mediterranean Basin and western North America. The genus contains annual/perennial, endemic/cosmopolitan, wild, and invasive species with basic numbers of x=6 (diploid) and x=7 (diploid/tetraploid/hexaploid). The latter display vastly greater speciation and geographic distribution. These attributes make *Phalaris* an ideal platform to study species diversification, dispersal, historic hybridization, polyploidy events, and chromosome evolution in the grasses.

This body of research presents the first molecular phylogenetic and phylogeographic reconstruction of the genus based on the nuclear ITS and plastid *trnT-F* DNA regions allowing species relationships and the importance of polyploidy in speciation to be assessed. Divergence dates for the genus were determined using Bayesian methods (BEAST, version 1.6.2) and historic patterns of dispersal were analyzed with RASP (version 2.1b). Self-incompatibility and the feasibility of hybridization between major groups within the genus were studied with a series of greenhouse experiments. Acetocarmine and fluorescent staining techniques were used to study the morphology of the chromosomes in a phylogenetic context and the nuclear DNA content (C values) was quantified using flow cytometry.

Four major clades were revealed in the genus with cytological and geographic affinities leading to the establishment of two subgenera and four sections in the first

comprehensive infrageneric treatment of *Phalaris*. Divergence dating revealed a Miocene emergence (20.6-8.4 MYA) for the genus which is concurrent with studies of other genera in the Aveneae tribe. The hypothesis stating that *Phalaris* originated in the Mediterranean Basin and dispersed to the New World via a western route leading to a secondary center of diversification in western North America was supported by phylogeographic and cytological analyses. An empirical study comparing the weight, length, and width of the florets by morphological type and cytotype revealed significant differences that support a dispersal advantage among the New World and Arundinacea species. The x=6 species displayed greater intraspecific C value variation, higher DNA content per haploid chromosome set, and a distinct karyotype compared with the x=7 species indicating a complex history of chromosome evolution.

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DEDICATION

To my parents, for inspiring my interest in nature and science.

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ATTRIBUTION

Dr. Khidir Hilu, Department of Biological Sciences, Virginia Tech, Blacksburg, VA, 24060. Dr. Hilu was my advisor and chair of my advisory committee. He is a co-author on all publications resulting from the research in my dissertation.

Dr. Riccardo Baldini is a collaborator on the *Phalaris* project from the University of Florence. Dr. Baldini provided the images used to generate Figures 1.1, 1.2, and 3.1 and is a co-author on the publications resulting from the research in chapters 1 and 3.

Rohit Kumar and Nicholas Tatalovich were undergraduate researchers in the lab who generated sequence data for a preliminary investigation of *Phalaris* and are co-authors on the manuscript resulting from the research in chapter 1.

INTRODUCTION

The Grass family (Poaceae) has played a significant role in the history of our planet both for human civilization and the biosphere as a whole. The Poaceae is the fourth largest angiosperm family in terms of diversity and the most dominant in terms of abundance (Shantz, 1954; Brooks & al., 2004). They have spread and adapted to many habitats taking advantage of the ecological diversity found across continents. Polyploidy is prominent in the grasses and is considered to be a significant factor leading to speciation and successful colonization of diverse habitats (Stebbins, 1985; Hunziker & Stebbins, 1987; De Wet, 1987; Levy & Feldman, 2002; Hilu, 2006). Currently, a staggering 80-90% of all grasses are believed to be of polyploidy origin (De Wet 1987; Hilu 2004). Grasses were the staple food crop for early humans and still are in most civilizations today.

Traditionally, *Phalaris* has been placed in the Aveneae tribe of the Pooideae subfamily (Clayton & Renvoize, 1986). Molecular phylogenetics studies have revealed that the Aveneae and Poeae tribes form a complex rather than being two discreet clades, but *Phalaris* was reconstructed in a lineage of mostly Aveneae species (Döring & al., 2007; Quintanar & al., 2007, Schneider & al., 2009). The majority of *Phalaris* species possess a basic chromosome number of x=7, a common cytotype in the Aveneae tribe. Within the Aveneae tribe, the x=6 cytotype is confined to *Phalaris*, although other genera possess basic chromosome numbers of x=2, 4, 5, 8, and 9 (Hilu, 2004). A study assessing the relationship of the x=6 and x=7 species is a necessary step to examine chromosome evolution in *Phalaris* and learn more about this case of apparent aneuploidy.

Phalaris L. (Poaceae, Aveneae) includes 21 natural species distributed throughout temperate and subtropical regions of the Old and New World in addition to an artificial hybrid (Baldini 1995). The earliest records of *Phalaris* by Dioscorides dates back to the first century AD (Matthioli, 1554). *Phalaris* species were recognized by Bauhin in 1623, but the first modern descriptions came from Linnaeus (1753) and (1778). The taxonomic and nomenclatural history of the genus have been discussed by Trinius (1828), Steudel (1855), Paunero (1948), Anderson (1961), Clayton and Renvoize (1986), Baldini and Jarvis (1991), and Baldini (1993, 1995). Tsvelev (1973, 1983) established an infrageneric treatment, but it only included species found in the former Soviet Union. Previous treatments have not consistently recognized the distinctions between diploid and polyploid species leading to publications where *P. caesia* is referred to as the "hexaploid form" of *P. arundinacea* (McWilliam and Neal-Smith 1962). Neither Anderson (1961) or Baldini (1995) proposed an infrageneric classification for the genus. Rauschert (1969) raised *P. arundinacea* to a generic level, Phalaroides Wolf, based on morphological information. All prior studies were based on intuitive assessments using morphological features; a comprehensive molecular phylogeny and infrageneric treatment for the genus are lacking.

Phalaris is characterized by laterally compressed ovate spikelets with a single terminal floret and two lateral sterile florets that are modified into reduced sterile lemmas. Sterile lemmas are usually subulate and shorter than the fertile one, rarely chaffy, but sometimes appear as little fleshy scales, with the lower ones reduced to insignificant knobs (both are obscure in *P. paradoxa* and *P. coerulescens*). The fertile lemmas are coriaceous with the margin not overlapping, acute, and awnless. The palea is

coriaceous, bi-nerved, and awnless. There are either two reduced lodicules or none at all; fertile florets usually contain three stamens and two stigmas with the caryopsis described as plump with a reticulate pericarp (Baldini 1995). Variation in the relative size and shape of the sterile lemmas has been shown to be effective for *Phalaris* taxonomy (Anderson, 1961; Baldini, 1993, 1995) and will complement the molecular phylogenetic reconstructions in this study. The florets are subtended by two lower persistent glumes that are subequal, large, awnless, usually flattened, and dorsally keeled. Anderson (1961) recognized four primary floret types in *Phalaris* and Baldini (1995) further divided the fourth type into three, and consequently recognized a total of six floret morphotypes. Baldini (1995) underscored their potential usefulness in the systematics of the genus. The reproductive structures of grasses have been linked with their widespread distribution (Davidse, 1987; Clayton, 1990) and may prove to be useful when examining the biogeographical history of the genus.

Many species such as *P. maderensis, P. rotgesii, P. platensis, P. californica*, and *P. lemmonii* are endemics with limited distribution, but other members of the genus are cosmopolitan and even invasive (Baldini, 1993, 1995). *Phalaris arundinacea* has a significant ecological impact as an invasive species in North America (Galatowtsch,& al., 1999; Lavergne & Molofsky, 2004; Thomsen & al., 2012). It was originally introduced in the Midwestern region of North America to restore damaged wetlands, but quickly established itself as an aggressive invasive because of its high degree of adaptability (Merigliano & Lesica, 1998; Casler & al., 2009). Despite the aggressiveness of *P. arundinacea* in certain regions, a variegated variety of *P. arundinacea* ‘picta’ was developed and naturally became a popular as an ornamental for its hardiness in many

agricultural zones and aesthetic appeal which ranges from white and green to red, white and green striping (Grounds, 2004). Several species of *Phalaris* are used as forage and ornamentals; however, *P. arundinacea* and *P. canariensis* are the only two that regularly appear in literature. *Phalaris canariensis* is used as bird feed and the aesthetically pleasing inflorescence is enjoyed in gardens as well as indoors when arranged with dried flowers (Grounds, 2004).

The genus possesses species that are both self-compatible and incompatible. *Phalaris coerulescens* has been used as a model organism to study the S-Z allele system responsible for self-incompatibility in grasses (Hayman, 1956; Liu & al., 1997). Self-incompatibility has been linked with promoting genetic variation and increases the chances of hybridization occurring (Allen & Hiscock, 2008). Hybridization is often linked with polyploid formation and is an important mechanism for speciation in the grasses. *Phalaris* species may be of auto- or allopolyploid origin making ancestral hybridization events a likely factor for speciation in the genus. A study examining self-incompatibility across the genus is lacking.

Cytological investigations in *Phalaris* have been sporadic over the years, often focusing on a single species or pair of related species and usually aimed at obtaining counts with nondifferential staining techniques rather than making comparisons in an evolutionary context. Jenkin & Sethi (1932) carried out cytological studies of artificial crosses they generated between *P. arundinacea* and *P. aquatica*. McWilliam (1962) followed up the the work of Jenkin & Sethi (1932) by crossing the hexaploid *P. caesia* with *P. aquatica*. Development of hybrids and octoploids was typically conducted with the goal of developing better forage crops. The artificial octoploid *P. davesii* was

developed for this purpose (Baldini, 1995). Parthasarathy (1938) conducted a descriptive study of a few members of *Phalaris* and related genera and Ambastha (1955) later continued cytological investigations of *Phalaris* calling attention to discrepancies in chromosome counts throughout publications of the era, notably the different ploidy levels and cases of aneuploidy reported in *P. arundinacea*. Anderson (1961) reported chromosome counts for several species and considered the chromosome number when presenting his hypothesis of phyletic relationships in the genus. A study placing characteristics of the chromosomes in phylogenetic context is lacking.

The nuclear genome size varies greatly within the angiosperms (Bennett & Smith, 1976, 1991). The origin and nature of this variation has garnered attention for many years (Stebbins, 1976; Cavalier-Smith, 1985; Bennett & Smith, 1976, 1991; Bennett, 1987, 1998; Leitch et al., 1998; Leitch & Hanson, 2002). Kadir (1976) assessed the genome size of several *Phalaris* species finding a large degree of variation, but methodology has improved since then warranting another assessment. Examining the genome size in *Phalaris* is another necessary step toward understanding its evolutionary history and shedding light on the origin of the x=6 cytotype.

The diversity of features found in the canary grasses make them an ideal platform to study trends in biogeography, floret morphology, genome size, cytology, breeding systems and the role these factors have played in the success of the genus. Establishing the first molecular phylogeny for the genus is a critical first step toward uncovering the evolutionary history of *Phalaris*.

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Chapter 1

Canary grasses (*Phalaris*, Poaceae): Molecular phylogenetics, polyploidy and floret evolution

Stephanie Voshell, Riccardo Baldini, Rohit Kumar, Nicholas Tatalovich, Khidir Hilu

ABSTRACT

The 21 wild species of *Phalaris* L. (Poaceae) are distributed in temperate areas of both hemispheres and in the mountains of tropical Africa and South America. The genus contains annual and perennial diploids based on x=6 and 7 and polyploids based on x=7, with the x=6 cytotypes displaying less species diversification. *Phalaris* presents a potentially valuable case study for speciation in conjunction with chromosomal evolution and biogeography in the Poaceae due to its global distribution, notable variation in morphological traits, and the key role of polyploidy in its evolution. We reconstructed the first phylogenetic tree for the genus using nuclear ITS and plastid *trnT-F* sequences to assess species relationships and map trends in floret differentiation and patterns of polyploid evolution. The study demonstrated a single origin of the x=6 chromosome number and revealed the sister relationship of this lineage to the monophyletic x=7 lineage. The clades recovered in the analyses display geographic affiliations and demonstrate diploid-polyploid associations. A trend in sterile lemma reduction is evident, with members of the early diverging x=6 lineage displaying relatively large and lanceolate sterile lemmas, followed by gradual reduction in size, culminating in almost obsolete sterile lemmas in one of the terminal x=7 clades.

INTRODUCTION

Phalaris L. (Poaceae) comprises 21 wild species (Baldini, 1995) distributed in temperate areas of both Northern and Southern hemispheres and the mountains of tropical Africa and South America (Fig. 1.1). *Phalaris* is placed either in the traditional Aveneae tribe (Clayton & Renvoize, 1986) or in a primarily Aveneae lineage in a recent molecular phylogenetic study of the Pooideae (clade 1, Schneider & al., 2009). We will follow the traditional Aveneae classification in this paper. The genus contains annual and perennial diploids based on x=6 and x=7 chromosome numbers and polyploid species based on x=7 (Parthasarathy, 1938; Ambastha, 1956; McWilliam & Neal-Smith, 1962; Stebbins, 1971; Kadir, 1974; Hunziker & Stebbins, 1987). *Phalaris* includes important forage crop species such as *P. canariensis*, and some weedy and invasive species such as *P. arundinacea*.

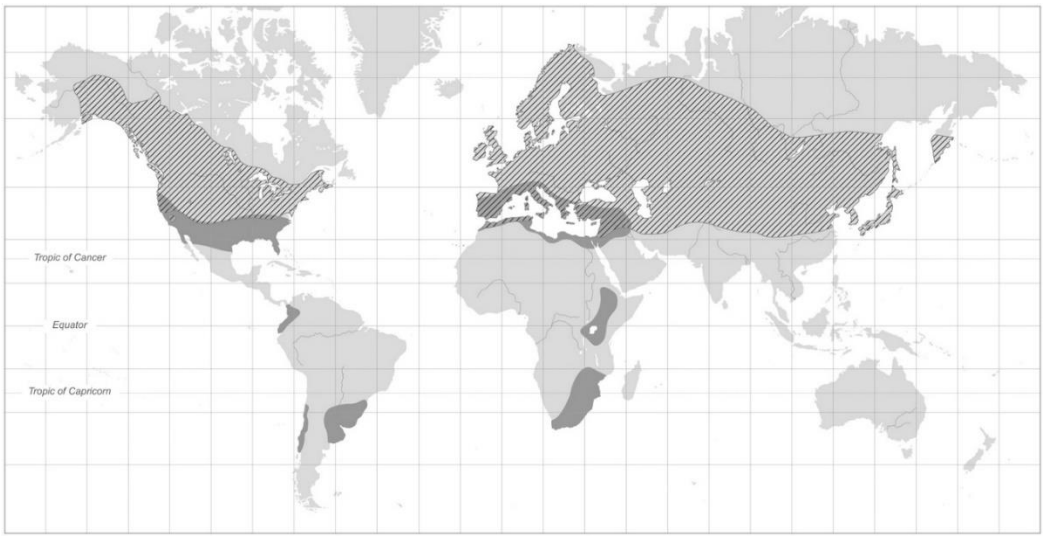

Fig. 1.1. Geographic distribution of canary grasses (*Phalaris*). Area in stripes denotes the distribution of *P. arundinacea* and solid areas represent other *Phalaris* species. Note the Mediterranean association, disjunct distribution in Africa and South America, and wide distribution in the Northern Hemisphere.

Phalaris is characterized by laterally compressed ovate spikelets with a single fertile floret and two proximal sterile lemmas. The florets are subtended by two lower persistent glumes that are subequal, large, awnless, usually flattened, and dorsally keeled. *Phalaris paradoxa* and *P. coerulescens* deviate in having spikelets assembled into deciduous clusters of one fertile and up to 6-7 sterile spikelets. The sterile lemmas of *Phalaris* are subulate and shorter than the fertile one. The fertile lemmas are coriaceous, acute, awnless, and the margins are not overlapping. The palea is coriaceous, bi-nerved, and awnless. Anderson (1961) recognized four floret types in *Phalaris* and Baldini (1995) further divided the fourth type into three, resulting in a total of six floret types. Baldini (1995) underscored the potential usefulness of floret types in the systematics of the genus. The six floret types are illustrated in Figure 1.2.

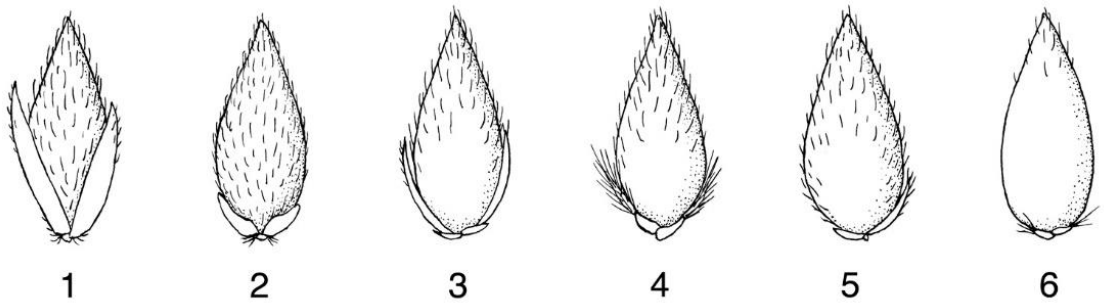

Fig. 1.2. A diagrammatic illustration of six floret types recognizable in species of *Phalaris* following Anderson (1961) and Baldini (1995). Note the central fertile floret and the two lateral sterile lemmas that display successive reduction in size.

Phalaris along with *Anthoxanthum* and *Hierochloe* possess a distinctive spikelet structure and are members of the traditional subtribe Phalaridinae Rchb. within the Aveneae (Clayton & Renvoize, 1986; Soreng & al., 2003). Soreng & Davis (2000) recovered *Phalaris*, *Anthoxanthum* and *Hierochloe* in one clade based on phylogenetic analysis of chloroplast restriction data and morphological characters. *Phalaris* appeared in an individual clade sister to these two genera in phylogenetic studies based on DNA sequences (Döring & al., 2007; Quintanar & al., 2007). Although Quintanar & al. (2007) consider *Phalaris* as an isolated genus of uncertain phylogenetic relationship in the *Aveneae*, their *trnT-F* tree resolved *Anthoxanthum+Hierochloe* and *Briza+Airopsis* in subclades diverging directly after *Phalaris*, implying close phylogenetic relationships. The taxonomic and nomenclatural history of *Phalaris* and its infrageneric delimitation has been discussed in Paunero (1948), Anderson (1961), Clayton & Renvoize (1986), Baldini & Jarvis (1991), and Baldini (1993, 1995). In a morphological study focused on *Phalaris* in Spain, Paunero (1948) recognized four species and two sections: sec. *Baldingera* (Gaertn.) Paunero comprising *P. arundinacea*, and sec. *Euphalaris* Paunero, encompassing the remaining three species. Anderson (1961) recognized 15 species and Baldini (1995) and Kodela & al. (2009) listed 22 species for the genus (including the artificial hybrid species *P. daviesii* S. T. Blake); neither study proposed an infrageneric classification. Rauschert (1969) and Valdés & Scholz (2006) raised *P. arundinacea* to a generic level, *Phalaroides* Wolf, based on morphological data. All the previously mentioned taxonomic studies were based on intuitive assessments using morphological features. A phylogenetic study for the genus based on structural and/or molecular information is lacking.

Phalaris grows in diverse habitats ranging from wild communities to disturbed areas, generally at low altitudes in open grounds, old fields, sandy soils, and waste beds (Baldini, 1995). *Phalaris arundinacea, P. rotgesii, P. caesia, P. peruviana,* and *P. lindigii* occupy lacustrine habitats. A number of species, such as *P. arundinacea,* can grow in permanently aquatic habitats, and populations of *P. aquatica* and *P. coerulescens* are subject to seasonal flooding.

Anderson (1961) and Baldini (1995) proposed the European-Mediterranean and Macaronesian regions as the primary area of speciation and Southwestern USA as a secondary center of differentiation for *Phalaris*. The North European *P. arundinacea* is the most widespread species, extending across the circumboreal region (Fig. 1.1). Merigliano & Lesica (1998) proposed a "native" status for this species in a small section of western North America and indicated that the North American populations are a mix of native strains and agronomic cultivars (see also Barkworth, 2007). Casler & al. (2009) analyzed AFLP markers and chloroplast DNA sequence data of *P. arundinacea* with Unweighted Pair Group Method Using Arithematic Averages (UPGMA) and revealed higher genetic diversity in Europe and a limited gene pool in western North America. They concluded that the presence of *P. arundinacea* in western North America is potentially the result of limited natural migration. The Neotropics and southern temperate South America represent additional areas of speciation for *Phalaris*. The Neotropics contain the two endemic perennials *P. lindigii* and *P. peruviana*, while southern temperate South America has the two annual endemics *P. amethystina* and *P. platensis*. Further, North and South America share the disjunct populations of *P. angusta*

whereas a vicariance biogeography has been proposed for *P. lemmonii-P. platensis* (Henrard, 1938).

The objectives of this study were to use DNA sequence information from the nuclear ribosomal internal transcribed spacer (ITS) and the plastid *trnT-F* region from *Phalaris* species to reconstruct a phylogenetic tree and use it for the assessment of species relationships and polyploid and floret evolution.

MATERIALS AND METHODS

Species Sampling

The ITS and *trnT-F* regions were sequenced from 39 and 30 accessions, respectively, representing 20 of the 22 species of *Phalaris* recognized by Baldini (1995; Appendix A). Plant material for *P. elongata*, which is now considered as a variety of *P. aquatica* (Oram & al., 2009), was unavailable, and the synthetic allopolyploid *P. daviesii* was excluded since the focus of the study was on the wild species. The two genomic regions were chosen based on their documented utility in phylogenetics at this level and their differential mode of inheritance (bipaternal vs. maternal) that may potentially help discern patterns of polyploid evolution. For the ITS dataset, more than one accession per species was used whenever feasible; the number of accessions was increased when molecular variation was detected, such as with *P. minor*, *P. paradoxa*, *P. caroliniana,* and *P. californica* (see Appendix A). For the *trnT-F*, the number of accessions was reduced to mostly one per species due to the low level of variation in nucleotide substitutions encountered in this genus. Accessions for a species with identical DNA sequences were represented by one accession in the data set to speed up phylogenetic

analyses. Sources and geographic origin of the material and chromosome counts are noted in the Appendix A.

DNA Isolation, Amplification and Sequencing

For fresh material, genomic DNA was isolated following Doyle & Doyle (1990). In the case of herbarium samples (often 20 to 60 years old), the DNA isolation method was modified to optimize the procedure. In those cases, the ground leaf material was extracted two to three times in CTAB+BME and the supernatant solutions collected from each extraction were combined to increase the amount of DNA recovered. Additionally, the solution was allowed to remain at -20 °C overnight at the DNA precipitation step. The ITS region was amplified using the universal primers ITSA and ITSB (Downie & Katz-Downie, 1996) and the Polymerase Chain Reaction (PCR) method described in Woods & al. (2005). The *trnT-F* region was amplified using universal primers and the protocol from Taberlet et al. (1991) with a 50 °C annealing temperature. The region was either amplified in two segments using the primers *trnA/trnC* and *trnC/trnF,* or in three sections in cases of degraded genomic DNA using the primer combinations *trnA/trnB, trnC/trnD,* and *trnE/trnF*. Amplification products for both genomic regions were resolved on 0.8% TAE-agarose gels, excised, and cleaned using QIAquick PCR purification or QIAquick Gel Extraction kits (Qiagen, Valencia, CA) or the Promega Wizard SV Gel & PCR Clean-Up System (Promega, Madison, Wisconsin). Cycle sequencing was performed using the ABI PRISM ® Big Dye terminator Cycle Sequencing kit v. 3.1 (Applied Biosystems Inc., Foster City, CA), and the products were resolved using Applied Biosystems 3730 automated sequencer at the Core Sequencing

Facility at Virginia Bioinformatics Institute, Virginia Tech, or at the DNA Analysis Facility of Duke University.

Outgroup Selection

Anthoxanthum monticola and *Hierochloe equiseta* were selected as outgroup in the ITS phylogenetic analyses. In the case of the *trnT-F* analyses, sequences for *H. australis* were substituted for *H. equiseta* due to unavailability of genomic DNA and GenBank sequences for the latter species. *Briza minor* alone and in combination with the above outgroup taxa were also considered for rooting the tree, but the phylogenetic structure (resolution and support) was reduced in comparison with those based on *Anthoxanthum* and *Hierochloe*. Traditional and current systematic studies have demonstrated that the three genera are closely related (see introduction).

Sequence Alignment and Phylogenetic Analyses

The ITS and *trnT-F* sequences were manually aligned using Quickalign (Müller, 2006). Gaps were inserted at the cost of two or more substitutions and coded into the alignment matrix file using SeqState (Müller, 2005) and selecting the Simple Coding option (Simmons & Ochoterena, 2000). Double peaks detected in the ITS phenograms were assigned IUPAC ambiguity codes. The two data sets were analyzed separately and in combination using maximum parsimony (MP) in PAUP* (Swofford, 2002) and Bayesian inference (BI) in Mr. Bayes version 3.0b4 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003).

In the MP analysis, heuristic searches were performed with all characters equally weighted. Stepwise addition for 1,000 replicates was implemented with TBR branch swapping, MULPARS on, and steepest descent off. A strict consensus tree was computed from all shortest trees. Maximum parsimony bootstrap (BS) values as a measure of clade support (Felsenstein, 1985) were obtained by conducting searches of 1,000 iterations with 10 random sequence replicates. In the BI analysis, the Markov Chain Monte Carlo (MCMC) method (Larget & Simon, 1999; Lewis, 2001) was implemented. Optimal models of sequence evolution and starting likelihood parameters were determined using Modeltest (Posada & Crandall, 1998), and the GTR+G model was shown to be the best fit model for both ITS and *trnT-F* data sets. Four Markov chains were simultaneously run for 1,000,000 generations, starting with a random tree, and with trees and parameters being sampled every 1,000 generations. The analyses reached stationarity where the average standard deviation of split frequencies between runs was ≤ 0.01prior to the end of the runs. The first 2,500 'burn in' trees were discarded, and the rest were used to compute a 50% majority-rule consensus tree. The posterior probabilities were calculated and stored for each tree.

To determine the potential of combining the ITS and *trnT-F* sequence data for phylogenetic analyses, we used a partition homogeneity test (PHT; Farris & al., 1995). The whole *trnT-F* data set (30 accessions/20 species) and only the corresponding ITS sequences were used in the test to achieve completely overlapping data sets. The PHT was performed in PAUP* with 100 replicate partitions and 1,000 trees per replicate. Significant heterogeneity was found between the two genomic regions (P=0.01), implying significant incongruence between the two data sets. We also performed the

PHT using only variable characters as recommended by Cunningham (1997) but the outcome remained the same. Although the results suggest that the two data sets are not compatible, we opted to analyze a combined data set composed of completely overlapping sequences, i.e., ITS sequences for accessions that do not have corresponding *trnT-F* sequences were excluded. Our reasoning for the combined analyses was that the PHT tends to be overly sensitive to noise and differences in tempo of evolution of genomic regions and, as a result, might not accurately reflect congruencies (Dolphin & al., 2000; Barker & Lutzoni, 2002; Darlu & Lecointre, 2002). Furthermore, our partitioned analyses demonstrated that differences between the topologies of the trees derived from the two regions do not represent hard incongruence (Seelanan & al., 1997).

To evaluate trends in floret character evolution among species, we mapped on the phylogenetic trees the following six *Phalaris* floret types (Fig. 1.2) recognized in Baldini (1995) based on sterile lemma structure: Type 1, two chaff-like; Type 2, two reduced coriaceous: Type 3, two equal or subequal; Type 4, two equal or subequal feathery; Type 5, one well developed sterile lemma; and Type 6, two obsolete sterile lemmas. Chromosome numbers were also mapped on the tree to reflect shifts in basic chromosome number (x=6 and x=7) as well as ploidy levels.

RESULTS

ITS Data Set

The ITS region varied in length from 588 bp (*P. rotgesii*) to 602 bp (*P. arundinacea*). After insertions of fourteen gaps of 1-3 bp in length, the alignment was 732 characters long. Following the exclusion of portions at the ends of the alignment due

to missing data, the number of characters used in the analysis was reduced to 618 including indels. There were 169 variable characters, out of these 142 were parsimony informative (PI). The MP analysis recovered 113417 equally parsimonious trees of 231 steps, with CI and RI values of 0.831 and 0.952, respectively.

Tree topologies obtained from the MP and BI analyses are completely congruent and therefore will be discussed together. The monophyly of *Phalaris* received maximum BS and PP support (Fig. 1.3). A split into two clades is evident at the base of the genus. One clade (99% BS, 1.00 PP) comprises *P. truncata* sister to *P. canariensis* plus *P. brachystachys* (100% BS, 1.00 PP). All members of this clade have a basic chromosome number of x=6 as a synapomorphy and possess floret type 1 (*P. canariensis*) and type 2 (*P. truncata* and *P. brachystachys).* The second clade (97% BS, 1.00 PP) includes species having x=7. Species in this clade form three strongly supported lineages, which we will refer to informally as lineage 1, 2 and 3. Lineage 1 (95% BS, 1.00 PP) represents species endemic to North and South America (*P. californica, P. caroliniana, P. amethystina, P. platensis,* and *P. lemmonii*) and one species (*P. angusta*) found in both North and South America. *Phalaris angusta* appears in a clade with *P. lemmonii, P. amethystina and P. platensis* (89% BS, 1.00 PP) sister to two clades representing *P. californica* and *P. caroliniana*. Members of this group share floret type 3 as a synapomorphy. Lineage 2 (100% BS, 1.00 PP) includes a diploid (*P. rotgesii*), a tetraploid (*P. arundinacea*), and a hexaploid (*P. caesia),* as well as *P. peruviana* (chromosome number unknown) in a polytomy (Fig. 1.3). Members of this group possess floret type 4. Lineage 3 (96% BS, 1.00 PP) depicts the tetraploid *P. aquatica* and *P. lindigii* (chromosome number unknown) as sister to a polytomy (100% BS, 1.00 PP)

of three subclades that includes diploid and tetraploid species: 1) two accessions of *P. paradoxa*, four accessions of *P. minor*, and the accession of *P. maderensis* (97% BS, 1.00 PP), 2) two accessions of *P. paradoxa* plus the single accession of *P. appendiculata* (86% BS, 1.00 PP), and 3) two *P. coerulescens* accessions (90% BS, 1.00 PP). Members of lineage 3 possess floret types 4 (*P. lindigii*), 5 and 6 (Fig. 3). The number of accessions for *P. minor* and *P. paradoxa* was increased to examine initial results pointing to the emergence of different accessions of these species in more than one clade.

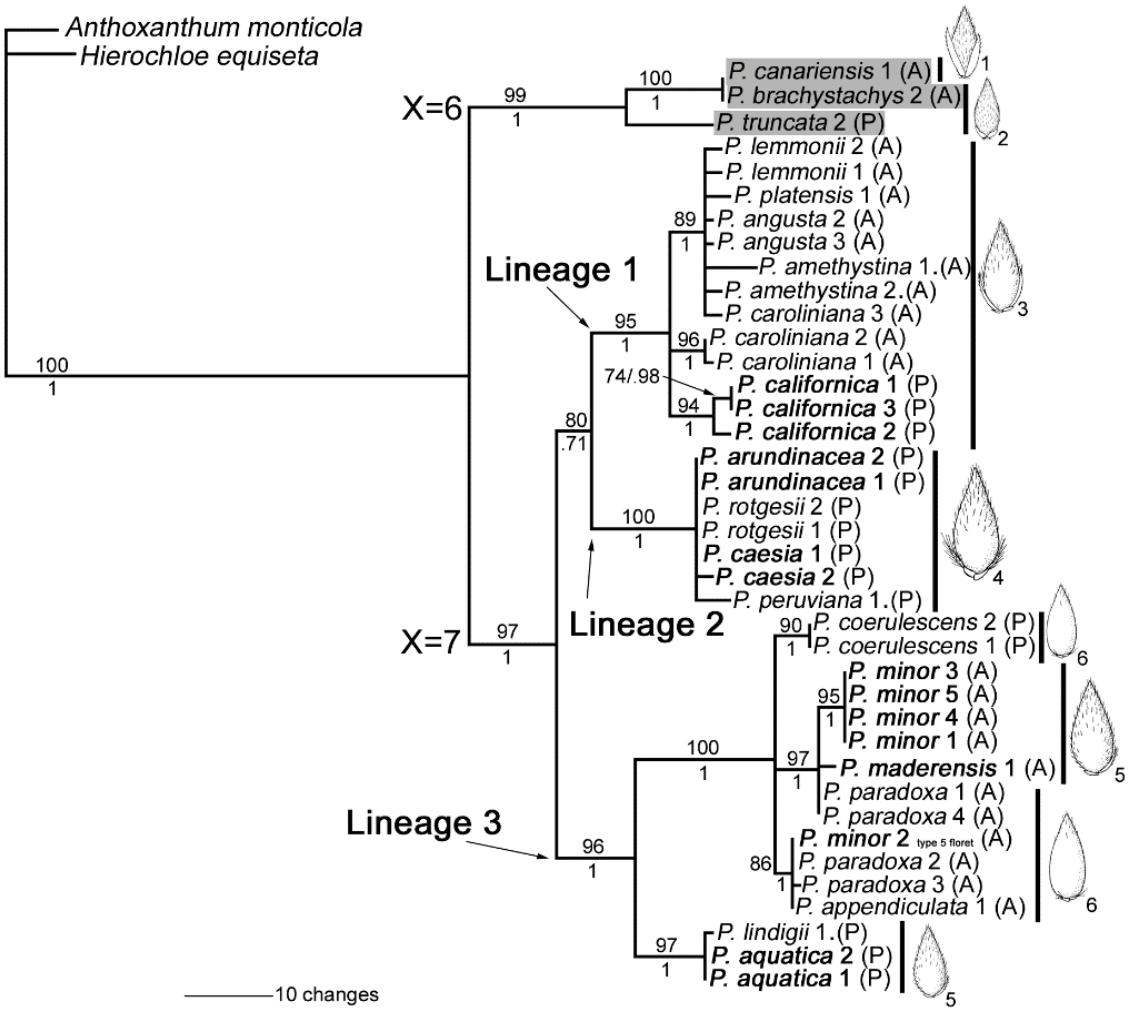

Fig. 1.3. ITS phylogram based on Bayesian inference. Parsimony bootstrap values are noted above the line and Bayesian posterior probabilities are below. Shaded section of the tree highlights species with *x*=6, and names shown in bold denote polyploid species. Floret types follow Fig. 2. • = unknown chromosome number. A = annual and P = perennial habit.

trnT-F Data Set

The *trnT-F* region varied in length from 1669 (*P. californica*) to 1650 (*P. paradoxa*) nucleotides. Twenty-two gaps of 1-27 nucleotides were required in the alignment. The exclusion of the 3' and 5' ends of the alignment due to missing sequences and the removal of the poly A and C regions reduced the data set to 1764 characters including indels. Of these characters, only 169 (10%) were variable and 80 were parsimony informative. The MP analyses generated 1120 equally parsimonious trees of 202 steps and CI and RI scores of 0.881 and 0.907, respectively. Posterior probability support increased for all clades when compared to the MP bootstrap values. The topology of the BI tree is identical to the *trnT-F* MP consensus tree topology, but with higher resolution. Consequently, the BI tree is presented with both PP and BS values; nodes that collapsed during the MP analysis are noted on the tree (Fig. 1.4).

As in the ITS analysis, the monophyly of *Phalaris* is supported by maximum PP and 94% BS (Fig. 1.4). *Phalaris californica* and *P. peruviana* emerge as consecutive sisters to remaining species in the BI tree but support for this topology is not significant (Fig. 1.4). These nodes collapsed in the MP strict consensus tree, revealing a basal polytomy with the major clades (Fig. 1.4). The x=6 species appear sister to lineage 1 of the x=7 species, but with weak support for this relationship (63% BS, 0.87 PP). However, the internal structure of the x=6 clade is identical to that obtained in the ITS tree, but with lower BS and PP support. In lineage 1 (93% MP, 1.00 PP), *P. caroliniana* is sister to a polytomy of *P. angusta, P. lemmonii + P. platensis, and P. amethystina*. Lineage 2 is recovered with low support and includes *P. lindigii*, a member of lineage 3 of the ITS phylogenetic tree. Lineage 3 is not recovered as such with the *trnT-F* data.

The tetraploids *P. aquatica*, *P. minor* and *P. maderensis* emerge in a strongly supported group (98% BS, 1.00 PP); unlike the ITS topology, they appear sister to lineage 2, but without significant support (<50% BS, 0.83 PP). Among remaining members of lineage 3, the diploids *P. coerulescens* and *P. paradoxa* form a clade with maximum BS and PP support. One accession of *P. paradoxa* and *P. appendiculata* form a group with 96% BS and 1.00 PP support.

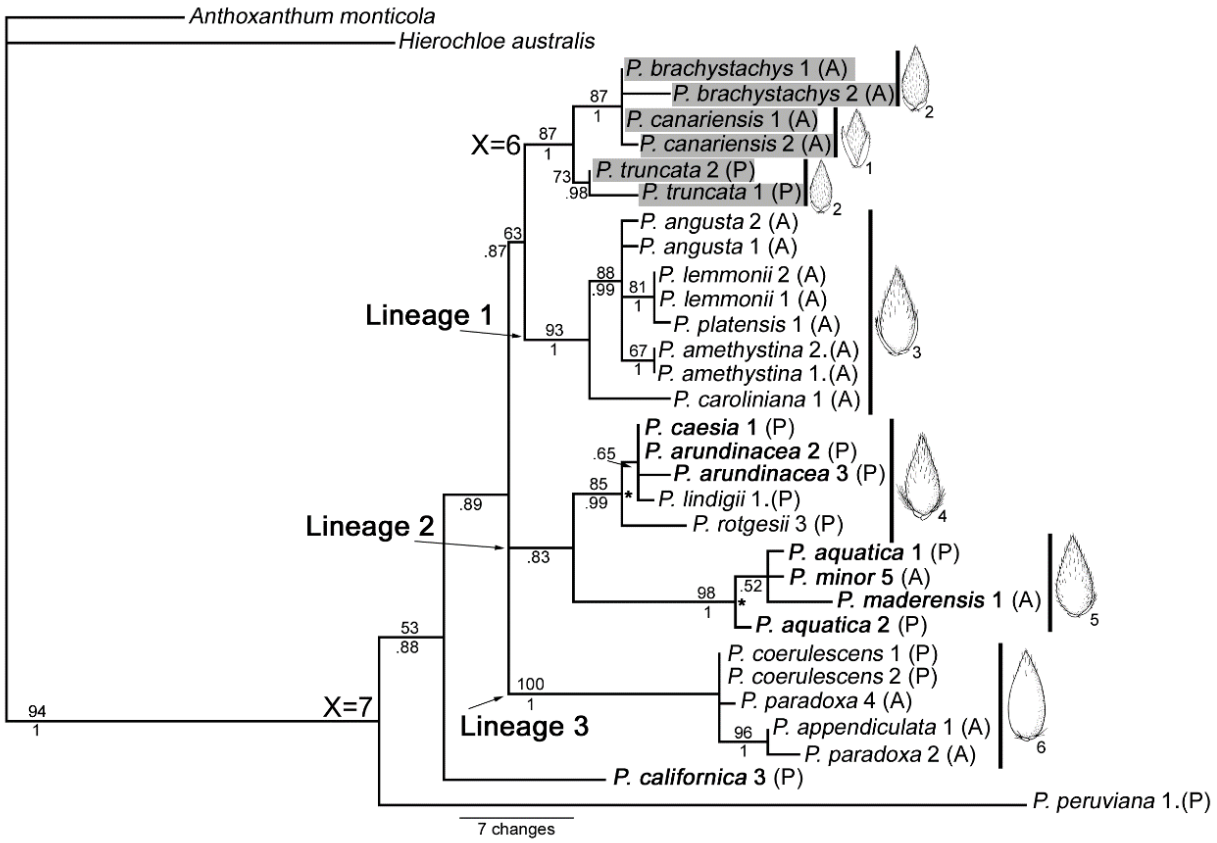

Fig. 1.4. *trnT-F* phylogram generated from Bayesian inference. Parsimony bootstrap results are above the line and Bayesian posterior probabilities are below. Shaded section of the tree highlights species with x=6, and names shown in bold denote polyploid species. * = clade collapsed in the strict consensus maximum parsimony tree. • = unknown chromosome number. Floret types follow Fig. 2. A = annual and P = perennial habit.

Combined ITS and trnT-F Data Set

The combined ITS/*trnT-F* analyses resulted in 4 most parsimonious trees of 457 steps. The CI and RI values were 0.799 and 0.901, respectively. The tree derived from the combined data set is similar in overall topology to the one based on the ITS data, but with higher resolution (Fig. 1.5). Differences include the sister relationship of lineage 2 to lineage 3 rather than lineage 1 as seen in the ITS tree (Fig 1.3); neither relationship receive convincing support. There is higher resolution in members of lineage 1, with *P. californica* being sister to remaining members. The *P. minor/P. maderensis* clade gained strong support as sister to the *P. paradoxa/P. coerulescens* group.

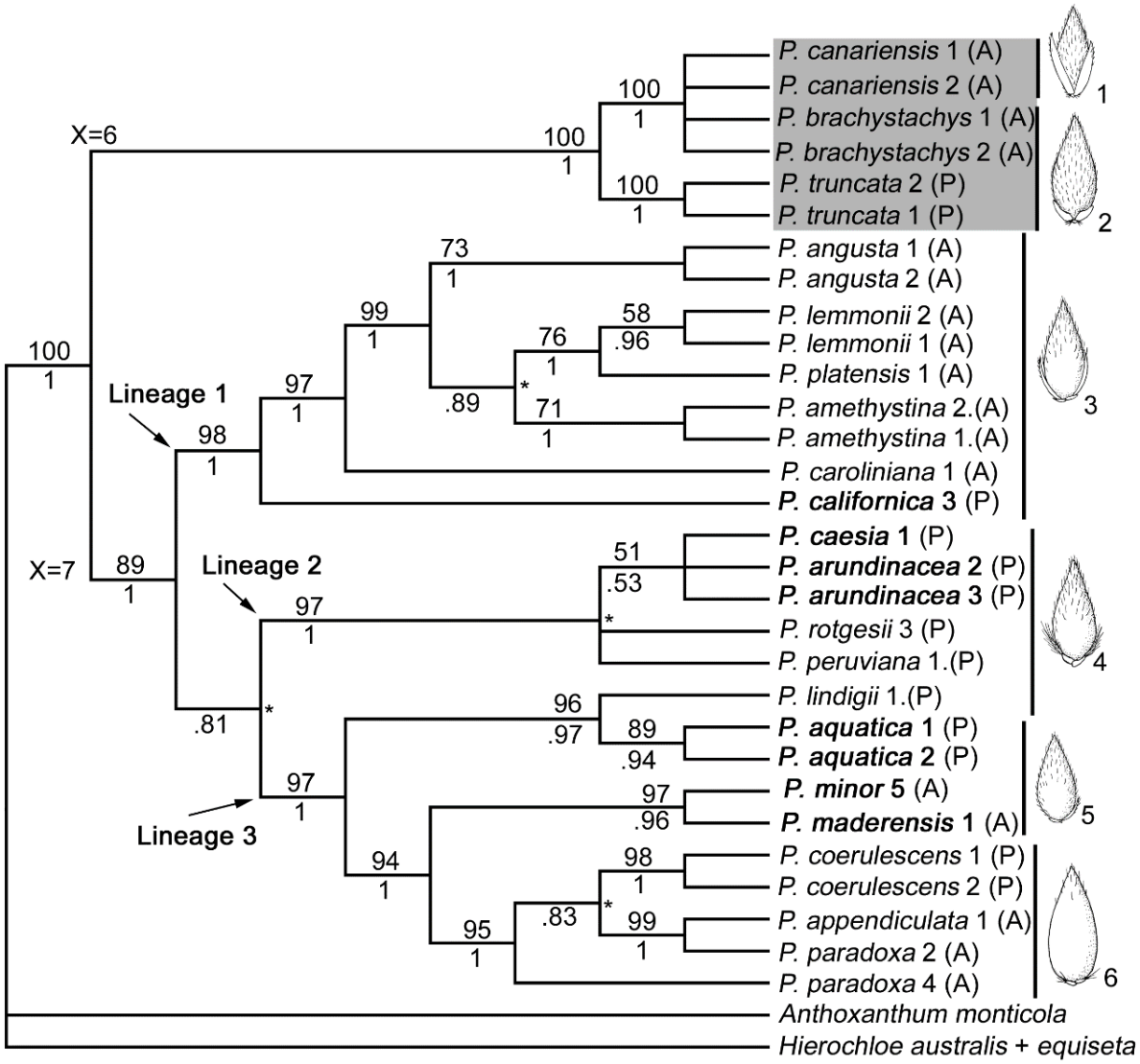

Fig. 1.5. Combined ITS and *trnT-F* phylogeny based on maximum parsimony and Bayesian inference. Shaded section of the tree highlights species with *x*=6, and names shown in bold denote polyploid species. * = nodes collapsed in the strict consensus maximum parsimony tree. • = unknown chromosome number. Floret types follow the structure defined in Fig. 2. A = annual and P = perennial habit.

DISCUSSION

ITS vs. trnT-F Signal

The *trnT-F* alignment is approximately 3 times larger than the ITS alignment. However, the number of PI characters in the *trnT-F* is only 80 compared with 142 in the ITS. This difference in amount of informative characters could account for the lower resolution and support in the *trnT-F* based trees. Despite differences in phylogenetic signals, the CI and RI scores obtained from the analyses of the two regions are quite comparable and reflect a relatively low degree of homoplasy. The differential evolution of these two regions in different plant groups has been documented in several studies (Taberlet & al., 1991; Baldwin, 1993; Alvarez & Wendel, 2003; Neves & al., 2005). Regardless of low phylogenetic signal in the *trnT-F* and lower resolution of the tree, the region provided useful information on species relationships and potential patterns of hybridization and polyploid evolution in *Phalaris*.

Phylogenetic Relationships

MP and BI analyses of ITS and *trnT-F* partitioned and combined data clearly demonstrate the single origin of the x=6 species within this monophyletic genus (Figs. 1.3-1.5). Further, both ITS and combined analyses recovered this lineage as sister to the remaining species of *Phalaris* (Figs. 1.3, 1.5). Morphologically, the x=6 species differ from the x=7 in having either type 1 (chaff-like sterile lemma) or type 2 florets (reduced coriaceous sterile lemma; Fig. 1.2). However, the annuals *P. canariensis* and *P. brachystachys,* although not sharing the same floret type, consistently appear in a

strongly supported clade separate from the perennial *P. truncata.* These morphological features add further support for the distinction of these *x*=6 species from the rest of *Phalaris*.

Similarly, the monophyly of the *x*=7 *Phalaris* species is also strongly supported (95%, 1.00 PP ITS and 89%, 1.00 PP combined). Although the *trnT-F* data did not recover this group as a monophyletic unit, most of its lineages correspond to those resolved in the ITS phylogeny (Figs. 1.3, 1.4). The lower resolution in the *trnT-F* tree in the BI analysis (Fig. 1.4) is probably a reflection of the low phylogenetic signal in this genomic region. Support for the backbone of the tree in general is weak in the *trnT-F* tree compared with trees based on the ITS or combined data (Figs. 1.3-1.5). Therefore, the conflict between the *trnT-F* and ITS tree topologies represents a case of soft incongruence (Seelanan & al., 1997), and consequently, expanding the plastid data set might clarify this situation. The emergence in the *trnT-F* tree of *P. californica* and *P. peruviana* as sister to remaining *Phalaris* may be due to long branch attraction (Felsenstein, 1978), particularly for the latter species (Fig. 1.4). However, both *P. peruviana* and *P. californica* share with the outgroup species unique SNP mutations and six indels in the *trnT-F* alignment. These shared characters might imply historic intergeneric hybridization events and subsequent lineage sorting (Doyle, 1992; Wendel & Doyle, 1998), and could have contributed to the conflicting placement of *P. californica* and *P. peruviana*.

As noted earlier, three major lineages are evident for the *x*=7 species. Lineage 1 (Figs. 1.3-1.5) represents all North American and most South American species, and includes four diploids (*P. angusta, P. lemmonii, P. platensis,* and *P. caroliniana*), one

tetraploid (*P. californica*), and one species with unknown chromosome number (*P. amethystina*). Members of this lineage share floret type 3 (Figs. 1.2, 1.5). All species are annuals except for the tetraploid *P. californica*, which is a perennial. The emergence of *P. californica* as sister to remaining species may imply that perennial habit and tetraploidy are ancestral states. However, the inconsistent placement of *P. californica* in the partitioned and combined analyses renders such a conclusion difficult to support at this point. Additional molecular markers are needed to illustrate the mode of habit and polyploid evolution in this lineage.

Lineage 2, as defined in the ITS and combined analyses, encompasses the broadly distributed *P. arundinacea* (tetraploid), along with the European Mediterranean *P. caesia* (hexaploid) and *P. rotgesii* (diploid), and South American *P. peruviana* (chromosome number unknown). The monophyly of these four species receives strong BS and maximum PP support in all analyses except for the 85% BS support in the *trnT-F* based tree (Figs. 1.3-1.5). The four species also share the unique morphological features of floret type 4 (Fig. 1.2). This clade stands out as an excellent example of evolution through polyploidy and its association with geographic expansion. The diploid *P. rotgesii* is found in small, scattered populations confined to Corsica Island (Europe). The only tetraploid in its clade, *P. arundinacea*, is a robust species distributed in most temperate habitats in the Northern Hemisphere (Fig. 1.1). The hexaploid *P. caesia* is distributed in southern France, northern Spain, Portugal, the Middle East, and northern, eastern and southern Africa (Baldini, 1995). Thus, we propose that the addition of a third genome copy has not resulted in increased geographic expansion but in the occupation of

certain habitats such as the afroalpine grasslands and European lacustrine areas (Baldini, 1995).

The *trnT-F* phylogeny suggests that the morphologically variable diploid *P. rotgesii* is a potential progenitor of both polyploid species. The chromosome number and evolutionary origin of *P. peruviana* is currently unknown. This Neotropical species differs from other members of its clade by the narrow ovoid panicle, acuminate and keeled glumes and pubescent sterile lemmas (Baldini, 1995). The phylogenetic relationship of this South American species to other species in its clade remains ambiguous due to the polytomy. The presence of only one diploid species in this lineage and the existence of unique morphological synapomorphies within this diploid/tetraploid/hexaploid complex imply that autopolyplodization may have played a role in its evolution. Soltis & al. (2007, 2010) indicated that there are more autopolyploids than have been recognized. However, a conclusive assessment awaits a chromosome count for *P. peruviana*.

Lineage 3 of the x=7 clade is strongly supported in the ITS and combined data analyses (96% and 97% BS, 1.00 PP). It consists of species endemic to temperate Europe, some of which are introduced to North America, and one species, *P. lindigii*, exists in South America. Members of this group exhibit diploid-tetraploid relationships (*P. lindigii* lacks a chromosome count). The phylogenetic relationships resolved in this clade depict an intriguing pattern of polyploid evolution. The tetraploid *P. aquatica* and the South American *P. lindigii* are strongly supported as sister to remaining species of this lineage, which includes both diploids and tetraploids (Figs. 1.3, 1.5). A conclusion on polyploid evolution within this lineage requires chromosome counts for *P. lindigii*,

which is known only from a few collections. *Phalaris lindigii* displays intermediate features between *P. aquatica* and *P. arundinacea,* such as a cylindrical panicle and keeled glumes as in *P. aquatica* and two equal sterile lemmas and a truncate ligule as in *P. arundinacea*. In the *trnT-F* based tree, *P. lindigii* grouped with *P. arundinacea* in lineage 2 (Fig. 1.4). This may imply that *P. lindigii* is a hybrid between *P. arundinacea* (maternal) and *P. aquatica* (paternal). Natural and artificial hybrids between these two species have been reported, and in the case of the artificial hybrids success was higher with *P. arundinacea* as the maternal parent (Jenkin & Sethi, 1932).

The remaining tetraploids in lineage 3 (*P. minor* and *P. maderensis*) form a strongly supported clade based on *trnT-F* data, and appear in a clade with one accession of diploid *P. paradoxa* based on the ITS information (Figs. 1.3, 1.5). The latter relationship, which received near maximum BS and PP support, points to the potential paternal genome donation of the diploid *P. paradoxa* to the two tetraploids. The appearance of one of the accessions of *P. minor* with other accessions of *P. paradoxa* in different clades of the ITS tree may imply multiple origins for the tetraploid *P. minor*. We observed several haplotypes in both ITS and *trnT-F* for diploid *P. paradoxa,* which lends support to the potential multiple origin of tetraploid *P. minor*. *Phalaris paradoxa* and *P. minor* overlap in geographic distribution (Baldini, 1995), increasing the likelihood of derivation of one of the genomes of the tetraploid *P. minor* from the former species. All the tetraploids in this lineage possess floret type 5 (floret with one developed lemma) as a synapomorphy, while the diploids share floret type 6 (sterile lemmas nearly obsolete; Fig. 1.2). *Phalaris paradoxa* is a highly variable species (Anderson, 1961) with three recognizable subtypes of sterile spikelets (glumes clavate, reduced and normal). *Phalaris*

appendiculata is considered one of the morphological extremes of *P. paradoxa* (Baldini, 1995). We therefore treat *P. appendiculata* as a morphotype of *P. paradoxa* without any taxonomic rank.

The tetraploid *P. maderensis* is a rare species found on the island of Madeira off the western coast of Africa near Morocco. Baldini (1995) proposed that *P. maderensis* is derived from *P. minor* since the two share similar floret morphologies. This study suggests a close relationship between the two; however, only one accession of *P. maderensis* was available due to its rarity. Lineage 3 deserves detailed study with denser sampling at the population level, additional sequences and chromosome banding to determine the pattern of species and polyploid evolution in this complex clade.

Floret Evolution in Phalaris

Phalaris stands out as the most specialized genus in the subtribe *Phalaridinae* sensu Clayton & Renvoize (1986). All three genera of the tribe possess one central fertile floret and two lower fertile or sterile ones (sterile lemmas). In *Hierochloe,* the lower florets are either male or sterile but are as large as the central fertile floret, compared with *Anthoxanthum* where the lower florets are empty, rarely one or both are male, though are as large as those found in *Hierochloe*. Lower florets in *Phalaris* have undergone extreme reduction in size and are always sterile. Within *Phalaris*, the six floret types (Fig. 1.2) follow a successive trend of reduction when mapped on the phylogenetic trees (Figs. 1.3, 1.5). This trend of reduction starts from the x=6 clade where the sterile lemmas tend to be relatively broad (types 1 and 2) to the obsolete sterile lower lemmas (type 6 floret) found in *P. paradoxa and P. coerulescens*. Therefore, transitional reduction in floret

structure is evident from *Hierochloe* to *Anthoxanthum* and continued with *Phalaris*, where it reaches an extreme state deeper in the evolution of the latter genus.

Patterns Associated with Polyploidy

Polyploidy is quite prevalent in the *Poaceae*, with about 80% of the species considered to be of polyploid origin (Stebbins, 1985; Hunziker & Stebbins, 1987; Levy & Feldman, 2002; Hilu, 2006). Polyploidy has also been considered as an important factor in the diversification of the family (Levy & Feldman, 2002). Hilu (2006) demonstrated a correlation between polyploidy, perennial habit and increase in species number in grass genera. The perennial habit provides a time buffer for newly formed polyploids to regain fertility. Polyploid evolution and species diversification in *Phalaris* occurred only in association with the x=7 basic chromosome number. The x=6 species, although possessing both annual (*P. canariensis* and *P. brachystachys*) and perennial (*P. truncata*) habits, remained diploid. This is particularly striking in the case of *P. truncata* where self-incompatibility has been documented (Oram, 2004; Heslop-Harrison, 1982), a breeding system that promotes allopolyploid evolution.

The emergence of a basal split in *Phalaris* into two major clades each composed strictly of x=6 or x=7 species implies early evolution of aneuploidy. Although x=7 is a common number in the traditional Aveneae, aneuploid derivatives (x=2, 4, 5, 6, 8, and 9) from that number are not uncommon, with x=6 confined to *Phalaris* (Hilu, 2004). Variation in basic chromosome number within genera have been reported, such as x=4, 5, 7 and 9 in *Milium*, and x=5 and 7 in *Briza* (Stebbins, 1982; Watson & Dallwitz, 1982; Stebbins, 1985; De Wet, 1987; Hunziker & Stebbins, 1987). Avdulov (1931), Ambastha

(1956) and Kadir (1974) postulated that *Phalaris* species with *2n*=12 might have been derived from those with *2n*=14 by the fusion of two chromosomes, thus assuming *x*=7 as the ancestral state. Closest genera recovered in the same lineage (Schneider & al., 2009), such as *Briza*, *Anthoxanthum*, *Hierochloe,* and *Airopsis*, possess basic chromosome numbers of 5, 7, and 4, respectively (Clayton & Renvoize, 1986; Hilu, 2004). Therefore, either *x*= 6 evolved *de novo* in *Phalaris,* or alternatively a common ancestor to the genus with *x*=6 is extinct.

The association between geographic distribution, ecological adaptation and basic chromosome number in the genus is striking. Members of this *x*=6 clade are confined to Mediterranean Europe in terms of natural distribution. The expansion of the annuals *P. canariensis* and *P. brachystachys* to North America was the result of relatively recent human introduction (Barkworth, 2007). In contrast, the perennial *P. truncata* is a rare species with fragmented distribution in Mediterranean Europe (Baldini, 1993). Therefore, low species diversification, lack of polyploidy, and restricted geographic distribution could imply that the *x*=6 group is an evolutionary dead-end lineage. On the other hand, in the case of *x*=7 clade, species diversification, polyploidy evolution (possibly both auto- and allopolyploidy), and expansion to other continents and new habitats are evident (Fig. 1.1). The tetraploid *P. arundinacea* has the widest modern distribution, particularly in eastern parts of Asia where other *Phalaris* species are not found. The *x*=7 lineage also shows the evolution of new diploids (*P. lemmonii, P. caroliniana, P. platensis* and *P. angusta*) and tetraploids (*P. californica*) in North and South America. Geographically, *P. paradoxa* and *P. minor* overlap in distribution and show a strong presence in California along with *P. aquatica*. Remaining diploid and

tetraploid species with x=7 exhibit wide geographic distribution, although in some cases, such as the hexaploid *P. caesia*, the distribution is disjunct. Regardless, an ecological advantage appears to have been conferred with polyploidy superimposed on a basic chromosome number of x=7. These hypotheses can be tested in an eco-physiological study. With the apparent early split in the genus into the x=6 and x=7 lineages and the confinement of the former lineage to the Mediterranean region, it appears that Mediterranean Europe is likely to be the center of origin for the genus, rendering western North America, as a secondary center of diversity and polyploidy. However, a biogeographic study is underway to evaluate this hypothesis.

Phalaris therefore presents a potentially valuable case study in speciation, chromosomal evolution, and biogeography in the grass family due to its global distribution, notable variation in morphologies, and the key role of polyploidy in its evolution.

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Appendix A. *Phalaris* species used, geographic origin of the material, information on the herbarium vouchers, and GenBank numbers are noted. The number appended to each species name is used throughout this study to identify the multiple accessions of the species. *2n* = somatic chromosome number.

Species, chromosome number, origin, voucher, GenBank accession (ITS, *trnT-F*)

***Phalaris amethystina* Trin., *2n*=14: 1,** Bio-Bio, Chile, V. Finot & R. Solis 2247 (CONC-CH), JF951053, JF951091; **2**, South America, 108407 (SGO), JF951060, JF951092. ***P. angusta* Nees ex Trin., *2n*=14: 1,** Louisiana, USA, R.D. Thomas 87875 (VPI), JF951112,--; **2**, Brazil, USDA 310292, JF951054, JF951093; **3**, Rio Grande do Sul, Brazil, 3455246 (US), JF951055,--. ***P. appendiculata* L., *2n*=14: 1,** Ethiopia, USDA 331404, JF951071, JF951107. ***P. aquatica* L., *2n*=28: 1**, Californica, USA, S. Boyd 11476 (RSA), JF951076, JF951113; **2**, Netherlands, USDA 284200, JF951056, JF951094. ***P. arundinacea* L., *2n*=28: 1,** Virginia, USA, D.W. Ogle 97462 (VPI), JF951077,--; **2**, Canada, USDA 387928, JF951075, JF951095; **3**, Kazakhstan, USDA 435303, --, JF951096. ***P. brachystachys* Link, *2n*=12: 1**, California, USA, Lowell Ahart 10286 (CSCH),--, JF951114; **2**, Algeria, USDA 239820, JF951057, JF951097. ***P. caesia* Nees, *2n*=42: 1,** Montpellier, France, M. Kerguelen 8983 (FI), JF951061, JF951115; **2**, Ethiopia, E. DeWilde 6804 (BR), JF951062,--. ***P. californica* Hook. & Arn., *2n*=28: 1**, California, USA, D. Keil s.n. (OBI), JF951063,--; **2**, California, USA, J.D. Prouty 857 (CSCH), JF951078,--; **3**, California, USA, S.H. Bicknell s.n. (HSC), JF951064, JF951098. ***P. canariensis* L., *2n*=12: 1**, Egypt, USDA 251274, JF951058, JF951100; **2**, Brazil, USDA 163357,--, JF951099. ***P. caroliniana* Walt., *2n*=14: 1**, Georgia, USA, R.K. Godfrey 68477 (VPI), JF951079, JF951101; **2**, Lousiana, USA, R.D. Thomas 133507 (VPI), JF951080,--; **3**, Texas, USA, T. Boulware 15 3320327 (US), JF951065,--. ***P. coerulescens* Desf., *2n*=14: 1**, Sardinia, Italy, R.M. Baldini s.n. (FI), JF951081, JF951116; **2**, United Kingdom, USDA 239340, JF951066, JF951102. ***P. lemmonii* Vasey, *2n*=14: 1**, California, USA, D. Bramlet 2290 (RSA), JF951082, JF951117; **2**, California, USA, D. Keil & C.D Oyler 25383 (OBI), JF951067, JF951103. ***P. lindigii* Baldini, *2n*=unknown: 1**, Carchi, Ecuador, P. Peterson et al. 3237425 (US), JF951068, JF951104. ***P. maderensis* Menezes, *2n*=28: 1**, Madeira Island, Portugal, J.A. Carvalho s.n. (FI), JF951083, JF951118. ***P. minor* Retz. *2n*=28: 1,** Tuscany, Italy, R.M. Baldini s.n. (FI), JF951084,--; **2**, Calabria, Italy, R.M. Baldini s.n. (FI), JF951085,--; **3**, California, USA, J. Gregory 857 (SD), JF951086,--; **4**, Turkmenistan, Konovalovat 16726 (BR), JF907187,--; **5**, South Africa, USDA 208404, JF951069, JF951105. ***P. paradoxa* L., *2n*=14: 1**, Tuscany, Italy, Z.R.A. Da Silva s.n. (FI), JF951087,--; **2**, California, USA, J.R. Reeder 5665 (RSA), JF951088, JF951119; **3**, Sardinia, Italy, R.M. Baldini s.n. (FI), JF951089,--; **4**, Cyprus, USDA 239845, JF951070, JF951106. ***P. peruviana* H. Scholz & Gutte, *2n*=unknown: 1**, Peru, P. Gutte & G. Miller (Type specimen), JF951072, JF951108. ***P. platensis* Henrard ex Wacht, *2n*=14: 1**, USDA 239339, JF951073, JF951109. ***P. rotgesii* (Husnot) Baldini, *2n*=14: 1**, Corsica, France, R.M. Baldini 12/8 (FI) JF951090,--; **2**, Corsica, France, R.M. Baldini 15/1 (FI),

JF951074, JF951110. ***P. truncata* Guss ex Bertol., *2n*=12: 1**, Tuscany, Italy, R.M. Baldini 15/22 (FI),--, JF951120; **2**, Tunisia, USDA 535561, JF951059, JF951111. ***Briza minor* L., *2n*=10: 1**, Hsiao et al. 1995, L36510. ***Hierochloe australis* (Schrad.) Roem & Schult., 2n=14: 1**, Quintinar et al. 2007, DQ631447.1. ***Hierochloe equiseta* Zotov, *2n*=14: 1**, Gardner et al. unpublished, AY705901.1. ***Anthoxanthum monticola* Mez, *2n*=10: 1**, Gillespie et al. 2008, DQ353953.1; **2**, Kim et al. 2008, EF577511.1.

Chapter 2

Canary Grasses (*Phalaris*, Poaceae): Biogeography, molecular dating and the role of floret structure in dispersal

Stephanie Voshell, Khidir Hilu

ABSTRACT

Canary grasses (*Phalaris*, Poaceae) include 21 species, widely spread throughout the temperate and sub-tropical regions of the world with two centers of diversity: the Mediterranean Basin and western North America. The genus contains annual and perennial, endemic, cosmopolitan, wild, and invasive species with diploid, tetraploid, and hexaploid cytotypes. As such, *Phalaris* presents an ideal platform to study diversification via historic hybridization and polyploidy events, and geographic dispersal in grasses. We present the first empirical phylogeographic study for *Phalaris* testing current, intuitive hypotheses on the centers of origin, historic dispersal events and diversification within a geological timeframe. Bayesian methods (BEAST v. 1.6.2) were used to establish divergence dates, and dispersal-vicariance analyses (RASP v. 2.1b) were implemented for ancestral node reconstructions. Our phylogeographic results indicate that the genus emerged during the Miocene epoch (20.6-8.4 MYA) in the Mediterranean Basin followed by dispersal and vicariance events to Africa, Asia and the Americas. We propose that a diploid ancestor of *P. arundinacea* migrated to western North America via the Bering Strait, where further diversification emerged in the New World. It appears that polyploidy played a major role in the evolution of the genus in the Old World, while diversification in the New World followed a primarily diploid pathway. Dispersal to various parts of the Americas followed different routes. Fertile florets with hairy

protruding sterile lemmas showed significant correlation with wider geographic distribution.

INTRODUCTION

The grass family (Poaceae) is the fourth largest angiosperm family and ecologically the most dominant, covering about 20-40% of the land surface (Shantz, 1954; Brooks & al., 2004). It has been proposed that polyploidy and hybridization have played substantial roles in the diversification, ecological success and geographical expansion of this wind-pollinated family (Stebbins, 1985; Hunziker & Stebbins, 1987; Levy & Feldman, 2002; Hilu, 2006). The widespread distribution of grasses has also been correlated with their reproductive structures (Davidse, 1987; Clayton, 1990). Canary grasses (*Phalaris*) represent an ideal genus in the Poaceae to correlate polyploidy, hybridization, long-distance dispersal, and adaptation to varied habitats. The genus is remarkable in its geographic pattern of distribution that includes endemics on several continents as well as cosmopolitans that thrive in a multitude of habitats. The 21 species not only diversified in both the Mediterranean region and North America, but have also expanded into Africa, Asia, and South America (Fig. 2.1). They have occupied both wild and disturbed areas of temperate and sub-tropical regions, ranging from lacustrine habitats, sandy soils, waste bed, open fields, and wetlands (Baldini, 1995). The genus contains *P. arundinacea,* a cosmopolitan species that has become invasive in North America, and is currently used as a model organism in plant invasion studies (Galatowitsch & al., 1999; Lavergne & Molofsky, 2004; Thomsen & al., 2012). An empirical phylogeographic study to determine area of origin and to assess the historic

pattern of distribution and dispersal of canary grasses across continents and into varied habitats is lacking. This information would be valuable for examining the relationship between diversification, geographic expansion, emergence of endemics, and the role of morphological features in this wind-pollinated genus. Furthermore, a deeper understanding of the patterns of dispersal of *P. arundinacea* may prove useful to future studies of invasiveness.

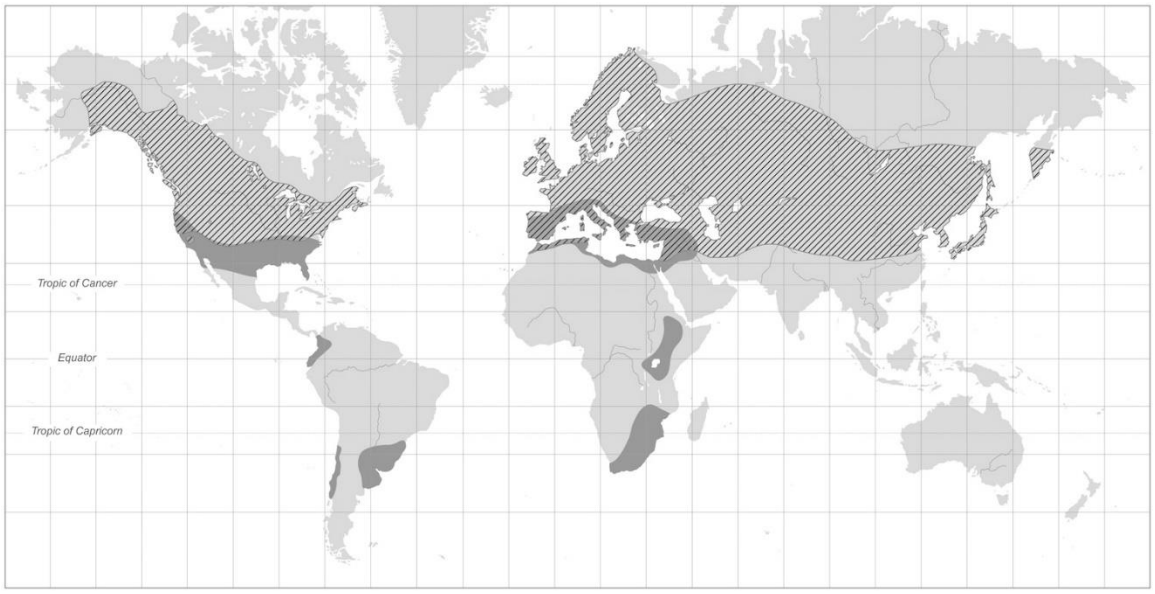

Fig. 2.1. Global distribution of *Phalaris* species. Dark grey areas represent endemic populations and the striped area shows the distribution of cosmopolitan *P. arundinacea*.

Schneider & al. (2009) place *Phalaris* in a mostly-Aveneae lineage in a phylogenetic reconstruction of the subfamily Pooideae (GPWG II 2011). In recent treatments of *Phalaris,* Anderson (1961) and Baldini (1993, 1995) circumscribed the species on the basis of morphological features and chromosome numbers. *Phalaris* species are characterized by laterally compressed ovate spikelets comprised of a single fertile floret subtended by two sterile florets, called here sterile lemmas (Fig. 2.2). The

florets are subtended by two glumes, which are awnless, generally flattened and dorsally keeled (Anderson, 1961). They display six distinct floret types (Fig. 2.2; Table 2.1) with sterile lemmas ranging from broad and glabrous to narrow and pubescent or completely reduced (Voshell & al., 2011). We hypothesize that the differences in length and hairiness of the sterile lemmas may have implications for long distance dispersal. Fig. 2.2 displays the different floret types and details their structure.

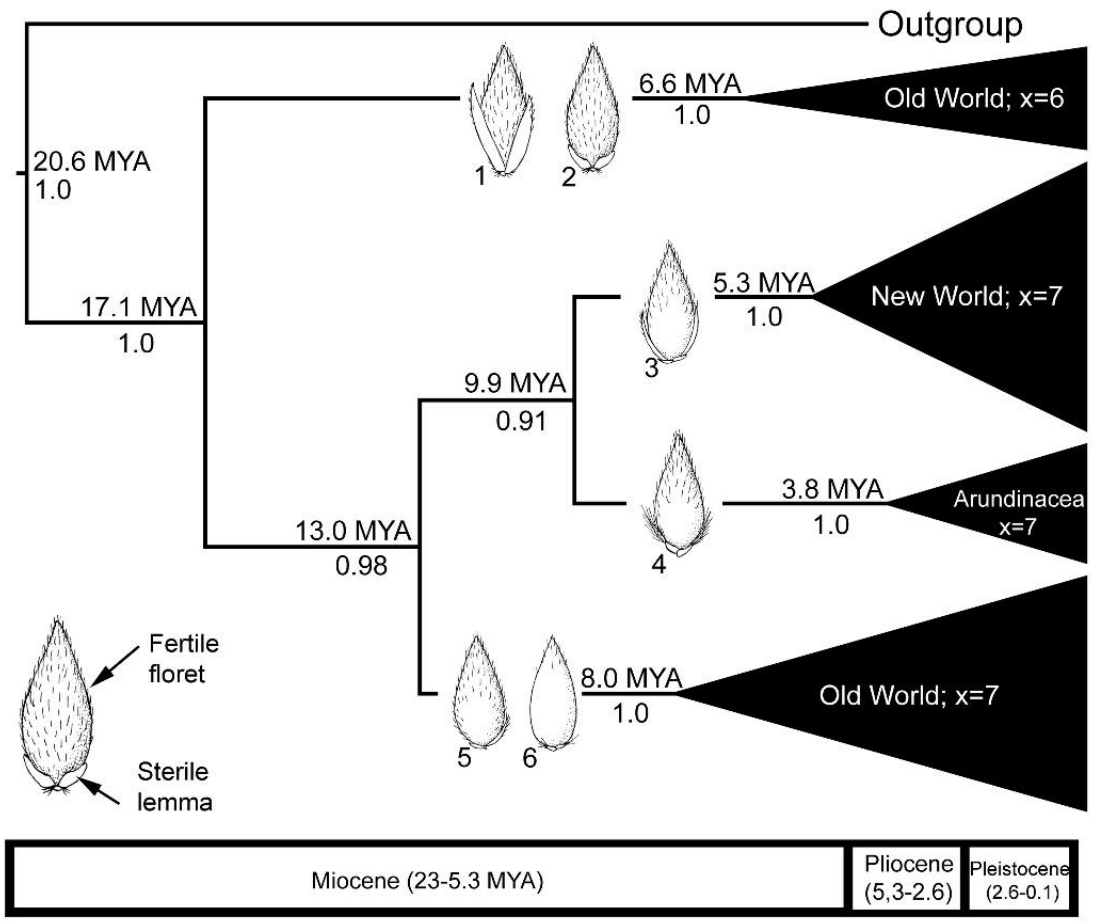

Fig. 2.2. Estimated dates of divergence within *Phalaris* based on the ITS dataset using BEAST v1.6.2. Divergence dates are shown above the lines and posterior probabilities are noted below the lines. The primary floret types for each major lineage of *Phalaris* are located near their respective clades. Note the presence of the two structures representing the sterile lemmas at the base of the large oval fertile floret in types 1-4, the presence of only one sterile lemma in type 5, and their absence in type 6. Annotation of floret parts are marked on a diaspora in the bottom left corner.

In a previous molecular phylogenetics study of the genus (Voshell & al., 2011), the ITS data provided a robust phylogeny with two strongly supported major lineages (1.00 PP, 100 BS) corresponding to the x=6 and x=7 species. The x=7 species in turn fell into three strongly supported clades (Voshell & al., 2011): the New World species emerged in one clade whereas the Old World species formed two clades, one with tetraploid *P. arundinacea* and its diploid and hexaploid relatives and another comprising the remaining Old World species (Voshell & al., 2011).

To establish an accurate phylogeographic picture within a geological timeframe for the genus, information on current and ancestral geographic distribution, dates of divergence, and a reliable phylogenetic history are essential. Divergence dates are critical for interpreting the biogeographic results in the context of historic climate and land topography that would have impacted potential routes of dispersal. Establishing divergence dates within the grass family has been quite challenging due to the limited availability of identifiable macrofossils (Prasad & al., 2005). This has led to reliance on a minimal number of fossil ages for calibration in molecular dating (Drummond & al., 2006; Inda & al., 2008; Ho & al., 2011; Molak, 2012; Hoffmann & al., 2013). Grass pollen in strata dated between 70 and 60 MYA (million years ago) has been found in both South America and Africa, indicating Paleocene emergence of grasses when the two continents were still connected (Linder, 1987; Jacobs & al., 1999). Pollen and macrofossil evidence have been used to constrain the divergence date for the Poaceae between 55 and 70 MYA (Kellogg, 2001). However, Bremer (2002) pushed the date to 75 MYA using the non-parametric rate smoothing method (NPRS; Sanderson, 1997). The divergence of the two major clades of the Poaceae, the BEP (Bambusoideae,

Ehrhartoideae, Pooideae) and PACMAD (Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, Danthonioideae) clades, is estimated to have been around 55-57 MYA, during the early Eocene (Crepet & Feldman, 1991; Bremer, 2002; Bouchenak-Khelladi & al., 2010). Within the BEP clade, the stem of the Pooideae, the subfamily containing *Phalaris*, is estimated at 44 MYA, limiting possible routes of geographic dispersal to the topology of the earth during the middle Eocene at the very earliest (Bouchenak-Khelladi & al., 2010).

Identification of grass fossils to the generic level is difficult due to the complex and often miniscule differences in key features (Jacobs & al., 1999). Two fossils from the Miocene layers of the Florissant formation in Colorado were initially identified as *Phalaris*, although these two records are questionable (Anderson, 1961). The first fossil was originally assumed to belong to *Melica* (Brues & Brues, 1909) but was later transferred to *Phalaris* by MacGinitie (1953). However, this record is questionable since it possesses more than one floret per spikelet (Anderson, 1961). The second fossil was assumed to be a member of *Phalaris* based on the solitary, reduced floret (Cockerell, 1913). Although it is difficult to ascertain that this fossil is a direct ancestor of *Phalaris*, it has been used as evidence for pooid diversification in the Americas (Anderson, 1961; Stebbins, 1981). The layer in which the fossils were recovered was dated to the Miocene (Brues & Brues, 1909), indicating that diversification of the Pooideae had already occurred in the Americas (Stebbins, 1981).

The Mediterranean Basin has been proposed as the center of origin for the genus based on the observation that the region contains the highest number of species and cytotypes, with western North America recognized as a second center of diversification

(Anderson, 1961; Baldini, 1993, 1995; Voshell & al., 2011). All three cytotypes (2x=14, 28, 42) are native to the Mediterranean, while the New World species are primarily diploids with the exception of limited native populations of tetraploid, cosmopolitan *P. arundinacea* and endemic *P. californica.* While human influence in terms of selective breeding is minimal in *Phalaris,* geographical distribution has been impacted by both intentional and unintentional transport of a few species. Only *P. aquatica*, *P. minor*, and *P. canariensis* have been extensively cultivated as forage and seed crops outside their natural distribution (Baldini, 1993, 1995). The most influential species in terms of environmental impact, *P. arundinacea,* has become a major invasive pest in many places around the world, notably the North American wetlands (Lavergne & Molofsky, 2004, 2007; Jakubowski & al., 2013). It was once believed to have been introduced to North America only by humans, but accounts of early collections in the far northwest region (Merigliano & Lesica, 1998) and recent molecular studies (Casler & al., 2009; Jakubowski & al., 2013) have indicated that a population of *Phalaris* existed prior to European settlement. This population may have resulted from a historic dispersal event from the Old World, presumably via the Bering Land Bridge (Milne, 2006). Aside from this small population, all *P. arundinacea* found in the Americas is likely the result of human introductions from Europe.

The objectives of this study were to conduct a molecular phylogeographic assessment of the genus using nuclear ITS and plastid *trnT-F* DNA sequences to evaluate current intuitive hypotheses on Mediterranean origin and discern ancestral distribution patterns and dispersal routes responsible for the modern distribution of *Phalaris*. As such, we envisioned three possible dispersal routes to the New World: 1) the Atlantic

Ocean, 2) the Bering Strait, or 3) independent migration events through both eastern and western routes. These hypotheses were evaluated based on the age of the genus as determined by Bayesian age estimation methods, consideration of land topology and climate during the time of divergence, and the results of the dispersal-vicariance analyses. Potential impacts of the structure of the diaspore on geographic expansion and ecological success were also evaluated.

MATERIALS AND METHODS

Taxon sampling and DNA sequencing

The 19-species datasets used by Voshell & al. (2011) were expanded by increasing sample density at the intraspecific level. New sequence data were obtained following the same procedures outlined in Voshell & al. (2011), and the accessions were deposited in GenBank. We used *Anthoxanthum monticola*, *Hierochloe equiseta* and *Hierochloe australis* as outgroups because of documented close phylogenetic affinity to *Phalaris* (Döring & al., 2007; Quintanar & al., 2007; Schneider & al., 2009) and prior effectiveness in tree resolution (Voshell & al., 2011).

DNA alignment and tree reconstruction

Sequences were manually aligned with Quickalign v1.6.0 (Müller, 2004). In the ITS data set, a small number of polymorphisms were detected (2 in *P. californica*, 6-10 in the Arundinacea clade, and 3 in *P. aquatica*) and were assigned IUPAC ambiguity codes in the alignment. Indels were not used as characters in the analyses. Accessions with identical sequences were represented by a single sequence in the analyses. After

removing identical accessions, there were 43 accessions left in the ITS alignment and 38 in the *trnT-F* alignment, including the two outgroup species. jModelTest v. 2.1.1 (Darriba & al., 2012) was used to determine the optimal substitution model for the Bayesian analyses based on the AIC criterion. The GTR+G model was recommended for both the ITS and *trnT-F* data sets. BEAUTI v.1.6.2 (Drummond & Rambaut, 2007) was used to prepare files for BEAST v1.6.2 (Drummond & Rambaut, 2007) to generate phylogenetic trees using Bayesian inference. The resulting trees were used for the biogeography and divergence dating analyses. Tracer v. 1.5 (Rambaut, 2009) was used to calculate the Effective Sample Size (ESS) of each run in BEAST and check for convergence through visual examination of plotted posterior probability estimates. The analyses were run for 10 million generations with the aforementioned models and four gamma rate categories; all other options were left at the default settings. Due to potential long branch attraction (Felsenstein, 1978) between *P. californica* and the outgroup species in the *trnT-F* data set (Voshell & al., 2011), BEAST was unable to correctly root the tree so we constrained the *Phalaris* species to be monophyletic to resolve the issue. We felt justified in making this constraint since *Phalaris* was shown to be monophyletic in our previous study using maximum parsimony as well as Bayesian inference implemented in MrBayes (Voshell & al., 2011). The sequence data for *P. peruviana* contained a large proportion (>50%) of missing data, and the remaining parts of the sequences were of poor quality. *Phalaris peruviana* is only known from the single type specimen, and consequently obtaining additional DNA material was not feasible. Thus, the species was excluded from all analyses.

Divergence dating

Since crown node fossils for *Phalaris* were unavailable, the time tree of life website (Hedges & al., 2006; Bouchenak-Khelladi & al., 2010) was used to estimate the age of the stem node to 20.6 MYA (the point where *Phalaris* diverged from the outgroup genus *Hierochloe*). The node ages were calculated in BEAST v1.6.2 (Drummond & Rambaut, 2007) by estimating the rate using the relaxed uncorrelated lognormal clock, the Yule tree prior, and the previously mentioned substitution models. A 10% burn-in was used when generating all max credibility clade consensus trees with TreeAnnotator v.1.6.2 (Drummond & Rambaut, 2007). The stem node on the maximum clade credibility tree was calibrated to 20.6 MYA using the timescale feature in FigTree v1.3.1 (2009). The ITS dataset was used for divergence dating since it provided a much more robust tree. The ITS dataset included twice the number of parsimony informative characters (128 vs. 64) despite its being significantly shorter (604 vs. 1492) than the *trnT-F* dataset. ITS datasets have proven to be effective in divergence dating studies (Baldwin & Sanderson, 1998; Oberprieler, 2005). Nevertheless, we also carried out a dating analysis on the *trnT-F* data set to check on divergence dates for nodes that are common between the topology of the two trees, following the same procedure noted above.

Biogeographical analysis

For the biogeography analysis, only the natural distribution of each species was considered as best understood from herbarium vouchers, historical records, and early floral surveys (Baldini, 1993; 1995). Consequently, Australia was excluded because all species of *Phalaris* in that continent are naturalized (Kodela & al., 2009).

Although various methodologies are available for phylogeographic analyses, the event-based dispersal-vicariance (DIVA; Ronquist, 1997; 2001) is the widely used method despite potential issues with the reliability of the phylogeny and ancestral area optimizations (Yan & al., 2010). Therefore, we opted to use the RASP program, since the Bayes-DIVA and S-DIVA methods it is based on are not sensitive to the previously mentioned shortcomings (Nylander & al., 2008; Yan & al., 2010; Yan & al., 2011). The S-DIVA and BBM methods were implemented in RASP (Yan & al., 2010) to infer potential ranges of ancestral nodes based on the *trnT-F* and ITS sequence data. RASP calculates the probability that the species at each node were present in a given region, thus presenting the most likely ancestral range (Ali & al., 2012). Ten geographic regions were recognized by dividing the natural distribution of the species into subsets representing distinct geographic areas of diversity (Baldini, 1993; 1995): A – western North America, B – eastern North America, C – western South America, D – eastern South America, E – Europe north of the Mediterranean Basin, F – Mediterranean Basin, G – eastern Africa, H – southern Africa, I – Middle East, and J – eastern Europe and northern Asia (Figs. 2.3-2.4). RASP calculates the ancestral node probabilities based on the average frequencies of all trees; therefore, all trees generated in BEAST were included in the analysis to minimize uncertainty. For both the BBM and S-DIVA methods all post burn-in trees were included and the maximum number of areas was set to five, leaving the other values at the default setting. RASP only allows analysis with a single outgroup and consequently *Anthoxanthum monticola* was chosen since it is found within the same range as *Phalaris*. *Hierochloe equiseta* and *H. australis* were kept in the analysis, but their distribution was not listed since they are native to New Zealand and

Australia, two regions that are entirely outside the native range of *Phalaris* (Biodiversity Occurrence Data). The most likely state option was chosen on the trees and the nodes where dispersal and vicariance events were likely to have occurred were highlighted.

Floret structure/distribution analysis

Florets of *Phalaris* species were grouped into two categories depending on the presence or absence of hairy protruding sterile lemmas based on examination of seed collections and data from Baldini (1993; 1995). The distribution of each species was coded as either 1 (confined to the Mediterranean Basin) or 2 (extend beyond the Mediterranean Basin). A one way ANOVA was performed on the data set using JMP (JMP 9, 2010) to determine if floret structure had a significant impact on species distribution beyond the Mediterranean Basin. A Chi-square test was also conducted in JMP 9 to test for variation between floret types and distribution.

RESULTS

The alignment of the ITS and *trnT-F* datasets was 604 and 1,492 characters in length, respectively. The ITS dataset contained 128 parsimony informative characters while the *trnT-F* dataset only contained 64 despite having greater than double the number of total characters. A poly C region in the *trnL-F* region as well as a conserved region toward the 3' end of the *trnT-L* region that contained missing data were excluded from the analysis. The posterior probability estimate plots generated in Tracer v. 1.5 for the biogeography analyses indicate that each run had reached convergence; the ESS values

obtained for the ITS and *trnT-F* analyses were respectively 2492 and 2474. Visual examination of the run used for date estimation in Tracer 1.5 suggested convergence was achieved; the ESS value was 668 for the ITS analyses. The date estimation based on the ITS data (Fig. 2.2) revealed that the major clades diverged in the Miocene epoch, with further speciation occurring within the last 5 million years during the Pliocene and Pleistocene. Similarly, the *trnT-F* dating analysis demonstrates comparable dates for the crucial clades for phylogeography, namely the New World species, Arundinacea group, and Old World x=7 (Fig. 2.5).

The S-DIVA and BBM results were not significantly different and, consequently, we chose to present the output from the BBM analyses (Fig. 2.3-2.4). The S-DIVA analysis suggests a complex series of dispersal and vicariance events during the history of the genus, primarily in the Arundinacea and New World clades (Figs. 2.3-2.4). The ITS analysis recovered 27 dispersal and 6 vicariances (Fig. 2.3); the *trnT-F* analysis yielded 20 dispersals and 6 vicariances (Fig. 2.4). The RASP analyses for both datasets (Figs. 2.3-2.4) place the Mediterranean region as the ancestral distribution throughout the backbone of the tree, suggesting that it is the most likely area of origin for the genus.

The ITS tree (Fig. 2.3) recovered the x=6 species in a clade of Mediterranean origin sister to all x=7 species. Within the x=7 clade, the Old World species emerged in a clade sister to the New World and Arundinacea clades, demonstrating that the most likely ancestral distribution for the entire x=7 clade is the Mediterranean Basin (node 80, 1.00 PP). The phylogeographic analyses of the ITS dataset showed the Mediterranean Basin and North America as the ancestral distribution for all the New World species (node 65, 0.99 PP). The initial dispersal events within the genus occurs at node 80-ITS (1.00 PP),

which represents the split between the Old and New World x=7 species. Among the Old World species, the results indicate that the main dispersal events occurred when *P. minor* or its common ancestor expanded its range eastwards into the Middle East (node 73-ITS, 1.00 PP; Fig. 2.3) and when *P. appendiculata* expanded to eastern Africa (node 74-ITS, 0.58 PP; Fig. 2.3). Dispersal events were detected at nearly every node in the Arundinacea and New World clades in both trees. A vicariance event appeared at node 70-ITS (1.00 PP; Fig. 2.3), which represents the divergence of *P. lindigii* from *P. aquatica*. The other vicariance events are at nodes 47 (0.23 PP), 46 (0.73 PP), 44 (0.40 PP), and 48 (0.95 PP) in the ITS analysis (Fig. 2.3). These nodes represent divergences within the New World clade.

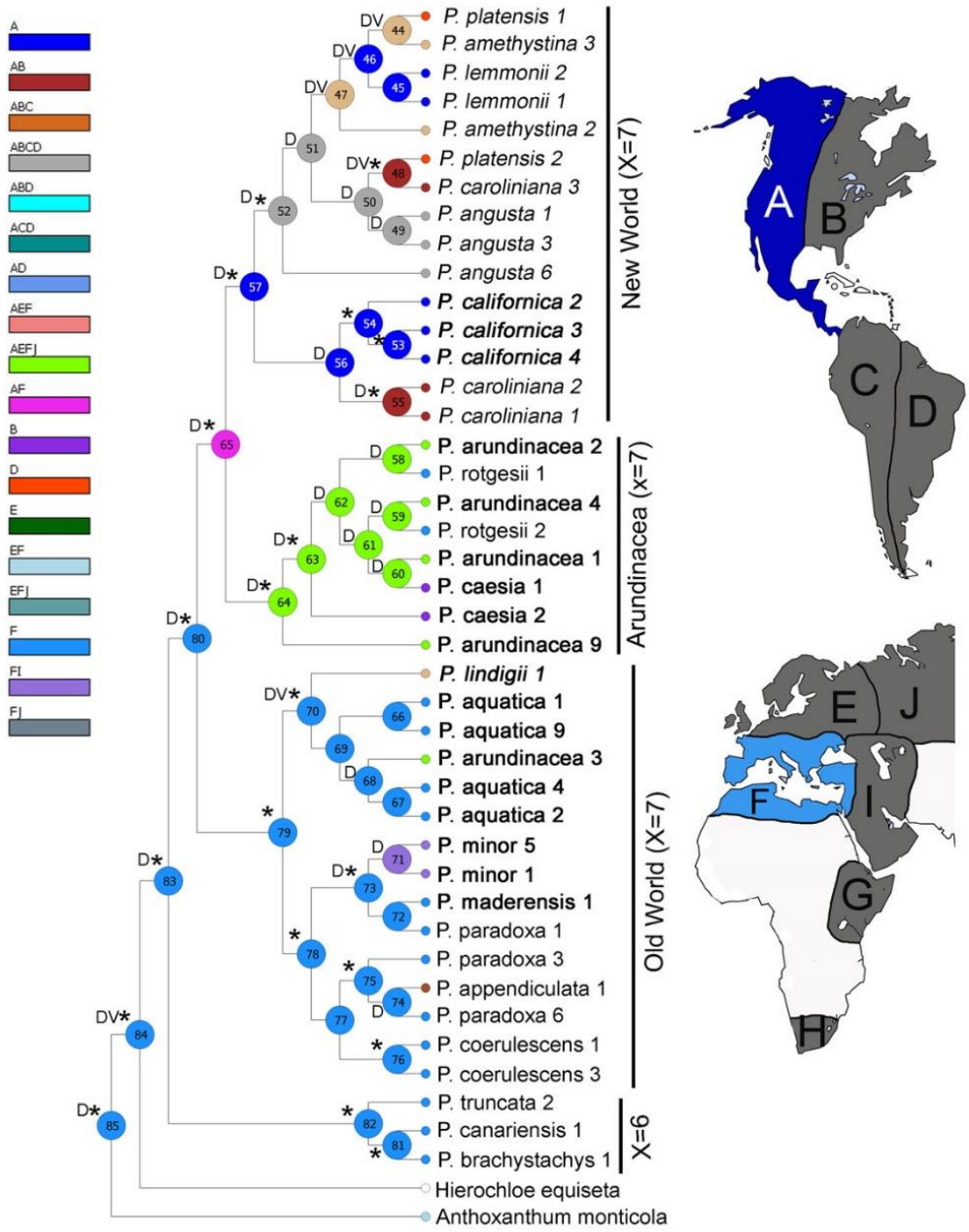

Fig. 2.3. Ancestral node reconstruction and dispersal-vicariance analysis generated in RASP from the ITS data set. Asterisks denote nodes with 0.95-1.00 PP support. Polyploid species are in bold, New World species are italicized, and Old World species are in regular type face.

The backbone of the *trnT-F* tree (Fig. 2.4) depicted *P. californica* as sister to remaining *Phalaris* species. The diploid Old World species emerged as a monophyletic lineage sister to a strongly supported clade containing the Old World polyploid species and *P. rotgesii,* plus another lineage representing a monophyletic x=6 clade and the New World species (Fig. 2.4). However, relationships among the major clades (Arundinacea + Old World polyploids, x=6 clade, New World diploids) lacked support indicating soft incongruence (Fig. 2.4). We have noted some indel and substitution synapomorphies between *P. californica* and the outgroup species in the *trnT-F* region which might explain this topology. Because of the incongruence between the ITS and *trnT-F* tree topologies, we opted not to conduct an analysis on the concatenated data set.

Despite the incongruence between the *trnT-F* and ITS trees, the *trnT-F* phylogeographic tree also depicted the Mediterranean Basin as the most likely ancestral distribution at the major basal nodes along the backbone until the divergence of the New World species (node 46; 0.92 PP; Fig. 2.4). Similar to the ITS analysis, the primary dispersals in the Old World clade are found in the *trnT-F* tree at nodes 54 (1.00 PP) and 64 (0.40 PP), which correspond to the expansion of *P. minor* into the Middle East and *P. appendiculata* southward into Africa. There are no dispersal events in the x=6 species, yet dispersal events occur at nearly every node in the Arundinacea and New World clades. Node 49 (0.94 PP) displays a vicariance event representing the divergence between the x=6 and New World species (Fig. 2.4). Two other vicariances are found at node 39 (0.88 PP) and 56 (1.00 PP), representing the split between *P. platensis*, *P. lemmonii*, and *P. lindigii* from the Arundinacea clade, respectively.

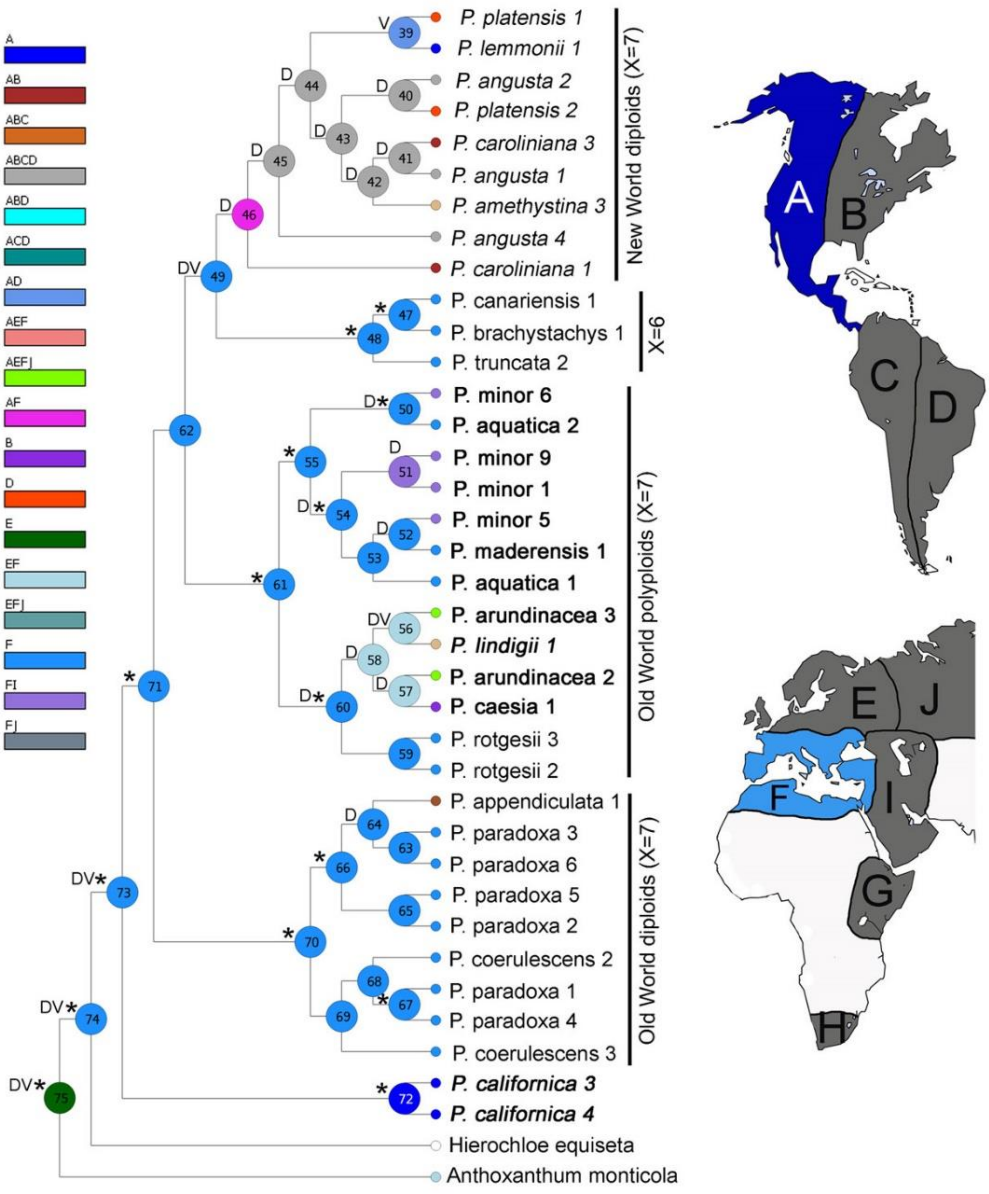

Fig. 2.4. Ancestral node reconstruction and dispersal-vicariance analysis generated in RASP from the *trnT-F* data set. Asterisks denote nodes with 0.95-1.00 PP support. Polyploid species are in bold, New World species are italicized, and Old World species are in regular type face.

The one way ANOVA performed in JMP (JMP 9, 2010) indicated that the structure of the floret (presence or absence of hairy protruding sterile lemmas) are highly correlated ($P < .0001$) with distribution outside the Mediterranean Basin. The chi-square test demonstrated a significant difference between the distribution of the two main floret types noted above ($P < 0.0011$, $N=19$, $DF =1$).

DISCUSSION

Divergence dating

To understand the historic phylogeographic patterns for canary grasses, it is essential to place the origin of the genus and the dispersal events across continents within the geological time scale. The Time Tree project points to the Miocene (20.6 MYA) as the date of the canary grasses stem node based on comparison of *Hierochloe* and *Phalaris* (Hedges & al., 2006; Bouchenak-Khelladi & al., 2010). Despite this calibration being based on a previous estimation, it represents a reasonable range for the divergence of *Phalaris* considering other Pooideae divergence dating studies (e.g. Bell & Donoghue, 2005; Inda & al., 2008). A biogeographical assessment of the Loliinae, a more recently diverging member of the same subfamily, estimated the age of the stem node at 13 MYA, placing them in the mid Miocene (Inda & al., 2008). Other dating and biogeographical studies of the Pooideae also concluded that its members first appeared in Eurasia during the Miocene (Soreng, 1990; Schippmann, 1991; Bell & Donoghue, 2005; Blattner, 2006; Inda & al., 2008).

Our results show that the major diversification event in the genus occurred during mid-Miocene (~13.0 MYA ITS, ~11.4 MYA *trnT-F;* Fig. 2.2, 2.5). The Miocene experienced a warming trend, especially at higher latitudes, allowing for expanded distribution further north than presently possible (Wing, 1998; Graham, 1999; Tiffney & Manchester, 2001; Knorr & al., 2011). These conditions would have allowed *Phalaris* to survive at higher latitudes, rendering migration higher north via birds, mammals or the ocean feasible. During the Miocene, grasslands and grazing mammals became dominant (Janis & al., 2004). Later in the Pleistocene epoch, North America was connected to Asia by the Bering Land Bridge, which facilitated animal migration (Janis & al., 2004), allowing for potential seed dispersal and further coevolution of grasses and herbivores (Stebbins, 1981). Studies have shown that transcontinental dispersals are not as rare as once believed (Vargas & al., 1998; Coleman & al., 2003; Blattner, 2006; Inda & al., 2008). Pockets of unglaciated land existed at that time along the route connecting Northern Asia and North America, serving as refugia for plant life (Abbott & al., 2000). These studies and our dating results suggest that it was possible for *Phalaris* to enter North America via the Bering Land Bridge. Diversification similar to that found in *Phalaris* has been reported for the *Poa* (Poaceae) species. Hoffmann & al. (2013) reported rapid diversifications of *Poa* in various parts of the world, including isolated areas in the Andes. Their estimated dates of diversification are 2.5-0.23 MYA, concurring with the time of species diversification in the *Phalaris* terminal branches in both ITS and *trnT-F* analyses (Fig. 2.2, 2.5).

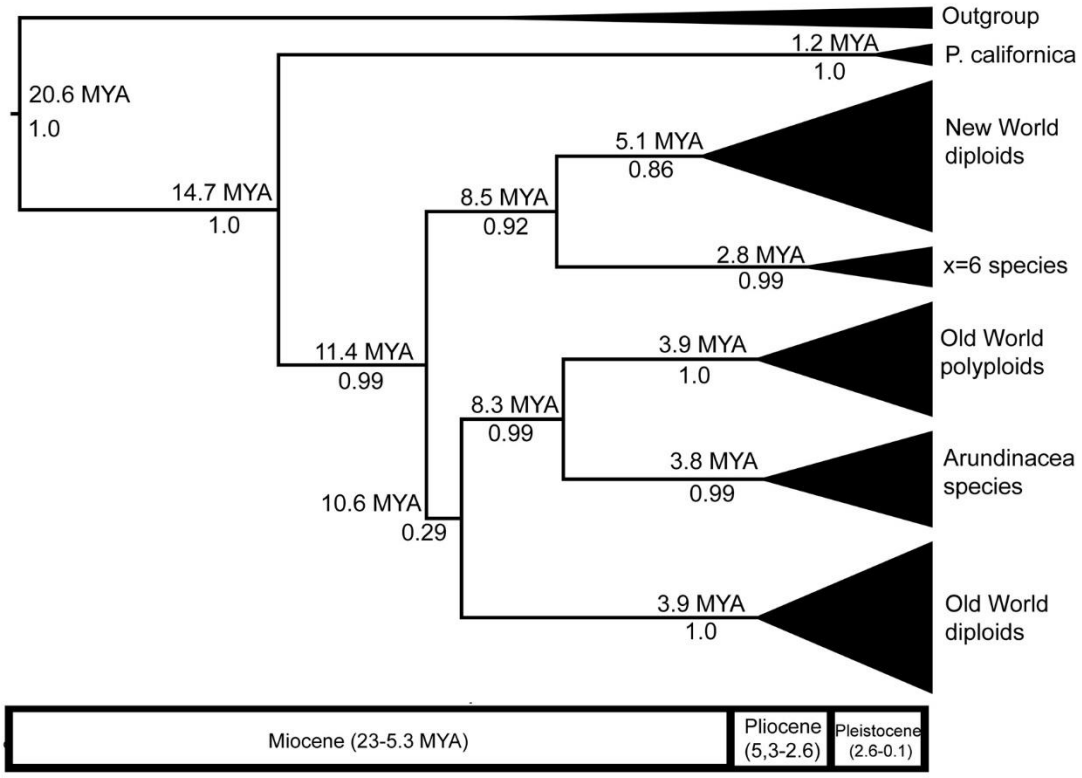

Fig. 2.5. Estimated dates of divergence within *Phalaris* based on the *trnT-F* dataset using BEAST v1.6.2. Divergence dates are shown above the lines and posterior probabilities are noted below the lines.

Geographic distribution and diversification

Both ITS and *trnT-F* analyses unequivocally support the Mediterranean Basin as the center of origin for *Phalaris* (Figs. 2.3-2.4) since the node for its split from the outgroup was reconstructed as Mediterranean. This finding was evident despite topological differences between the *trnT-F* tree and ITS trees. The results show very few dispersal events within the Old World clades, but a high degree of radiation for the Arundinacea and New World species. This relatively limited distribution of the Old World species may suggest narrow ecological adaptability to climates beyond the

Mediterranean, inability to disperse, or a combination of both. The analyses suggest vicariance events for several New World species with disjunct populations, implying that factors such as historic climate change or physical barriers may have led to the isolation and speciation events (Vuilleumier, 1971).

Although two hypotheses were proposed for the migration route to North America, the Bering Land Bridge route, but not the Atlantic route, is supported by our results since only the western North America and Mediterranean regions were recovered in the ancestral clade reconstruction (Figs. 2.3, 2.4; node 65 and 46 respectively). Based on the phylogenetic and phylogeographic position of *P. arundinacea* and the primarily diploid nature of the New World species, we propose that a diploid ancestor to the Arundinacea and New World clades migrated to western North America during the late Miocene. Chromosome evolution from diploidy to polyploidy, not the other way, is the pattern generally observed in grasses and other plant families (Levy & Feldman, 2002), making this assumption more likely. This event was the foundation for the diversification of diploid canary grasses across the New World. As such, it implies a narrow genetic base for speciation in the Americas. This might explain the low resolution in the New World clade encountered during tree reconstruction.

The presence of non-invasive, "native" tetraploid *P. arundinacea* in northwestern North America (Merigliano & Lesica, 1998) suggests subsequent migration events of modern tetraploid individuals from the Old World during the Pleistocene via the Bering Land Bridge. Casler & al. (2009) indicated that "native" *P. arundinacea* displays little genetic variation, pointing to a founder effect phenomenon caused by limited migration out of Europe. Lavergne & Molofsky (2007) propose that frequent recent human

introductions of *P. arundinacea* to the New World relieved the genetic bottleneck, possibly contributing highly invasive novel genotypes.

Phalaris californica represents a distinct evolutionary case in New World *Phalaris*. The origin of this single native New World tetraploid species of *Phalaris* needs to be evaluated in light of its placement in both the bipaternal ITS and the maternal *trnT-F* phylogenetic trees. In the ITS tree, a clade of *P. californica* + *P. caroliniana* accessions 1 and 2 emerged as sister to the New World species (all diploids). This clade was sister to the Arundinacea clade (Fig. 2.3), underscoring a deeper common ancestry between *P. californica* and tetraploid *P. arundinacea*. In contrast, *P. californica* appeared sister to all remaining *Phalaris* species in the *trnT-F* tree. *Phalaris californica* shares a unique 5 bp indel with the outgroup species (*Hierochloe* and *Anthoxanthum*). This shared synapomorphy could either be the result of homoplasy, or more likely were gained from a common ancestor. If we consider the latter case, then *P. californica* may have evolved from intergeneric hybridization event at the tetraploid level between *P. arundinacea* and a closely related Pooideae species, with the latter being the maternal parent. Intergeneric hybridization has been reported in the Poaceae (de Wet, 1987; Zapiola & Mallory-Smith, 2012).

After dispersal to western North America, *Phalaris* radiated throughout that continent and subsequently extended into South America. North American *P. caroliniana* and *P. angusta* are the only two species that extend from the southern half of North America to northern South America. Therefore, they are the best candidates for a common ancestry with the endemic South American species *P. platensis*, *P. amethystina, P. lindigii,* and possibly *P. peruviana*. *Phalaris caroliniana* is quite variable in both ITS

and *trnT-F* sequences, with accessions appearing in both North American subclades in all analyses (Figs. 2.3-2.4). Morphological intermediates between *P. caroliniana* and other New World species have been reported, implying possible interspecific hybridization (Baldini, 1995). Therefore, *P. caroliniana* might have played a role in the evolution of the South American species. The placement of accession *P. platensis* 1 (native to eastern South America) in the western rather than the eastern subclade (Figs. 2.3, 2.4) is questionable since the material originated from an experimental forage population in Australia.

In South America, endemic *P. lindigii* presents a very intriguing case phylogeographically. The species was recovered in all analyses inside the Old World clades that are entirely of Mediterranean origin (Figs. 2.3-2.5). A vicariance event for *P. lindigii* has consistently emerged (node 70-ITS, 1.00 PP; 56-*trnT*-F, 1.00 PP; Figs. 2.3, 2.4). Therefore, the phylogeography and geographic distribution are in disagreement. The species appeared within the Arundinacea complex maternally (Fig. 2.4) and as sister to *P. aquatica* in the ITS tree (Fig. 2.3). The same is true for *P. arundinacea* accession 3 collected in Peru, the general region where *P. lindigii* is found. These phylogenetic affinities suggest that *P. lindigii* as well as *P. arundinacea* accession 3 are hybrids between true *P. arundinacea* and *P. aquatica*, with the later being the paternal parent. These two species have been shown to hybridize in numerous breeding experiments, and that success of hybridization requires *P. arundinacea* being the maternal parent (Jenkin & Sethi, 1932). Covas & Cialzeta (1953) discuss the use of *P. arundinacea* x *P. aquatica* hybrids as forage crops in Argentina. The results of these experiments are consistent with our conclusion.

Floret structure and dispersal

Floret size plays an important role in reproductive success and dispersal of grasses (Harper & al., 1970). Species that produce a large number of small caryopses may benefit from higher fecundity and ease of dispersal, but would suffer from lower germination and survival rates since less energy is invested (Henery & Westoby, 2001; Westoby & al., 2002; Muller-Landau & al., 2008). Cheplick (1998) and Cousens & al. (2008) have suggested that lighter weight caryopses are likely favored for adhesive dispersal by animals. Further, it has even been proposed that small size is the best predictor of a diaspore's ability to remain attached to an animal (Römermann & al., 2005; Tackenberg & al., 2006). On the other hand, plants that produce fewer yet larger caryopses may have improved germination and survival rates, but lower fecundity and degree of expansion (Westoby & al., 2002; Moles & Westoby, 2004; Moles & Westoby, 2006; Muller-Landau, 2010). This correlation is observed in *Phalaris* where the floret of the x=6 Mediterranean species, such as *P. canariensis*, is 4-6 mm in length compared with the smaller 3-3.5 mm (Baldini, 1993; 1995) of the widely distributed and invasive x=7 *P. arundinacea*. Species possessing the x=6 cytotype of *Phalaris* have a much narrower natural distribution than the x=7 (Table 2.1). Numerous human introductions of the x=6 species to other continents were successful, indicating a physical reason such as caryopsis shape or size is responsible for the limited natural distribution, rather than inability to adapt to new environments.

Table 2.1. *Phalaris* species used, chromosome number (2*n* = somatic chromosome number), floret type, and geographic region(s) of distribution. Description of floret types are noted in the captions of Fig. 2.2 and in the text.

Species	2*n*=	Floret type	Geographic regions
Phalaris 74methystine Trin.	14	3	C
Phalaris angusta Nees ex Trin.	14	3	ABCD
Phalaris appendiculata Schult.	14	6	FG
Phalaris aquatica L.	28	5	F
Phalaris arundinacea L.	28	4	AEFJ
Phalaris brachystachys Link	12	2	F
Phalaris caesia Nees	42	4	FGH
Phalaris californica Hook. & Arn.	28	3	A
Phalaris canariensis L.	12	1	F
Phalaris caroliniana Walt.	14	3	AB
Phalaris coerulescens Desf.	14	6	F
Phalaris lemmonii Vasey	14	3	A
Phalaris lindigii Baldini	--	3	C
Phalaris maderensis Menezes	28	5	F
Phalaris minor Retz.	28	5	FI
Phalaris paradoxa L.	14	6	F
Phalaris peruviana H. Scholz & Gutte	--	3	AC
Phalaris platensis Henrard ex Wacht	14	3	D

Phalaris rotgesii (Husnot) Baldini	14	4	F
Phalaris truncata Guss. Ex Bertol	12	2	F

In addition to caryopsis size, Harper & al. (1970) has suggested that grass floret structure plays an important role in short and long distance dispersal. It has been shown that a bare caryopsis is incapable of acting as an effective dispersal unit (Davidse, 1987; Clayton, 1990). Prior studies of grasses have demonstrated that morphological features such as long hairs or basal bristles on the diaspore often represent an adaptation to adhesive dispersal (Rabinowitz & Rapp, 1981; Fischer & al., 1996; Cheplick, 1998; Cousens & al., 2008). In *Phalaris,* diaspores derived from florets with glabrous or pubescent protruding sterile lemmas (Fig. 2.2; types 3, 4 and 5) may have an advantage over smoother ones since they can adhere to mammal fur. The success of *Phalaris* species as forage grasses suggests frequent historic interaction with mammals, a situation that commonly leads to passive external transport (Stiles, 2000). Consequently, it follows that species with diaspores of floret types 3, 4 and 5 (Fig. 2.2) have a wider distribution due to their hairy protruding sterile lemmas than those with types 1, 2 and 6 (Fig. 2.2). Despite their large size, the sterile lemmas of floret type 1 do not remain firmly attached to the diaspore. Both the one way ANOVA and chi-square analysis comparing floret type and extent of geographic distribution underscored the highly significant correlation between the two ($P < .0001$, $P < 0.0010$, respectively). Therefore, species with hairy/protruding sterile lemmas (types 3 and 4) have dispersed beyond the Mediterranean Basin whereas those with reduced sterile lemmas (types 1, 2, 5, and 6) are confined to the Mediterranean.

The notable geographic expansion and success of *P. arundinacea* in dispersal might have been enhanced by its type 4 floret (Fig 2.2). The reduced sterile lemmas of this species are covered in tufts of hair, increasing the propensity for the caryopses to stick to mammal's hair and fur compared with the other floret types. Anderson (1961) originally proposed that all the New World species were direct descendants of the migration of *P. arundinacea*. The evolution of the type 3 floret structure with its long protruding sterile lemmas in the New World species may have assisted the rapid geographic radiation of *Phalaris* throughout the Americas.

Our phylogeographic study demonstrates unequivocally the origin of canary grasses in the Mediterranean Basin. We also demonstrate that diploid ancestral type of *P. arundinacea* might have migrated over the Bering Land Bridge to western North America during the Miocene, where a secondary center of diversification of primarily diploid species emerged. From there, speciation and geographic radiation appear to have occurred throughout the rest of North America and into both sides of South America where new endemics emerged. It appears that floret structure and caryopsis size might have played a varied role in dispersal as species with smooth diaspores display narrow geographic distribution whereas those with hairs cover wider geographic area. Further studies are needed to thoroughly sample the New World species and examine affinities between the eastern and western populations.

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DATA ACCESSIBILITY

DNA sequences: GenBank Accession nos: AY705901, DQ353953, DQ631447, EF577511, JF951055–JF951056, JF951058–JF951062, JF951064–JF951065, JF951067–JF951069, JF951071, JF951073–JF951084, JF951087, JF951089–JF951090, JF951093–JF951095, JF951098, JF951100–JF951102, JF951104–JF951107, JF951109–JF951115, JF951117–JF951119, KF753773–KF753800.

Individual specimen GenBank numbers and DNA alignment files from analyses: http://purl.org/phylo/treebase/phylows/study/TB2:S14877

Chapter 3

Canary grasses (*Phalaris*, Poaceae): Infrageneric treatment based on molecular phylogenetics and floret structure

Stephanie Voshell, Riccardo Baldini, Khidir Hilu

ABSTRACT

Phalaris L. (Poaceae, canary grasses) is a genus of 20 species found throughout the world with both endemic and cosmopolitan members. A variety of features in the genus underscore its importance for the study of polyploid evolution in relation to biodiversity, ecological niche expansion/contraction, endemism, and invasiveness. A formal and comprehensive infrageneric classification for the genus is lacking. This study utilizes molecular phylogenetics (nuclear ITS and plastid *trnT-F* regions), morphological features, primarily floret structure, and chromosome cytology to present the first comprehensive taxonomic classification for the genus. Two subgenera and four sections are established here to accommodate the 20 *Phalaris* species. Keys to the subgenera and sections, morphological descriptions, and lists of synonyms and species are provided.

INTRODUCTION

Recent molecular phylogenetic studies recognize *Phalaris* L. (Poaceae, grass family) as a member of the Aveneae/Poeae complex of the subfamily Pooideae (Döring & al., 2007; Quintanar & al., 2007; Schneider & al., 2009). *Phalaris* comprises 20 species found throughout the temperate and neo-tropical regions of the world. The genus possesses a wide range of variation in ploidy levels, habitat, habit, floret structure, and geographic ranges (Table 3.1; Fig. 3.1).

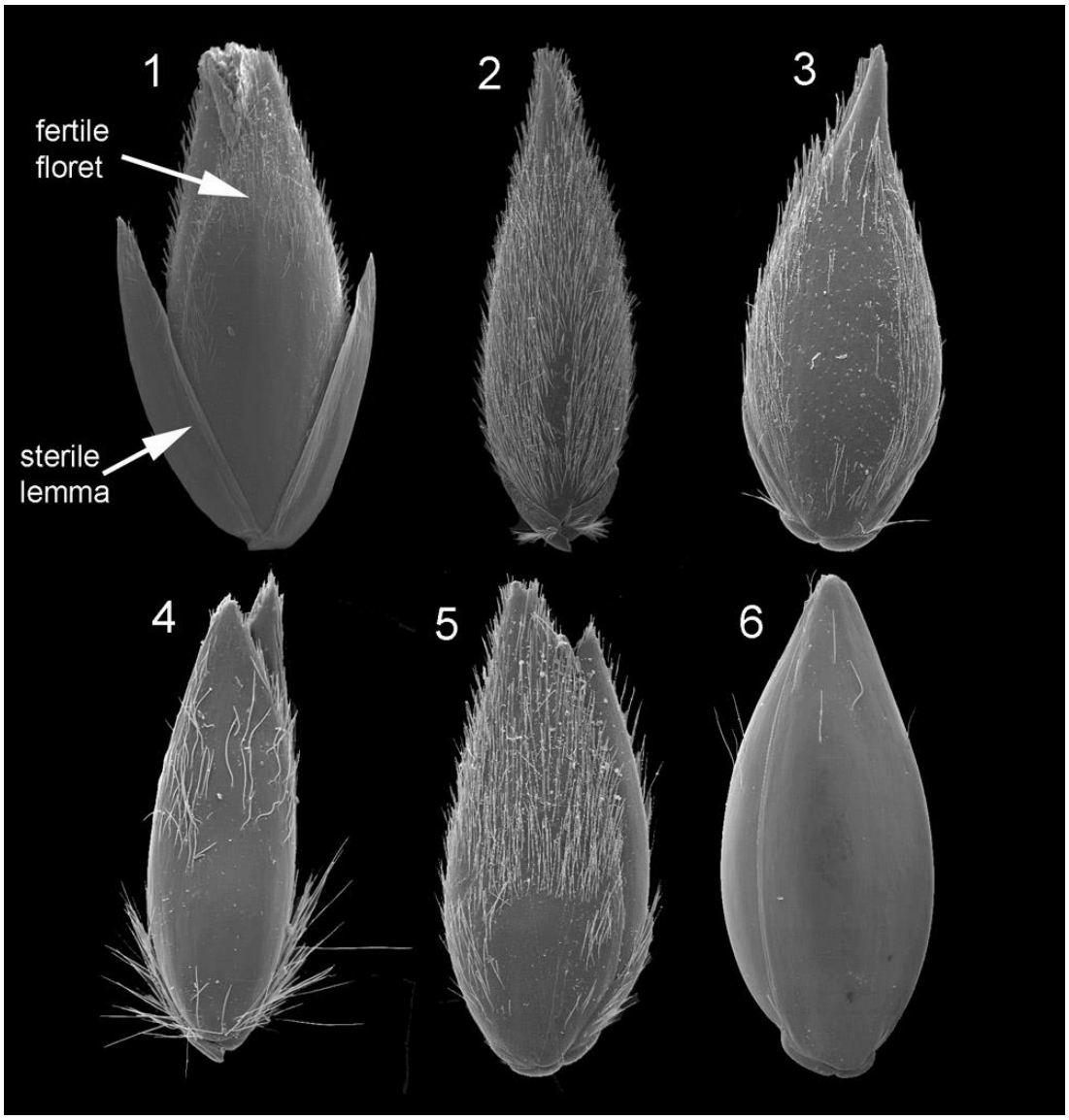

Fig. 3.1. SEM images of the six floret types recognized in *Phalaris*. The fertile floret and sterile lemmas are labeled on the type 1 floret. *Phalaris* florets range from 2-6 mm in length. SEM images provided by Riccardo Baldini (University of Florence).

It contains endemic species, such as *P. rotgesii* (Husnot) Baldini, *P. truncata* Guss. ex Bertol., *P. californica* Hook & Arn., *P. maderensis* (Menezes) Menezes, and *P. lemmonii* Vasey, as well as the cosmopolitan *P. arundinacea* L., which has become a model species

to study invasiveness. Although polyploidy has played a major role in the evolutionary success of the Poaceae (Stebbins, 1985; Hunziker & Stebbins, 1987; Levy & Feldman, 2002; Hilu, 2006), its role in *Phalaris* varied. The *x*=6 species as well as the New World *x*=7 species, except for *P. californica*, evolved through diploidy. In contrast, polyploidy played a role in the diversity of remaining Old World species with the emergence of tetraploid and hexaploid taxa. These attributes render *Phalaris* as an ideal model for experimental studies of the dimensions of biodiversity in the Poaceae. Despite these intriguing features, an infrageneric taxonomic treatment for the genus is lacking. Current phylogenetic and phylogeographic studies of *Phalaris*, (Voshell & al., 2011; Voshell & Hilu, 2014), when integrated with information from morphology, chromosome cytology and biogeography, provide solid grounds for the assessment of species relationships and infrageneric delimitation.

Table 3.1. Currently recognized *Phalaris* species and respective information regarding chromosome number and polyploid level, geographic range, habit, and floret type. A= annual, P= perennial.

species	chromosome number	range	habit	floret
Phalaris amethystina Trin.	x=7; diploid	S. America	A	3
Phalaris angusta Nees ex Trin.	x=7; diploid	N./S. America	A	3
Phalaris appendiculata Schult.	x=7; diploid	Mediterranean/Africa	A	6
Phalaris aquatica L.	x=7; tetraploid	Mediterranean	P	5
Phalaris arundinacea L.	x=7; tetraploid	Cosmopolitan	P	4
Phalaris brachystachys Link	x=6; diploid	Mediterranean	A	2
Phalaris caesia Nees	x=7; hexaploid	Mediterranean/Africa	P	4

Phalaris californica Hook & Arn.	x=7; tetraploid	N. America	P	3
Phalaris canariensis L.	x=6; diploid	Mediterranean	A	1
Phalaris caroliniana Walt.	x=7; diploid	N./S. America	A	3
Phalaris coerulescens Desf.	x=7; diploid	Mediterranean	P	6
Phalaris lemmonii Vasey	x=7; diploid	N. America	A	3
Phalaris lindigii Baldini	x=7; unknown	S. America	P	3
Phalaris maderensis (Menezes) Menezes	x=7; tetraploid	Maderia, Portugal	A	5
Phalaris minor Retz.	x=7; tetraploid	Mediterranean	A	5
Phalaris paradoxa L.	x=7; diploid	Mediterranean	A	6
Phalaris peruviana H. Scholz & Gutte	x=7; unknown	S. America	P	3
Phalaris platensis Henrard ex Wacht.	x=7; diploid	S. America	A	3
Phalaris rotgesii (Husnot) Baldini	x=7; diploid	Mediterranean	P	4
Phalaris truncata Guss. ex Bertol.	x=6; diploid	Mediterranean	P	2

Phalaris has a rich taxonomic history dating back to the first century AD. The first historical record of the genus is a brief, first century AD, description by Dioscorides (most likely in reference to *P. canariensis* L.), which was accompanied by a Byzantine era drawing (525 AD; Matthioli, 1554). Anderson (1961) noted that the crude nature of the information and the accompanied sketch make it impossible to identify the plant with certainty. Two species of *Phalaris* were named by Bauhin in 1623 prior to the advent of the binomial system: "*Phalaris major semine albo*" (*P. canariensis*) and "*Phalaris major semine nigro*" (Cf. *P. minor* Retz.). Linnaeus (1753) included five species in the first edition of Species Plantarum, and added five more in later editions (1755, 1763, 1767, 1771, 1781); some of these species were subsequently transferred to other genera (Baldini & Jarvis, 1991; Baldini, 1995). Lamarck (1778, 1783, 1805) circumscribed the genus to include 21 species, but due to his broad definition of the genus, over half the

species were later reassigned to other genera. Trinius (1828) described nine species and was the first to use the sterile floret features in the classification of the *Phalaris* species. His later revision (Trinius, 1840) listed 15 species and included a taxonomic key. Steudel published taxonomic treatments in 1841 and 1855, in which he examined a large list of names associated with the genus and assigned all but 25 as synonyms.

Although several regional treatments were subsequently produced, Anderson (1961) published the first worldwide revision since Steudel (1855); his treatment was based on morphological features and cytology and recognized 15 species. This treatment was followed by the comprehensive assessments of Baldini & Jarvis (1991) and Baldini (1993, 1995), where they recognized a total of 22 species including a synthetic octoploid. Baldini's (1995) treatment differed from the previous studies by including *P. appendiculata Schult., P. caesia* Nees, *P. daviesii, P. elongata* Braun-Blanq., *P. lindigii* Baldini, *P. peruviana* H. Scholz & Gutte, and *P. rotgesii*. *Phalaris daviesii* is an artificial octoploid hybrid derived from a cross between *P. minor* and *P. aquatica* L. (Blake, 1956) and is used only as a forage plant in Australia.

None of these studies addressed the infrageneric groupings of the species. Tsvelev (1973, 1983) was the first to publish an infrageneric treatment of the *Phalaris* species, although it was regional, covering the taxa in the former Soviet Union. He recognized eight species in four sections (*Bulbophalaris*, *Paraphalaris*, *Heterachne*, and *Phalaris*) and raised *P. arundinacea* to a generic level (*Phalaroides* Wolf). A recent molecular phylogenetic study showed that the entire genus, including *P. arundinacea*, is monophyletic (Voshell & al., 2011) and questioned the validity of the sections recognized by Tsvelev.

We present here the first comprehensive infrageneric classification for *Phalaris* based on molecular phylogenetics, reproductive morphological features, and biogeographic distribution.

MATERIALS AND METHODS

For the molecular phylogenetic analysis, a dataset was constructed by combining nuclear ITS and plastid *trnT-F* sequence data generated in Voshell & al. (2011) and Voshell & Hilu (2014). To account for the impact of polyploidy on phylogenetic reconstruction (Soltis & al., 2008), we eliminated the polyploid taxa from the combined data set and conducted the same analyses on the diploid-only data set. The concatenated ITS/*trnT-F* dataset differs from our previous studies in that we 1) excluded *P. peruviana* due to the excessive amount of missing data and the low quality of the sequences and 2) eliminated accessions with identical sequences, a step that resulted in improved resolution and support in the reconstructed tree. *Phalaris peruviana* is known only from the type specimen and the leaf sample received was in poor condition. The ingroup dataset was comprised of 18 species/accessions. *Anthoxanthum monticola* (Bigelow) Veldkamp, *Hierochloe equiseta* Zotov *and Hierochloe australis* (Schrad.) Roem. & Schult. were used as outgroup species because of their close phylogenetic proximity to *Phalaris* (Döring & al., 2007; Quintinar & al., 2007; Schneider & al., 2009) and prior documented effectiveness in *Phalaris* tree reconstruction (Voshell & al., 2011; Hilu & Voshell, 2014). Sequences were manually aligned in Quickalign v1.6.0 (Müller, 2004). The combined alignment of the ITS and *trnT-F* sequences was 2127 nucleotides in length. A poly C region in the *trnL-F* region as well as a conserved region toward the 3'

end of the *trnT-L* region were excluded because they contained an excessive amount of missing data. All sequences have been submitted to GenBank (Appendix B).

The partitioned and combined datasets were analyzed using Bayesian inference (Drummond & Rambaut, 2007) and RAxML (Stamatakis, 2014). The aligned sequences were analyzed in jModelTest v. 2.1.1 (Darriba & al., 2012) to select a suitable substitution model, and the GTR+G+I model was chosen based on the AIC criterion. For the Bayesian inference analysis, the data files were prepared using BEAUTI v.1.6.2 (Drummond & Rambaut, 2007) and the analyses were conducted in BEAST v.1.6.2 (Drummond & Rambaut, 2007). The Estimated Sample Size (ESS) was checked using Tracer v. 1.5 (Rambaut, 2009) and the plotted posterior probability estimates for all runs were visually inspected to check for convergence. The analysis was run for 10 million generations using the GTR+G+I substitution model and four gamma rate categories. All other parameters were left at the default settings and a 10% burn-in was used. BEAST was allowed to select the outgroup species and consequently the tree was rooted with *Hierochloe equiseta* and *Anthoxanthum monticola* as expected.

A phylogeny was also generated using a maximum likelihood analysis through the CIPRES portal using RAxML Version 8 (Stamatakis, 2014). *Anthoxanthum monticola* and *Hierochloe equiseta* were selected as outgroup taxa and 1000 replicates were run using the default settings and GTG+I+G model. PAUP* version 4.0b (Swofford, 2003) was used to convert the data into tree files with support values, which could be opened in FigTree v1.3.1 (2009). All trees were visualized and prepared for publication using FigTree v1.3.1 (FigTree v1.3.1, 2009).

RESULTS

Evidence from molecular phylogeny

In both the Bayesian and RAxML analyses, the phylogenetic trees based on the combined ITS and *trnT-F* datasets show maximum support for the monophyly of *Phalaris* (Fig. 3.2-3.3). These analyses also reveal a strongly supported split at the base of the tree into two clades representing the *x*=6 and the *x*=7 species (Figs. 3.2-3.3; 1.00 PP and 0.98 PP, respectively). In the Bayesian analysis, the *x*=7 lineage split into an Old World clade (1.00 PP) and a lineage containing two clades (0.98 PP): one representing the North American species, (1.00 PP) and the other encompassing *P. rotgesii*/*P. arundinacea*/*P. caesia*, which we will refer to at this point as the Arundinacea species. A topological incongruence has been detected between the maternal *trnT-F* and bipaternal ITS trees (Voshell & al., 2011; Voshell & Hilu, 2014). However, the topological conflict did not receive support in the *trnT-F* tree and thus it is considered as soft incongruence (Seelanan & al., 1997). The RAxML tree resolved all the major lineages found in the Bayesian tree with similar support; the two trees differ topologically in only two cases. The RAxML tree placed the Arundinacea clade as sister to the clade containing the remaining Old World species but with poor support compared with its placement in the Bayesian tree as sister to the New World species (<50 BS; Fig. 3.2). The second incongruence concerned the placement of *P. platensis,* which appears as sister to *P. lemmonii* in the RAxML tree instead of being sister to *P. angusta*. Support for the relationship was lacking or extremely low in both cases.

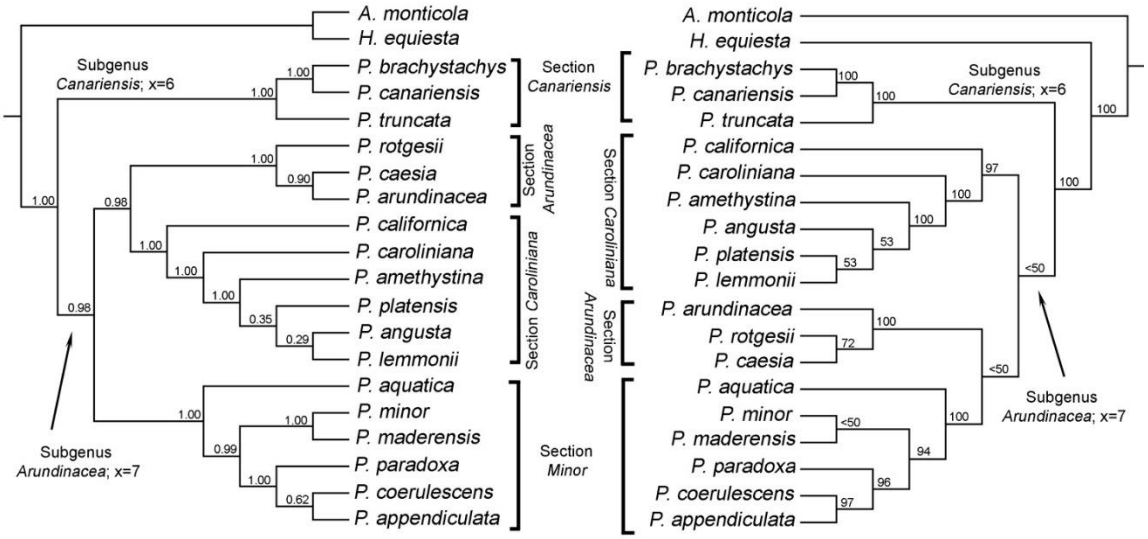

Fig. 3.2. Phylogenetic reconstruction of *Phalaris* based on combined ITS and *trnT-F* regions using Bayesian inference (left) and RAxML (right) with respective posterior probabilities and bootstrap values noted. The proposed subgenera and sections are identified and the basic chromosome numbers for the two major clades (subgenera) are cited.

Trees reconstructed from the diploid-only dataset recovered all the major lineages and revealed a tree topology identical to the Bayesian tree (Fig. S3.1). Again, the latter two trees differ from that of the RAxML tree only in the position of the Arundinacea group. The phylogenetic placement of the Arundinacea group has received very low support in all cases (Figs. 3.2-3.3, S3.1). Therefore, it appears that the inclusion of the polyploid species in the analyses has not impacted the topology of the trees obtained from the full data set.

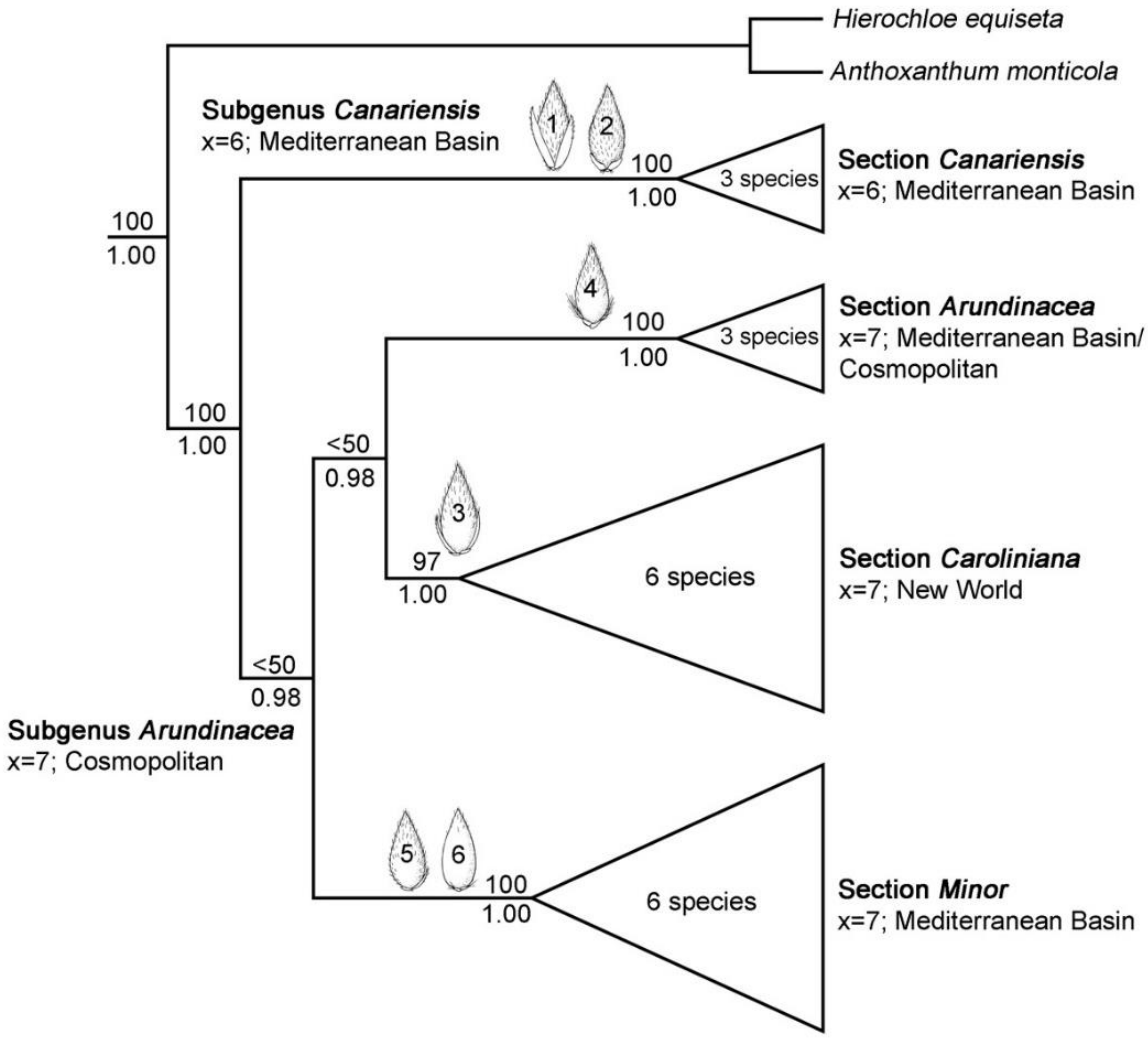

Fig. 3.3. Bayesian phylogenetic reconstruction of *Phalaris* based on combined ITS and *trnT-F* regions illustrating infrageneric classification, geographic affinities, and associated floret synapomorphies.

Evidence from morphology

The floral structure of *Phalaris* offers useful features for the taxonomic classification of the species. The spikelets consist of two glumes of similar size displaying variation in shape, size, vestiture, and presence/absence of a keel or a wing (Fig. 3.1). The glumes enclose a single fertile floret subtended by 1-2 highly reduced sterile ones (Fig. 3.1). In two rare cases, two fertile florets per spikelet were found in isolated populations of *P. aquatica* and *P. caesia* (Baldini, 1993).

Based on patterns of variation in sterile lemma structure, six floret types (Fig. 3.1) are recognized that can be used as synapomorphies for the clades resolved in the phylogenetic analyses, and subsequently in the infrageneric classification. Species in the *x*=6 clade (*P. canariensis, P. brachystachys* Link, *P. truncata*) have very distinctive broad, chaffy bract-like sterile lemmas (types 1-2; Fig. 3.1). In contrast, the sterile lemmas in the *x*=7 species range from thin and hairy to completely lacking (types 3-6; Fig. 3.1). Within the *x*=7 lineage, species of the New World clade (*P. californica, P. angusta* Nees ex Trin., *P. caroliniana* Walt., *P. platensis* Henrard ex Wacht., *P. amethystina* Trin., *P. lemmonii*) and the Arundinacea clade (*P. arundinacea, P. caesia, P. rotgesii*) two thin sterile lemmas with varied amounts of pubescence are displayed (types 3-4; Fig. 3.1). On the other hand, the Old World species (*P. paradoxa* L., *P. coerulescens* Desf., *P. minor, P. aquatica, P. maderensis*) possess either one thin sterile lemma or lack both (types 5-6; Fig. 3.1).

Reproductive characters also provide useful information on species delimitation. Among the three *x*=6 species (Table 3.1), *P. brachystachys* has been included under *P. canariensis* (Baldini, 1995). However, *P. brachystachys* differs morphologically from *P. canariensis* in having short and broad sterile lemmas and seeds that shatter at maturity compared with the distinctively long and narrow sterile lemmas and seeds that remain in the spikelet in *P. canariensis* (Baldini, 1995). In addition, morphological intermediates between the two species have not been reported, implying lack of gene flow. We thus treat the two as distinct species. In the *x*=7 lineage, *P. arundinacea*, *P. rotgesii*, and *P. caesia* are sometimes lumped under *P. arundinacea*. These three taxa also differ in size and hairiness of the floret. The species are reproductively isolated by polyploidy and thus should be consistently treated at the species level as proposed in Baldini (1995).

Phalaris paradoxa and *P. appendiculata* are morphologically the most variable species in the genus. *Phalaris appendiculata* was either lumped with *P. paradoxa* as a subspecies (Baldini, 1993, 1995) or treated as a distinct species. *Phalaris appendiculata* can be distinguished from *P. paradoxa* by the unique presence of complex clusters of single-floret fertile spikelets surrounded by numerous sterile spikelets. Phylogenetic analyses have revealed that the species are closely related (Voshell & al., 2011; Voshell & Hilu, 2014). Field and herbarium studies have pointed to some morphological intermediates between the two (Baldini, 1993, 1995), thus it is likely that a limited amount of gene flow still occurs among their populations in areas of geographic overlap (Baldini, 1995). *Phalaris paradoxa* has a broader range than *P. appendiculata*; the latter

is confined to northern Africa and Ethiopia (Baldini, 1995). Thus, *P. appendiculata* should be treated as a distinct species based on pronounced and unique reproductive morphology, geographic range, and the presence of molecular markers (substitutions and indels) not shared with *P. paradoxa*.

Phalaris lindigii is endemic to South America (Baldini, 1995). Recent studies (Voshell & al., 2011; Voshell & Hilu, 2014) have demonstrated the potential hybrid origin of this species from *P. arundinacea* and *P. aquatica*, two species that have been introduced to the region by human activities. *Phalaris lindigii* emerges sister to *P. arundinacea* in the maternal *trnT-F* tree and to *P. aquatica* in a bipaternal ITS tree (Voshell & Hilu, 2014). The species is now endemic to South America. Despite the potential hybrid origin of *P. aquatica*, the species is morphologically distinct in spikelet structure (Baldini, 1995; Voshell & al., 2011) and it will be treated at the species level at this point (Baldini, 1995).

The remaining species possess distinct morphologies and thus their taxonomic status has not been disputed (Anderson, 1961; Baldini, 1993, 1995). Consequently, we recognize 20 species in *Phalaris*.

DISCUSSION

Floret evolution in Phalaris

The key morphological features responsible for the placement of *Phalaris* in the "mostly Aveneae" lineage are the reduced floral elements and the arrangement of the sterile and fertile components of the spikelet. *Phalaris* appears in isolated positions in these molecular phylogenetic treatments (Döring & al., 2007; Quintanar & al., 2007). Nevertheless, its morphological association with *Anthoxanthum* and *Hierochloe* has

previously been underscored (Voshell & al., 2011). A trend in floral reduction exists in the *Phalaris*, *Anthoxanthum*, and *Hierochloe* complex (Clayton & Renvoize, 1986). The spikelets of these three genera are all comprised of a central floret, subtended by two lower ones. In *Hierochloe*, the lower florets are the same size as the central one, but are either male or sterile. *Anthoxanthum* displays further reduction with the lower florets being occasionally male, but usually empty. *Phalaris* demonstrates the greatest floret reduction, with the lower florets being much smaller than the central fertile floret, and are highly reduced to sterile lemmas, and in some cases completely lacking.

Despite prominent reduction in floral structure in *Phalaris*, six floret morphotypes (Fig. 3.1) have been detected (Anderson, 1961; Baldini, 1993, 1995; Voshell & al., 2011) that correlate with clade structure. The early-splitting x=6 lineage possesses the largest and most prominent sterile lemmas. Within the x=7 subgenus, the Arundinacea species display sterile lemmas which are reduced to hairy tufts while the New World species have long, thin sterile lemmas with less hair. The Old World species show the greatest degree of reduction with florets possessing either one sterile lemma or none at all.

The structure of the florets appears to have a biogeographic dimension. Voshell & Hilu (2014) carried out dispersal-vicariance analyses to discern geographic affinities for major clades. The study showed the Mediterranean region as the area for the origin of *Phalaris*, and the center of diversity for the *x*=6 and the Old World *x*=7 species. The study also suggests that a diploid ancestor of *P. arundinacea* migrated from the Mediterranean Basin to North America (Voshell & Hilu, 2014) over the Bering Land Bridge (~9-5 MYA) into western North America with subsequent dispersal and speciation to the rest of the New World. The small floret size and protruding sterile

lemmas associated with the Arundinacea and the New World species could have been instrumental in their dispersal via animals and for the subsequent speciation. In contrast, clades with species having larger florets exhibit relatively limited geographic distribution.

Considering the total evidence, the basal split in the trees obtained with the combined ITS and *trnT-F* data set show two distinct and highly supported lineages (100 BS. 1.00 PP, Fig. 3.2-3.3, RAxML and Bayesian) with distinct floret morphologies (type 1-2 vs. 4-6; Figs. 3.1, 3.3) and aneuploid cytotypes (x=6 vs. x=7). The three strongly supported clades in the x=7 lineage are each associated with distinct floret type and geographic affinities (Fig. 3.2-3.3). These biological entities stand out as reliable basis for the *Phalaris* infrageneric classification proposed below.

PROPOSED INFRAGENERIC CLASSIFICATION

We propose that *Phalaris* includes two subgenera representing the two, first-diverging major lineages corresponding to the x=6 and x=7 cytotypes. Within the x=7 lineage, the three major clades will be recognized at the sectional level. Brief descriptions for all these taxonomic units is provided, as well as the type species, synonyms, and species lists with updated synonymy. This information is mainly based on Baldini & Jarvis (1991) and Baldini (1993, 1995). Most names published for *Phalaris* can be found in websites such as IPNI (http://www.ipni.org) and TROPICOS (http://www.tropicos.org). For each taxon cited below, we report the homotypic (≡), heterotypic (=) and misapplied names (-).

Subgeneric classification

Gen. ***Phalaris*** L., Sp. Pl. 1: 54. 1753

Phalaroides Wolf, Gen. Pl. Vocab. Char. Def. 11. 1776

Typhoides Moench, Meth. 201. 1794

Baldingera P. Gaertn., B. Meyer & Scherb., Fl. Wetterau 1: 96. 1799

Digraphis Trin., Fund. Agrost. 127. 1822

Endallex Raf., Bull Soc. Genève 1: 220. 1830

Phalaridantha St.-Lag. In Cariot, Etude fl. Ed. 8, 2: 900. 1889, *nom. superfl.*

Subg. ***Canariensis*** Voshell, Baldini & Hilu, **subg. nov**.

Type: *Phalaris canariensis* L. "*Phalaris 1*" [cult.] (BM. Herb. Clifford 23), lectotype designated by Baldini & Jarvis (1991).

Description

Sterile lemmas chafflike, 2-3 mm long and narrow or 1/5-1/10 the length of the fertile floret and broad

Sect. ***Canariensis*** Voshell, Baldini & Hilu, **sect. nova**

Type: *Phalaris canariensis* L.

Description

Perennial rhizomatous with swelling base-stem, or annual herbs; ligule acuminate, cylindrical, often lacerate, 3-7 mm long; panicle 2-8 cm long, cylindrical, ovate to subovoid; glumes 6-10 mm long, winged; sterile lemmas chafflike, 2-3 mm long; fertile lemmas equal to subequal 4-6 mm long. Chromosome basic number: x=6.

Phalaris canariensis L., Sp. Pl. 54, 1753

(≡) *Phalaris ovata* Moench, Meth. 208. 1794, *nom. superfl.*

(≡) *Phalaris avicularis* Salisb., Prodr. 17. 1796, *nom. superfl.*

(≡) *Phalaris canariensis* L. subsp. *typica* Posp., Fl. Oest. Küst. 1: 59. 1897, *nom. inval.*

(=) *Phalaris canariensis* L. var. *debilis* Tocl & Rohlena, S.B. k. boehm. Ges. Wiss. 49: 1. 1902

(=) *Phalaris canariensis* L. var. *subcylindrica* Thell., Viert. Nat. Ges. Zurich 56: 271. 1912

(=) *Phalaris canariensis* L. forma *vivipara* Junge, Jahrb. Hamb. Wiss. Anst. 30: 123. 1912

(=) *Phalaris canariensis* L. forma *bracteata* Jansen & Wacht., Nederl. Kruidk. Archief. 6: 135. 1917

(=) *Phalaris canariensis* L. var. *villosula* Jansen & Wacht. Nederl. Kruidk. Archief. 52: 213. 1942

(=) *Phalaris canariensis* L. var. *tenuis* Jansen & Wacht., Nederl. Kruidk. Archief. 52: 213. 1942

(=) *Phalaris canariensis* L. var. *tenuis* Jansen & Wacht. forma *colorata* Jansen & Wacht., Nederl. Kruidk. Archeif. 52: 213. 1942

Phalaris brachystachys Link in Schrad., Neu. J. Bot. 1(3): 134. 1806

(≡) *Phalaris canariensis* L. var. *brachystachys* (Link) Posp., Fl. Oest. Küst. 1: 59. 1897

(≡) *Phalaris brachystachys* Link var. *typica* Paunero, Anales Jard. Bot. Madrid 8: 492. 1948

(=) *Phalaris quadrivalvis* Lag., Gen. Sp. Nov. 3. 1816

(=) *Phalaris nitida* C. Presl, Cyp. Gram. Sic. 26. 1820

(=) *Phalaris brachystachys* Link var. *robusta* Thell., Mém. Soc. Nat. Sc. Cherbourg 38: 88. 1912

Phalaris truncata Guss ex. Bert., Fl. Ital. 2: 777. 1835

(≡) *Phalaris brachystachys* Link var. *truncata* (Guss.) Paunero, Anales Jard. Bot. Madrid 8: 492. 1948

(≡) *Phalaris truncata* Guss. var. *typica* Maire & Weiller in Maire, Fl. Afr. Nord 2: 20. 1953

(=) *Phalaris truncata* Guss. forma *angustata* Trab. in Batt. & Trab., Fl. Algér. Monocot. 140. 1895

(=) *Phalaris truncata* Guss. var. *angustata* (Trab.) Maire & Weiller in Maire, Fl. Afr. Nord 2: 20. 1953

(=) *Phalaris truncata* Guss. var. *villiglumis* Trab. ex Maire in Maire, Fl. Afr. Nord 2: 20. 1953, *nom. inval.*

(-) *Phalaris aquatica* Auct. Pro parte, non L. (1755)

Subg. ***Arundinacea*** Voshell, Baldini & Hilu, **subg. nov**.

Type: *Phalaris arundinacea* L. "*arundinacea 3*" (LINN 78.7 [tetraploid, 2n=4x=28], lectotype designated by Baldini & Jarvis (1991).

Description

Sterile lemmas hairy or tufts of hair, if glabrous one or both sterile lemmas reduced.

Sect. ***Arundinacea*** Voshell, Baldini & Hilu, **sect. nov**.

Description

Perennial, rhizomatous herbs; ligule subtruncate, truncate and spathulate, 7-15 mm long; panicle 5-30 cm long, compact, lobate, to branched; glumes 2-7 mm long, 3 nerved, not winged to narrow winged; sterile lemmas 2, equal, 1-2.5(3) mm long, feathery; fertile lemmas 2-5 mm long, scarcely feathery to sparsely pubescent. Chromosome basic number: 2x=7.

Phalaris arundinacea L., Sp. Pl. 55. 1753

(≡) *Arundo colorata* Aiton, Hort. Kew. 1: 116. 1768

(≡) *Arundo riparia* Salisb. Prodr. 24. 1796, *nom. illeg.*

(≡) *Calamagrostis colorata* (Aiton) Sibth., Fl. Oxon. 37. 1794

(≡) *Typhoides arundinacea* (L.) Moench, Meth. 202. 1794

(≡) *Digraphis arundinacea* (L.) Trin., Fund. Agrost. 127. 1822

(≡) *Baldingera arundinacea* (L.) Dumort., Obs. Gram. Fl. Belg. 130. 1824

(≡) *Phalaridantha arundinacea* (L.) St. Lag. In Cariot, Etude fl. Ed. 8, 2: 900. 1889

(≡) *Phalaroides arundinacea* (L.) Rausch., Feddes Repert. 79(6): 409. 1969

(≡) *Phalaris arundinacea* (L.) var. *genuina* Hack., Bull. Herb. Boiss. 9: 646. 1899, *nom. inval.*

(≡) *Phalaris arundinacea* L. subsp. *typica* Paunero, Anales Jard. Bot. Madrid 8: 489. 1948, *nom. inval*.

(=) *Endallex arundinacea* Raf., Bull. Bot. Genéve 1: 220. 1830

(=) *Phalaris arundinacea* L. var. *arundinacea* forma *ramosa* Gaudin, Fl. Helv. 1: 160. 1828

(=) *Phalaris arundinacea* L. var. *arundinacea* forma *coarctata* Prahl ex Junge, Jarbh. Hamb. Wiss Anst. 30: 123. 1912

(=) *Phalaris arundinacea* L. var. *arundinacea* forma *ramifera* Junge, Jarbh. Hamb. Wiss. Anst. 30: 123. 1912

(=) *Phalaris arundinacea* L. var. *arundinacea* forma *minor* Jansen & Wacht., Nederl. Kruidk. Archief. 6: 141. 1917

(-) *Phalaris americana* Auct. Fl. Amer., non Ell. (1816)

Phalaris caesia Nees, Fl. Afr. Austral. 6. 1841

(≡) *Phalaroides arundinacea* (L.) Rausch. subsp. *caesia* (Nees) Tzvel., Novosti Sist. Vyssh. Rast. 10: 80. 1973

(≡) *Phalaroides caesia* (Nees) Holub, Folia Geob. Phytotax. 12(4): 428. 1977

(=) *Phalaris arundinacea* L. var. *thyrsoidea* Willk., Oesterr. Bot. Zeitschr. 40(4): 145. 1890

(=) *Phalaris arundinacea* L. var. *arundinacea* forma *thyrsoidea* (Willk.) Paunero, Anales Jard. Bot. Madrid 8: 489. 1948

(=) *Phalaris hispanica* Coincy, Morot Journ. De Bot. 8: 207. 1894

(=)*Phalaris arundinacea* L. subsp. *hispanica* (Coincy) Kerguélen, Bull. Soc. Bot. France 123: 322. 1976

(=) *Phalaroides hispanica* (Coincy) Holub, Folia Geob. Phytotax. 12(4): 428. 1977

(=) *Phalaris arundinacea* L. subsp. *oehleri* Pilger, Bot. Jahrb. Syst. 43: 91. 1909

(≡) *Phalaroides arundinacea* (L.) Rausch. subsp. *oehleri* (Pilger) Valdés & H.Scholz, Willdenowia 36: 664. 2006

(=) *Phalaris arundinacea* L. var. *leioclada* Maire, Bull. Soc. Hist. Nat. Afr. N. 32: 217. 1941

(-) *Phalaris arundinacea* sensu Auct. Fl. Afr., non L. (1753)

Phalaris rotgesii (Husnot) Baldini, Webbia 47(1): 13. 1993

Bas. *Baldingera arundinacea* (L.) Dumort. var. *rotgesii* Husnot, Graminées 87. 1899

(≡) *Baldingera arundinacea* (L.) Dumort. form. stat. *rotgesii* (Husnot) Foucaud & Mandon, Bull. Soc. Bot. France 3 sér., 47(7): 99. 1900

(≡) *Phalaris arundinacea* L. subvar. *rotgesii* (Husnot) Fiori in Fiori & Paol., Fl. Anal. Ital. 4: 14. 1907

(≡) *Phalaris arundinacea* L. prol. *rotgesii* (Husnot) Litard. Ex Briq., Prodr. Fl. Corse 1: 71. 1910

(≡) *Phalaris arundinacea* L. "race" *rotgesii* (Husnot) Jansen & Wacht., Nederl. Kruidk. Archief. 142. 1917

(≡) *Thyphoides arundinacea* (L.) Moench subsp. *rotgesii* (Husnot) Gamisans, Candollea 29: 44. 1974

(≡) *Phalaroides rotgesii* (Husnot) Holub, Folia Geobot. Phytotax. 12: 428. 1977

(≡) *Phalaroides arundinacea* (L.) Rausch. subsp. *rotgesii* (Husnot) Valdés & H.Scholz, Willdenowia 36: 664. 2006

Sect. ***Caroliniana*** Voshell, Baldini & Hilu, **sect. nova**

Type: *Phalaris caroliniana* Walt. "*South Carolina, McCormick Co., May 4, 1949, Wilbur H. Duncan 9468*" (US), neotype, designated by Anderson (1961).

Syn.: Sect. *Euphalaris* Asch. & Graebn. (1898), *pro parte*; *Euphalaris* Paunero, *nom. illeg.* (Paunero, 1948).

Description

Perennial rhizomatous and annual herbs; ligule 3-8 mm long, truncate-subtruncate to lanceolate, sometimes lacerate; panicle 2-20 cm long, cylindrical, ovoid to subovoid, sometimes branched at the base; glumes 3.5-8.0 mm long, keel winged to narrowly winged; sterile lemmas 2, equal, 0.8-3.5 mm long, scarcely feathery to densely pubescent; fertile lemmas 2.5-5.0 mm long, pubescent to scarcely pubescent, sometimes near the tip, or at the bottom. Chromosome basic number: x=7.

Phalaris caroliniana Walt., Fl. Carol. 74. 1788

(=) *Phalaris intermedia* Bosc ex Poiret, Encycl. Meth. Bot. Suppl. 1: 300. 1810

(=) *Phalaris microstachya* DC., Cat. Hort. Monsp. 131. 1813

(=) *Phalaris intermedia* Bosc ex Poiret var. *microstachya* (DC.) Vasey, Contr. US Nat. Herb. 3(1): 42. 1892

(=) *Phalaris americana* Ell., Sketch Bot. So. Car. Ga. 1: 101. 1817

(=) *Phalaris occidentalis* Nutt., Trans. Am. Philos. Soc., n.s., 5: 144. 1837

(=) *Phalaris trivilias* Trin., Mém. Acad. Sci. St-Pétersb., sér. 6, Sci. nat. 5(2): 55. 1839

Phalaris angusta Nees ex Trin., Sp. gram. 1, pl. 78. 1828

(≡) *Phalaris intermedia* Bosc var. *angusta* (Nees) Chap., Fl. South US 569. 1865

(=) *Phalaris angusta* Nees in Mart., Flor. Bras. 2: 391. 1829, non Nees ex Trin. (1828)

(=) Phalaris chilensis C. Presl, Rel Haenk. 1: 245. 1830

(=) *Phalaris intermedia* Bosc var. *angustata* Beal, Grasses in N. Amer. 2: 182. 1896

(=) *Phalaris angusta* Nees ex Trin. var. *angusta* forma *macra* Hack. Ex Jansen & Wacht. Nederl., Kruidk Archief. 6: 139. 1917

(=) *Phalaris angusta* Nees ex Trin. var. *angusta* forma *colorata* Jansen & Wacht., Nederl. Kruidk Archief. 6: 139. 1917

(=) *Phalaris angusta* Nees ex Trin. var. *robusta* Jansen & Wacht., Nederl. Kruidk Archief. 6: 139. 1917

(=) *Phalaris angusta* Nees ex Trin. var. *robusta* Jansen & Wacht. Forma *composita* Jansen & Wacht., Nederl. Kruidk Archief. 6: 139. 1917

(=) *Phalaris angusta* Nees ex Trin. var. *robusta* Jansen & Wacht. forma *interrupta* Jansen & Wacht., Nederl. Kruidk Archief. 6: 139. 1917

(=)*Phalaris angusta* Nees ex Trin. var. *robusta* Jansen & Wacht. forma *interrupta* Jansen & Wacht., mon. bracteata Jansen & Wacht., Nederl. Kruidk Archief. 6: 139. 1917

(-) *Phalaris intermedia* Auct., non Bosc (1810)

Phalaris amethystina Trin., Mém. Acad. Sci. St-Pétersb., sér. 6., Sci. nat. 5(2): 56. 1839

(=) *Phalaris berteroniana* Steud., Syn. Pl. Glumac. 1: 11. 1853

(=) *Phalaris robinsoniana* Steud., Syn. Pl. Glumac. 1: 11. 1853

(=) *Phalaris colchaguensis* Phil., Linnaea 33: 276. 1864

(-) *Phalaris angusta* sensu Trin. (1835), non Nees (1829)

(-) *Phalaris microstachya* sensu Desv. (1853), non DC. (1813)

(-) *Phalaris intermedia* sensu Johow (1896), non Bosc ex Poiret (1810)

Phalaris lemmonii Vasey, Contrib. US Nat. Herb. 3: 42. 1892

Phalaris platensis Henrard ex Wacht. in Heukels, Geillustr. Schoolfl. Nederl. 843. 1934

(≡) *Phalaris arechavaletae* Herter, Revista Sudam. Bot. 9: 105. 1953, *nom. superfl*.

(=) *Phalaris intermedia* Bosc forma *platensis* Arechav., Anales Mus. Nac. Montevideo 4: 298. 1896

(=) *Phalaris platensis* (Arechav.) Parodi in Valencia, Revista Argent. Agron. 4: 298. 1937, *homon. post*.

Phalaris californica Hook. & Arn., Bot. Beechey Voy. 1: 161. 1833

(-) *Phalaris amethystina* Auct. Fl. Amer., non Trin. (1839)

Phalaris peruviana H. Scholz & Gutte, Willdenowia 8: 379. 1978

Phalaris lindigii Baldini, Webbia 49(2): 317. 1995

Sect. ***Minor*** Voshell, Baldini & Hilu, **sect. nova**

Type: *Phalaris minor* Retz. "*Phalaris minor H.L. // aquatica Willd*." (n. 89/31.1962, LD).

Syn.: Sect. *Euphalaris* Asch. & Graebn. (1898), *pro parte*; *Euphalaris* Paunero, *nom. illeg.* (Paunero, 1948).

Description

Perennial rhizomatous often with swelling stem-base and annula herbs; ligule 3-8 mm long, cylindrical to subtruncate; panicle 3-15 cm long, cylindrical, ovato-lanceolate, ovato-spathulate; spikelets uniform, or in groups of 6-7, with 1 fertile floret surrounded at the bottom by (5)6-7 staminate florets (P. appendiculata, P. coerulescens) or 5-6 sterile spikelets (P. paradoxa); glumes 4-9 mm long, winged with margin entire or erose-dentate; sterile lemmas 1 or 2 inequal, 0.5-2 mm long, or both very reduced to obsolete (P. appendiculata, P. paradoxa, P. coerulescens); fertile lemmas 2.5-5 mm long. Chromosome basic number. 2x=7.

Phalaris minor Retz., Retz., Obs. Bot. 3:8. 1783

(≡) *Phalaris decumbens* Moench, Meth. 208. 1794, *nom. superfl.*

(≡) *Phalaris arundinacea* L. var. *minor* (Retz.) Paunero, Anales Jard. Bot. Madrid 8: 489. 1948

(≡) *Phalaris minor* Retz. var. *genuina* Maire & Weiller in Maire, Fl. Afr. Nord. 2: 23. 1953, *nom. inval.*

(=) Phalaris capensis Thunb., Prodr. Pl. Cap. 19. 1794

(=) *Phalaris aquatica* Thunb., Prodr. Pl. Cap. 19. 1794, non L. (1755)

(=) *Phalaris nepalensis* Trin., Sp. Gram. Ic. Tab. 80. 1828

(=) *Phalaris minor* Retz. var. *nepalensis* (Trin.) Bor, Grasses of India, Burma Ceylon and Pakistan 616. 1960

(=) *Phalaris brevis* Trin., Mém. Acad. St.-Pétersb. Sér. 6, Sci. nat. 5(2): 50. 1839

(=) *Phalaris ambigua* Fig. & De Not., Agrost. Aegypt. 10. 1853

(=) *Phalaris gracilis* Parl., Pl. Nov. 36. 1842

(=) *Phalaris minor* Retz. var. *gracilis* (Parl.) Parl., Fl. Ital. 1: 70. 1848

(=) *Phalaris minor* Retz. subsp. *gracilis* (Parl.) Arcang., Comp. Fl. Ital. 754. 1882

(=) *Phalaris minor* Retz. forma *gracilis* (Parl.) Asch. & Graebn. Syn. Mitteleurop. Fl. 2(1): 21. 1898

(=) *Phalaris minor* Retz. var. *comosula* Heldr., Bull. Herb. Boiss. 4: 396. 1898

(=) *Phalaris minor* Retz. var. *integra* Trab., Bull. Soc. Bot. Fr. 32(7): 394. 1885

(=) *Phalaris minor* Retz. var. *integra* Trab. forma *phaeosperma* Cavara, Bull. Orto Bot. Univ. Napoli 9: 42. 1927

(=) *Phalaris minor* Retz. forma *haematites* Duval-Jouve & Paris ex Trab. In Batt. & Trab., Fl. Alg. Monocot. 141. 1895

(=) *Phalaris minor* Retz. forma *subcylindrica* Web. & Thell. ex Jansen & Wacht., Nederl. Kruidk. Archief. 6: 131. 1917

(=) *Phalaris minor* Retz. var. *integra* Trab. forma *composita* Jansen & Wacht., Nederl. Kruidk. Archief. 6: 132. 1917

(=) *Phalaris minor* Retz. var. *integra* Trab. forma *bracteata* Jansen & Wacht., Nederl. Kruidk. Archief. 6: 132. 1917

(=) *Phalaris mauritii* Sennen, Diagn. Nouv. 243. 1936

(=) *Phalaris haematites* Duval-Jouve & Paris var. *granulosa* Sennen & Mauricio in Sennen, Diagn. Nouv. 244. 1936, *nom. inval*.

(-) *Phalaris aquatica* sensu Willd., Sp. Pl. 1: 326 (1797), pro parte, non L. (1755)

Phalaris aquatica L., Cent. I Pl. 4. 1755

(≡) *Phalaris nodosa* Murray in L., Syst. Veg. ed. 13: 88. 1774, *nom. superfl*.

(=) *Phalaris tuberosa* L., Mant. Pl. Alt. 557. 1771

(=) *Phalaris commutata* Roem. & Schult., Syst. Veg. 2: 403. 1817

(=) *Phalaris altissima* Menezes, Cat. Phanerogam. Madeira, Porto Santo 58. 1894

(=) *Phalaris tuberosa* L. var. *alata* Trab. in Batt. & Trab., Fl. Alg. Monocot. 141. 1895

(=) *Phalaris tuberosa* L. var. *hirtiglumis* Trab. in Batt. & Trab., Fl. Alg. Monocot. 140. 1895

(=) *Phalaris nodosa* L. var. *minor* Lojac., Fl. Sic. 3: 251. 1908

(=) *Phalaris elongata* Braun.-Blanq., Bull. Soc. Hist. Nat. Afr. N. 13: 21. 1922

(=) *Phalaris tuberosa* L. var. *clausonis* Maire & Trab., Bull. Soc. Hist. Nat. Afr. N. 24(7): 230. 1933

(=) *Phalaris stenoptera* Hack., Feddes Repert. 99: 333. 1908

(=) *Phalaris tuberosa* L. var. *stenoptera* (Hack.) Hitchc., Journ. Acad. Sci. 24: 292. 1934

(=) *Phalaris hirtiglumis* (Trab.) Baldini, Webbia 47(1): 20. 1993

(-) *Phalaris bulbosa* Auct. pro parte, non L. (1755)

Phalaris maderensis (Menezes) Menezes, Gram. Madeira 23. 1906

Bas. *Phalaris coerulescens* Desf. var. *maderensis* Menezes, Cat. Phanerogam. Madeira, Porto Santo 57. 1894

Phalaris coerulescens Desf., Fl. Atl. 1: 56. 1798

(≡) *Phalaris paradoxa* L. var. *coerulescens* (Desf.) Paunero, Anales Jard. Bot. Madrid 8: 486. 1948

(=) *Phalaris bulbosa* Cav., Icon. Descr. 1: 46, t. 64. 1791, non L. (1755)

(=) *Phalaris tuberosa* Link in Schrad., J. Bot. 4: 312. 1799 (1800), non L. (1771)

(=) *Phalaris variegata* Spreng., Neu. Entd. 2: 101. 1821

(=) *Phalaris coerulescens* Desf. var. *ovata* Parl., Pl. Nov. 33. 1842

(=) *Phalaris coerulescens* Desf. *tenuis* Asch. & Graebn., Syn. Mitteleur. Fl. 2(1): 17. 1898

(=) *Phalaris coerulescens* Desf. var. *villosula* De Not. Ex Parl., Fl. Ital. 1: 73. 1848

(=) *Phalaris coerulescens* Desf. var. *concolor* Lojac., Fl. Sic. 3: 251. 1908

(-) *Phalaris aquatica* Auct. pro parte, non L. (1755)

Phalaris paradoxa L., Sp. Pl., ed. 2, 1665. 1763

(≡) *Phalaris paradoxa* L. var. *typica* Paunero, Anales Jard. Bot. Madrid 8: 486. 1948, *nom. inval.*

(=) *Phalaris paremorsa* Lam., Fl. Fr. 3: 566. 1778

(=) *Phalaris paradoxa* L. var. *praemorsa* (Lam.) Coss. & Durieu, Expl. Sci. Algér. 2: 25. 1854

(=) *Phalaris paradoxa* L. forma *praemorsa* (Lam.) Paunero, Anales Jard. Bot. Madrid 8: 486. 1948

(=) *Phalaris paradoxa* L. var. *intacta* Coss. & Durieu, Expl. Sci. Algér. 2: 24. 1854

(=) *Phalaris paradoxa* L. var. *intermedia* Coss. & Durieu, Expl. Sci. Algér. 2: 24. 1854

(=) *Phalaris sibthorpii* Griseb., Spicil. Fl. Rumel. 2: 468. 1844

(=) *Phalaris paradoxa* L. var. *megastachys* Goiran, Nuov. Giorn. Bot. Ital., n.s., 17: 53. 1910

Phalaris appendiculata Schult. in Roem. & Schult., Mant. 2: 216. 1824

(=) *Phalaris rubens* Ehrenb. ex Trin., Mém. Acad. Sci. St.-Pétersb., sér. 6, Sci. nat. 5(2): 50. 1839

(=) *Phalaris obvallata* Trin., Mém. Acad. Sci. St.-Pétersb., sér. 6, Sci. nat. 5(2): 51. 1839

(=) *Phalaris pseudo-paradoxa* Fig. & De Not., Agrost. Aegypt. 11: 1853

(=) *Phalaris paradoxa* L. var. *appendiculata* (Schult.) Chiov. forma *appendiculata* Chiov., Ann. R. Ist. Bot., Roma 8(3): 328. 1908

(=) *Phalaris paradoxa* L. var. *appendiculata* (Schlt.) Chiov. forma *nana*, Ann. R. Ist. Bot., Roma 8(3): 328. 1908

Artificial Hybrids used as forage and crop, not naturalized

Phalaris daviesii S.T. Blake (*P. tuberosa* L. x *P. minor* Retz.), Proc. Roy. Soc. Queensland 67: 27. 1956 (2n=8x=56).

Phalaris tuberinacea Coville & Cialz. (*P. arundinacea* L. x *P. aquatica* L.), I.D.I.A. 68: 8. 1953 (2n=8x=56).

KEY TO SUBGENERA AND SECTIONS

1a. Sterile lemmas chafflike, 2-3 mm long and narrow or 1/5-1/10 the length of the fertile floret and broad……..subg. **Canariensis**/Sect. **Canariensis**

1b. Sterile lemmas hairy or tufts of hair, 1-3.5(4) mm long, if glabrous one or both sterile lemmas reduced……subg. .**Arundinacea**

Subg. Arundinacea: Key to the sections

1a. Sterile lemmas 2, equal, densely feathery, sparsely pubescent, never reduced or obsolete…….....2

1b. Sterile lemmas 1 or 2, inequal, not feathery, reduced or obsolete…….....3

2a. Sterile lemmas 2, equal, 1.5-2.5 mm long, densely feathery; fertile lemma 3-5 mm long, sparsely pubescent …….sect. **Arundinacea**

2b. Sterile lemmas 2, equal, pubescent, sometime feathery, 1.5-2 mm long; fertile lemmas 1.5-5 mm long……..sect. **Caroliniana**

3. Sterile lemmas 1 or 2, narrow, subequal, reduced to obsolete, glabrous, or pubescent, 0.5-2 mm long; fertile lemmas 0.3-5 mm long ……....sect. **Minor**

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Linnaeus, C. 1755. Amoenitates academicae 4. Lugduni, Netherlands: apud Wetstenium.

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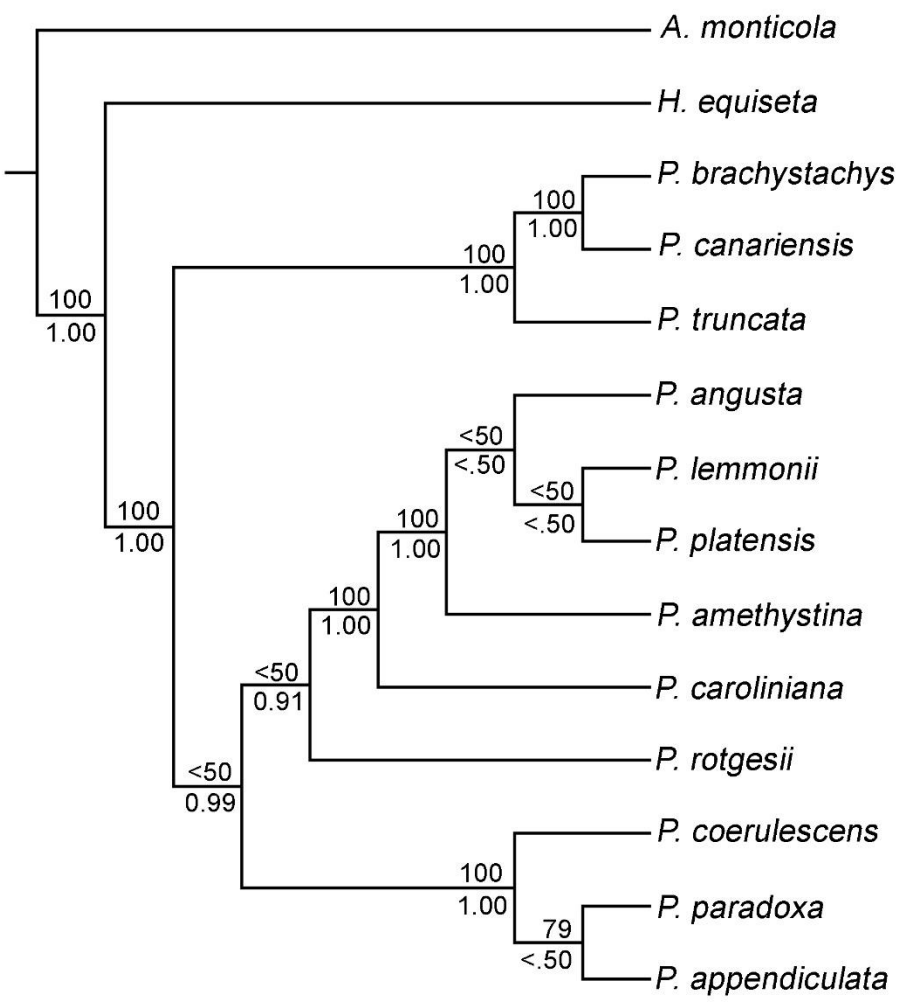


Fig. S3.1. RAxML phylogenetic tree based on the diploid-only ITS and *trnT-F* data set. The Bayesian Inference tree is completely congruent with the RAxML tree; posterior probabilities are noted below the lines.

Appendix B. Taxa used, their geographic origin, herbarium voucher information, and GenBank numbers.

Species, origin, voucher, GenBank accession (ITS, *trnT-F*)

Phalaris amethystina, South America, 108407 (SGO), JF951060, JF951092. ***P. angusta,*** Louisiana, USA, R.D. Thomas 87875 (VPI), JF951112, KF753786. ***P. appendiculata,*** Ethiopia, USDA 331404, JF951071, JF951107. ***P. aquatica*** Netherlands, USDA 284200, JF951056, JF951094. ***P. arundinacea*** Canada, USDA 387928, JF951075, JF951095. ***P. brachystachys*** California, USA, Lowell Ahart 10286 (CSCH), KF753780, JF951114. ***P. caesia*** Montpellier, France, M. Kerguelen 8983 (FI), JF951061, JF951115. ***P. californica*** California, USA, Hickman 1, KF753781, KF753789. ***P. canariensis*** Egypt, USDA 251274, JF951058, JF951100. ***P. caroliniana*** Georgia, USA, R.K. Godfrey 68477 (VPI), JF951079, JF951101. ***P. coerulescens*** United Kingdom, USDA 239340, JF951066, JF951102. ***P. lemmonii*** California, USA, D. Bramlet 2290 (RSA), JF951082, JF951117. ***P. lindigii*** Carchi, Ecuador, P. Peterson et al. 3237425 (US), JF951068, JF951104. ***P. maderensis*** Madeira Island, Portugal, J.A. Carvalho s.n. (FI), JF951083, JF951118. ***P. minor*** South Africa, USDA 208404, JF951069, JF951105. ***P. paradoxa*** Cyprus, USDA 239845, JF951070, JF951106. ***P. platensis*** Argentina, USDA 281598, KF753784, KF753799. ***P. rotgesii*** Corsica, France, R.M. Baldini 15/1 (FI), JF951074, JF951110. ***P. truncata*** Tunisia, USDA 535561, JF951059, JF951111. ***Hierochloe australis*** **(Schrad.) Roem & Schult.,** Quintinar et al. 2007, DQ631447.1. ***Hierochloe equiseta*** **Zotov,** Gardner et al. unpublished, AY705901.1. ***Anthoxanthum monticola*** **Mez,** Gillespie et al. 2008, DQ353953.1; Kim et al. 2008, EF577511.1.

Chapter 4

Canary grasses (*Phalaris*, Poaceae): breeding systems, floret morphology and genome size.

ABSTRACT

The canary grasses (*Phalaris*, Poaceae) include 20 species spread across the globe with both endemic and cosmopolitan members. This monophyletic genus boasts an impressive degree of variation between species. *Phalaris* species possess a basic chromosome number of either x=6 or x=7 with the latter having diploid, tetraploid, and hexaploid members. Six distinct floret types are present and species display variation in breeding system. The diversity of features found in the canary grasses make them an ideal platform to study trends in floret morphology, genome size, cytotype, breeding systems and the role these factors have played in the success of the genus. We present the first empirical study relating floret morphology to dispersal success. Our results show that the size and weight of New World caryopses are significantly smaller than those of other species supporting the hypothesis that these features influenced their dispersal success. C values have been determined for 27 accessions using flow cytometry revealing a larger 1C genome size with higher intraspecific variation for the x=6 species relative to the x=7 species. Self-incompatibility within the genus is highly variable with species ranging from being highly self-incompatible to capably of frequent self-pollination.

INTRODUCTION

The canary grass genus (*Phalaris*, Aveneae, Poaceae) is a small group of 20 species (Chapter 3) found throughout the world in a wide range of habitats. Some members of the genus are endemic while others have expanded around the world and are considered cosmopolitan and even invasive. There is a large degree of variation between species in traits such as basic chromosome number, ploidy, breeding system, genome size, floret structure, floret size and weight, and geographic range. These variables alone or in combination may explain the differential success of species in the genus to expand and adapt to new locations.

The floret structure of *Phalaris* has proven to be a useful trait for identification of the individual species (Anderson, 1961; Baldini, 1993, 1995). Six floret types have been established and strong affinities exist among the phylogenetic groups (Voshell & al., 2011). *Phalaris* spikelets each contain a single fertile floret subtended by two glumes. The structure and presence of one to two sterile lemmas on the florets are the distinguishing features for the six types (Chapter 1; Fig.1.2). Voshell & al. (2011) and Voshell & Hilu (2014) have shown that the species with the x=6 cytotype (type 1 and 2 florets) are sister to all x=7 species. There are three major clades within the x=7 group, the New World species (type 3 florets), the Arundinacea species (type 4 florets), and the Old World species (type 5 and 6 florets). Voshell & Hilu (2014) proposed that the weight and size of the florets of the New World species was related to their expansion out of the Mediterranean Basin, but empirical studies were not conducted. Diaspore (floret + caryopsis) size is well documented to play a critical role in the ability of a seed to be

dispersed via an animal vector (Cheplick, 1998; Cousens & al. 2008). The diaspores of the x=6 species (*P. canariensis*, *P. brachystachys*, and *P. truncata*) appear to be larger than their x=7 counterparts, but an empirical study is lacking.

The genus possesses species that are both self-compatible and self-incompatible. *Phalaris coerulescens* has been used as a model organism to study the S-Z allele system responsible for self-incompatibility in grasses (Hayman, 1956; Li & al., 1994, Bian & al., 2004). Self-incompatibility has been linked with promoting genetic variation and increases the chances of hybridization occurring (Allen & Hiscock, 2008). Hybridization is an important mechanism for speciation in the grasses. Several species of *Phalaris* may be of allopolyploid origin making ancestral hybridization events a likely factor for speciation in the genus. *Phalaris californica,* the only native New World tetraploid, possesses indels in the maternal *trnT-F* sequence not shared with other members of the genus. This failure could point to an ancestral intergeneric hybridization event (Voshell & al., 2011; Voshell & Hilu, 2014). Assessing the ability of potential diploid progenitors to interbreed is an important step toward assessing whether they gave rise to current tetraploid species. No polyploid populations have been observed in the x=6 species (Anderson, 1961; Baldini, 1995). The x=6 species have appeared as sister to all the x=7 species in the bipaternal ITS tree and sister to the x=7 Old World diploids in the maternal *trnT-F* tree, making its relationship to the other species difficult to assess (Voshell & al., 2011; Voshell & Hilu, 2014). It is unknown whether crosses between the x=6 and x=7 diploids are possible.

The term ‘C value’ describes the amount of DNA in the haploid nuclear genome in an organism before replication (Swift, 1950). Genome size often varies greatly

between closely related organisms and does not appear to relate to the complexity of life forms (Levin, 2002). The term 'C value paradox' was coined by Thomas (1993) for this reason. The nuclear genome size varies greatly within the angiosperms (Bennett & Smith, 1976, 1991). The origin and nature of this variation has garnered attention for many years (Stebbins, 1976; Cavalier-Smith, 1985; Bennett & Smith, 1976, 1991; Bennett, 1987, 1998; Leitch et al., 1998; Leitch & Hanson, 2002; Gurushidze & al., 2012). The differences in genome size are generally attributed to varying amounts of noncoding DNA and each new study about genome composition sheds light on the phenomenon (Schmidt & Heslop-Harrison, 1998; Petrov, 2001; Farahani & al., 2011; Pellicer et al., 2013). Bennett and Leitch (2011) have shown that infrageneric variation in genome size is common in plants and have highlighted its usefulness in assessing phylogenetic relationships. Kadir (1976) assessed the genome size of several *Phalaris* species finding a large degree of variation, but methodology has improved since then warranting another assessment. Aside from potential usefulness in phylogenetic studies (Leitch & Hanson, 2002), C values may also have ecological implications. Lavergne et al. (2010) noted a trend in genome size reduction and phenotypic traits related to invasiveness in *P. arundinacea*.

The goals of this study are to empirically assess the floret weight, length, and width by floret type, cytotype, and species and place the results in a biogeographical and phylogenetic context. C values will be determined and used to compare the genome size within species and among cytotypes to assess their phylogenetic utility. The third goal of the study is to collect data on the self-incompatibility of *Phalaris* species, attempt to generate artificial polyploids, and create hybrids between species. Information about the

breeding system and compatibility of species is important for a comprehensive picture of the evolutionary history of *Phalaris*.

MATERIALS AND METHODS

Plant Material

All plants used in this study were obtained from the USDA seed accessions or field collections by the author. Seeds were germinated in petri dishes and transplanted into pots containing basic soil mix. All plants remained inside a glass greenhouse for the duration of the study. Additional seeds were used for morphological assessments of floret weight, length, and width.

Self-incompatibility

To determine if individuals were self-incompatible, pollination bags were used to prevent cross pollination. Some inflorescences were not bagged and served as controls to assess the fertility rate of open pollination. Each inflorescence was bagged before the spikelets emerged from the sheath. Bags were folded shut at the bottom and stapled to prevent contamination. Plants of the same species were grouped together on the benches so cross-pollination between individuals without bagged inflorescences could occur. Previous greenhouse observations indicated that it was not necessary to manually cross-pollinate the plants for seed setting to occur. Most *Phalaris* species drop seed once the caryopsis develops; therefore, pollination bags were placed on the controls after the anthers withered so the seeds could be collected. All inflorescences were removed from the plants after they dried out and stored at room temperature for later analysis.

Inflorescences were collected from multiple individuals when possible, but multiple individuals were not available in all species leading to unequal sample sizes.

Self-incompatibility was assessed in two ways: 1) by determining if fully developed caryopses present or absent in each bag (Table 4.2) and 2) by counting the ratio of florets containing a caryopsis compared with the total spikelets per inflorescence for a subset of the collected bags (Table 4.3). Only florets containing caryopses that appeared mature and "solid" were considered fertile. Unfertile florets had a reduced, withered appearance and did not contain a mature caryopsis when dissected.

The number of individuals from which inflorescences were collected was uneven due to seed availability and plant deaths and reflects a small sample size. There were six individuals sampled for *P. platensis* and *P. canariensis*, five for *P. brachystachys* and *P. appendiculata*, three for *P. paradoxa*, two for *P. coerulescens*, and only one for *P. truncata* and *P. angusta*.

Artificial polyploids

Attempts to generate artificial polyploids followed McWilliam (1962). Vegetative clones were separated from the parent plant after roots were present and soaked in a 0.25% colchicine solution for 24 hours. Plants were rinsed and allowed to recover in cell packs with moist soil. They were kept in a shaded greenhouse on a mist bench until they established a full root system. Plant material was harvested three months later and flow cytometry was used to check the ploidy. The ploidy level was determined by comparing the results to those of known diploids of the same species (Table 4.1).

Hybridization

Artificial hybridization was attempted following Jenkins and Sethi (1932). All but 1-3 florets were removed from an inflorescence early in the morning before anthesis. The remaining florets were manually emasculated and pollen was applied from other species to receptive stigmas. Florets were bagged to prevent contamination and allowed to mature. After the inflorescences dried out, they were removed and kept at room temperature for three months. Seeds were placed in petri dishes with dry paper towels and kept at 4°C for one week to break dormancy. Initial attempts to germinate *P. californica*, which had been collected during the same season failed. A simple trial to see if a week in the 4°C refrigerator was sufficient to break dormancy was successful. The same procedure was used on all species in the study and resulted in germination. After the refrigeration period, water was added to the petri dishes and the seeds were germinated at room temperature. Seedlings were transplanted into moist soil in standard 6-cell packs after the first leaf appeared. A list of plant material generated in the study can be found in Table 4.4. The success of hybridization was checked by isolating DNA from leaf material following Voshell et al. (2011) and sequencing the ITS region to look for double peaks indicative of hybridization. Sequences were paired and aligned with data from the parent species in Quickalign v1.6.0 (Müller, 2004) and manually inspected for double peaks (Fig. 4.6).

Flow cytometry

Tillers were harvested the day before the flow cytometry procedure and kept in water overnight. The fleshy portion toward the base of the tiller was chopped with a

razor blade in chopping buffer (sodium citrate, MOPS, $MgCl_2$, Triton X-100) in a glass petri dish over ice. The chopped material was filtered through a 250 µm and 63 µm mesh screen and 500 µL of the flow through was saved and kept on ice. 0.25 mL of Ribonuclease A solution was added to 0.5 mL of the flow through of chopped leaf material two hours before the procedure and 0.125 ml of propidium iodide solution was added one hour before running the samples through the flow cytometer at the Virginia-Maryland Regional College of Veterinary Medicine Flow Cytometry Core Facility. Samples were measured with a BD FACSCallibur flow cytometer and the data were analyzed with FlowJo (Treestar, Inc). *Zea mays* was used as a standard because its relative genome size is similar to *Phalaris* and its genome has been sequenced providing a known number of base pairs to calibrate the results. C values were determined by comparing the highest peak on the graph with that of the standard.

Statistical Analysis

Statistical analyses were conducted in JMP Pro 10 (SAS Institute Inc. 2010) to test for normal distribution, compare means, and examine variance to determine whether differences in the C value and floret morphology data were significant. Oneway ANOVA was used to compare means and the Tukey-Kramer HSD was used to find significantly different pairs within each data set. Variance was assessed by examining the standard deviations. A P-value of <0.05 was considered significant.

RESULTS

Floret weight, length, and width by floret type and cytotype

The mean weight of 20 seeds randomly selected from each accession are presented in Figs. 4.1, 4.2a, and Appendix C. Floret type 1 weighed the most while floret type 3 weighed the least. Florets of *P. californica*, the only New World tetraploid, appeared larger than the other species so the analysis was conducted with and without the species. Type 3 florets remained the lightest group in both cases. A oneway ANOVA revealed significant difference ($P<0.0001$) between the mean weights of the florets by type (Fig. 4.2a). A pairwise comparison using the Tukey-Kramer HSD found the weight of type 1 florets to be different ($P<0.0001$) from types 2-6. Significant differences in weight were also found for floret types 2 and 3 ($P=0.0030$); types 2 and 4 ($P=0.0328$).

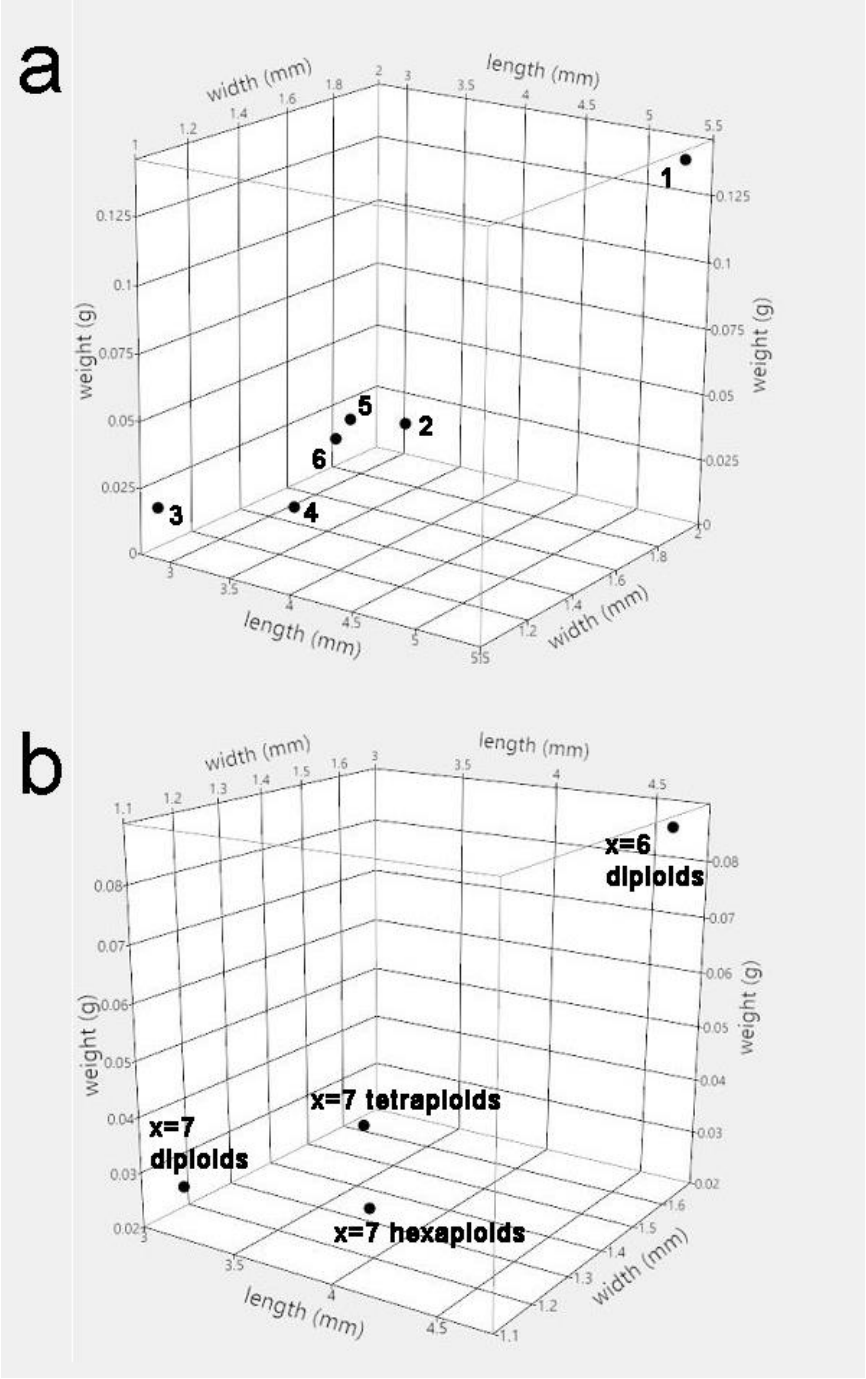
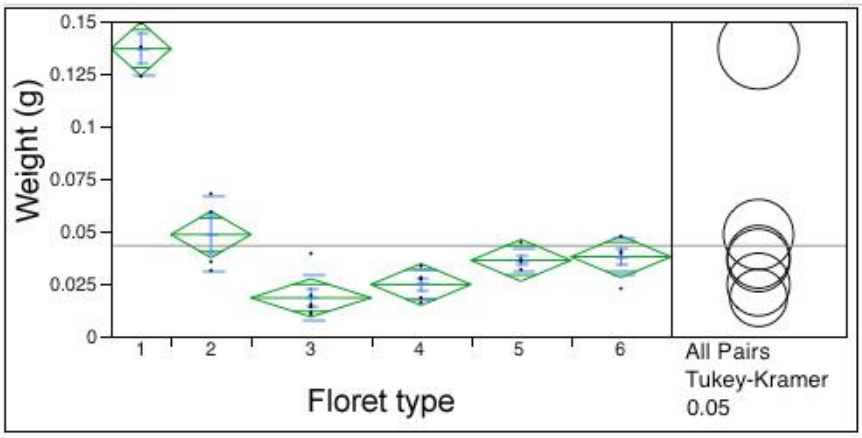


Fig 4.1. Summary of floret weight, length and width by floret type and cytotype. a) Average weight (g), length (mm), and width (mm) for floret types 1-6. b) Average weight (g), length (mm), and width (mm) for the four cytotypes (x=6 diploids, x=7 diploids, x=7 tetraploids, and x=7 hexaploids).

The weights of all species found exclusively in the Old World (mean 0.054g) were analyzed against those found in the New World (0.019g). The Old World florets display much greater variance with a standard deviation of 0.039 in contrast with 0.01 for New World species. The difference in the weight of each group was significant (P=0.0146). *Phalaris californica* was excluded from the analysis to determine if its large floret size would effect the results. The mean weight of the New World florets dropped to 0.017g with a standard deviation of 0.007. The weight of the two groups remained different (P=0.0134). A oneway ANOVA of the floret weights by cytotype (x=6 diploid, x=7 diploid, x=7 tetraploid, and x=7 hexaploid; Fig. 4.1b, Appendix C) found differences between groups (P=0.0006). The mean weight of 20 seeds was greatest in the x=6 diploid group, and lightest in the x=7 diploid group (Figs. 4.1, 4.2b).

The mean weights by species are reported in Appendix C. *Phalaris canariensis* and *P. brachystachys* (type 1 and 2 florets) were the heaviest species and *P. angusta* and *P. platensis* (type 3 florets) were the lightest.

a) Oneway analysis of floret weight by floret type



b) Oneway analysis of floret weight by cytotype

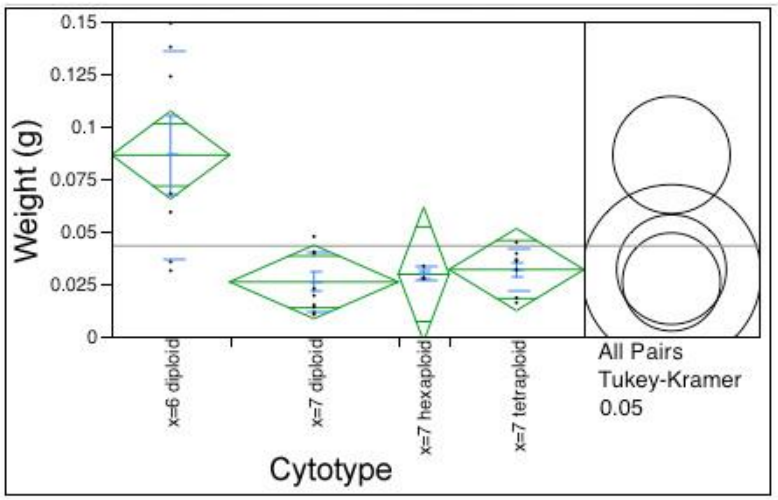
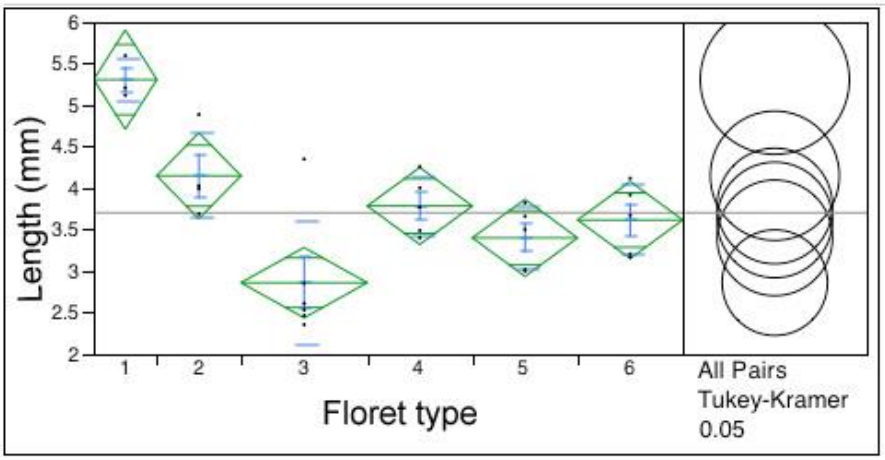


Fig. 4.2. Summary of floret weight statistics. Oneway ANOVA and Tukey-Kramer HSD analyses of floret weight by (a) floret type and (b) cytotype.

The mean floret lengths are presented in Figs. 4.1, 4.3a, and Appendix C. Type 1 florets were the longest and type 3 florets were the shortest. A oneway ANOVA revealed significant difference between the length of the florets by type ($P<0.0001$). Pairwise comparisons using the Tukey-Kramer HSD found the length of type 1 florets to be different from types 3-6. Differences in length were also found between floret types 2 and 3 ($P=0.0071$). Analysis of the length by cytotype found a significant difference between the groups ($P=0.0002$), but x=6 diploids were significantly longer than the x=7 tetraploids, but not hexaploids (Figs. 4.1, 4.3b). Mean floret lengths by species are reported in Appendix C. The trend in floret length is comparable to that of floret weight with x=6 *P. canariensis* and *P. brachystachys* having the longest florets while New World *P. angusta* and *P. plantensis* had the shortest.

a) Oneway analysis of floret length by floret type



b) Oneway analysis of floret length by cytotype

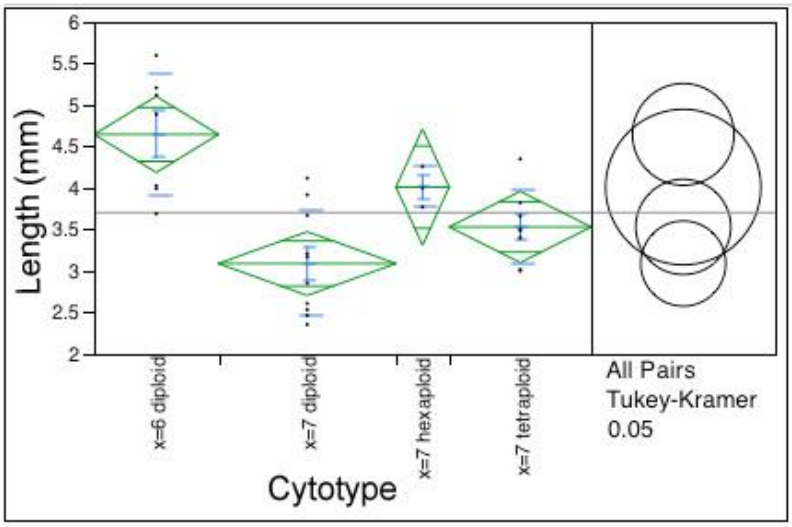
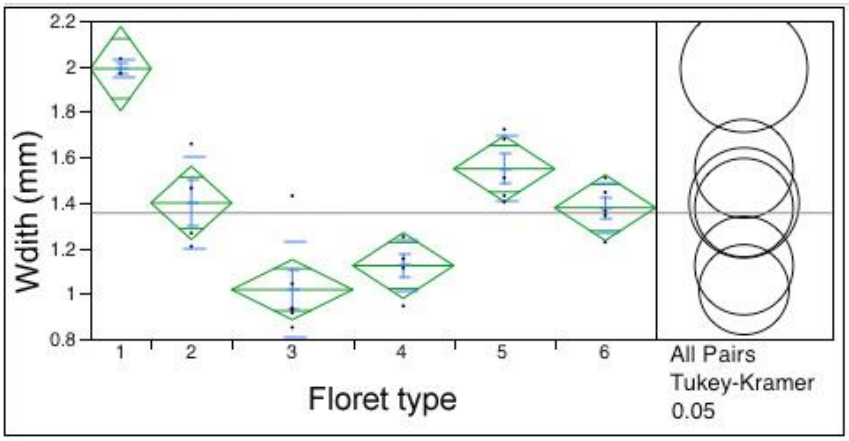


Fig. 4.3. Summary of floret length statistics. Oneway ANOVA and Tukey-Kramer HSD analyses of floret length by (a) floret type and (b) cytotype.

Analyses of floret width by floret type are presented in Figs. 4.1, 4.4a and Appendix C. Type 1 florets were the widest while type 3 florets were the narrowest. A oneway ANOVA revealed significant difference between the width of the florets by type ($P<0.0001$). Pairwise comparisons using the Tukey-Kramer HSD found the width of type 1 florets to be different from types 2-6. Differences in width were also found for floret types 5 and 3-4, types 2 and 3, and types 6 and 3. The width was compared by cytotype with a oneway ANOVA and significant difference was found between the groups ($P=0.0075$; Fig. 4.3b). The only pair displaying a significant difference in width was the x=6 diploid and x=7 diploid cytotype (Figs. 4.1, 4.4b). The averages widths for each species are reported in Appendix C. The width data differed from weight and length in that *P. canariensis* and *P. minor* were the widest while *P. angusta* and *P. arundinacea* were the narrowest.

a) Oneway analysis of floret width by floret type



b) Oneway analysis of floret width by cytotype

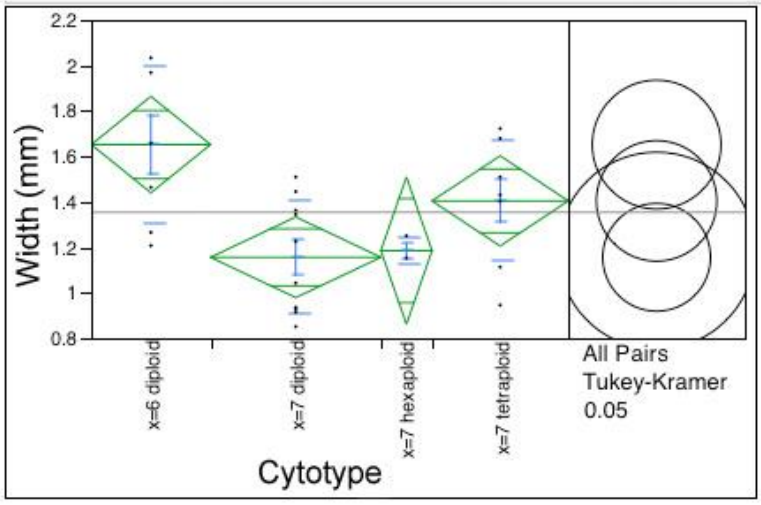


Fig. 4.4. Summary of floret width statistics. Oneway ANOVA and Tukey-Kramer HSD analyses of floret width by (a) floret type and (b) cytotype.

Flow cytometry

Genome size data obtained with flow cytometry is presented in Table 4.1. *Zea mays*, which has a known 2C genome size of 5.45 pg (Bennett & Smith, 1991) was used to calibrate the C values. Statistical analyses were conducted on both the total DNA content (2C) and the amount of DNA per set of chromosomes (1C) values which were obtained by plotting the speed of travel for the nuclei through the flow cytometer. All species used in the comparison were measured at the same time to eliminate technical errors from day to day calibration. The means for the total DNA content were 4.01 pg in the x=6 diploid group, 2.37 pg for the x=7 diploids, 8.39 pg for the x=7 hexaploid, and 7.07 pg for the x=7 tetraploids. The oneway ANOVA showed significant difference between the mean C values for the groups ($P<0.0001$). Fig. 4.5a displays the mean 2C values by cytotype and Fig. 4.5b displays them by species. The x=6 group displayed the greatest variance with a standard deviation of 1.24, followed by 1.13 in the x=7 tetraploids, and 0.79 in the x=7 diploids. The Tukey-Kramer HSD was used to compare individual groups and showed significance for the following pairs: x=7 hexaploid/x=7 diploid ($P<0.0001$), x=7 tetraploid/x=7 diploid ($P<0.0001$), x=7 hexaploid/x=6 diploid ($P=0.0030$), x=7 tetraploid/x=6 diploid ($P<0.0001$), x=6 diploid/x=7 diploid ($P=0.0117$). The x=7 hexaploid and x=7 tetraploid groups were not significantly different.

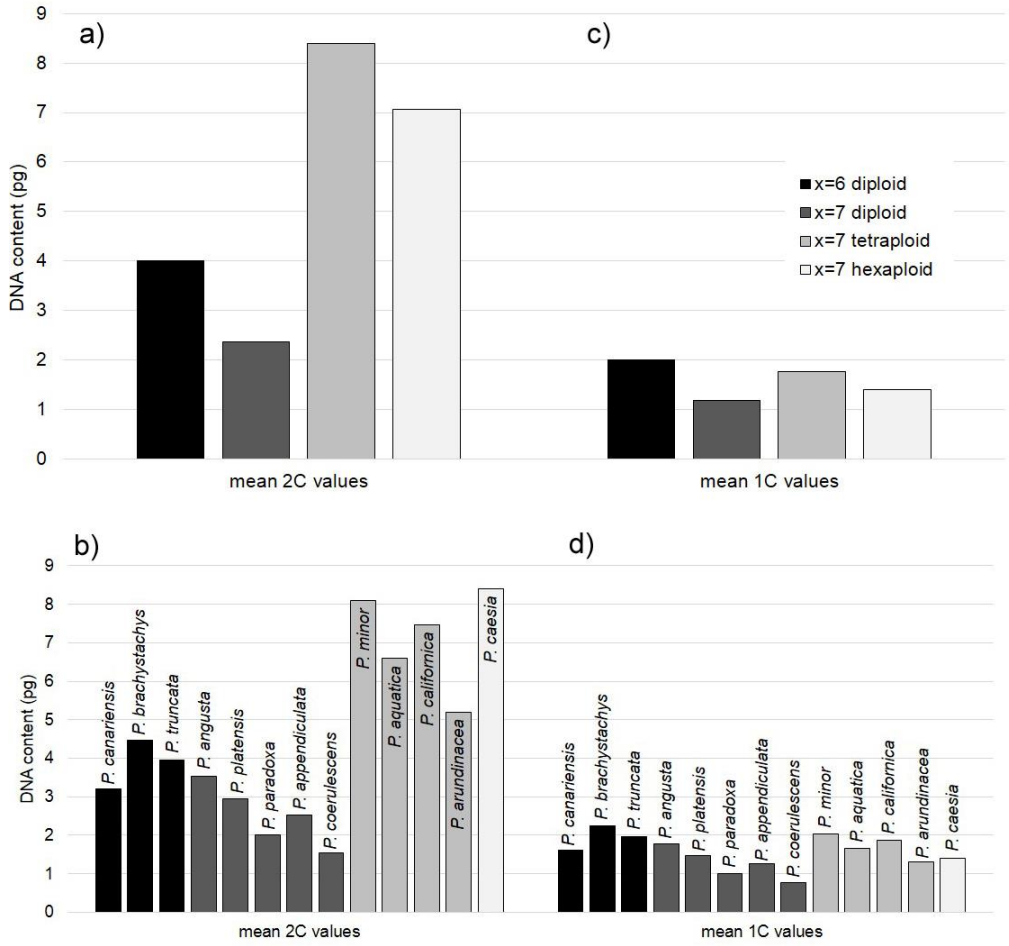


Fig. 4.5. Mean C values by cytotype and species for *Phalaris*. a) 2C values by cytotype b) 2C values by species c) 1C values by cytotype d) 1C values by species.

Table 4.1. 2C values calculated from flow cytometry.

Species	accession	2C value	cytotype
Phalaris angusta	310292-A	3.36	x=7 diploid
Phalaris angusta	210292-B	3.69	x=7 diploid
Phalaris appendiculata	331404-A	2.52	x=7 diploid
Phalaris aquatica	284200-A	7.13	x=7 tetraploid
Phalaris aquatica	575	6.04	x=7 tetraploid
Phalaris arundinacea	DP1	5.20	x=7 tetraploid
Phalaris caesia	284179	8.39	x=7 hexaploid
Phalaris brachystachys	239820-A	4.11	x=6 diploid
Phalaris brachystachys	202678-A	3.61	x=6 diploid
Phalaris brachystachys	380967-1	5.03	x=6 diploid
Phalaris brachystachys	380967-2	5.12	x=6 diploid
Phalaris californica	CAL1	7.05	x=7 tetraploid
Phalaris californica	CAL3	7.89	x=7 tetraploid
Phalaris canariensis	163357-A	1.68	x=6 diploid
Phalaris canariensis	251274-A	4.70	x=6 diploid
Phalaris coerulescens	239340-A	1.34	x=7 diploid
Phalaris coerulescens	318840-2	1.68	x=7 diploid
Phalaris coerulescens	236530-1	1.59	x=7 diploid
Phalaris minor	208404-A	7.72	x=7 tetraploid
Phalaris minor	239845-A	8.47	x=7 tetraploid
Phalaris paradoxa	SP165-3	1.68	x=7 diploid

Phalaris paradoxa	239845-A	2.35	x=7 diploid
Phalaris paradoxa	239845-2	2.01	x=7 diploid
Phalaris platensis	239339-A	3.19	x=7 diploid
Phalaris platensis	281598-1	2.68	x=7 diploid
Phalaris truncata	240196-6	5.03	x=6 diploid
Phalaris truncata	535561-A	2.85	x=6 diploid

The means for the 1C values (total DNA content divided by number of chromosome sets) were 2.01 pg in the x=6 diploid group, 1.19 pg for the x=7 diploids, 1.40 pg for the x=7 hexaploid, and 1.77 pg for the x=7 tetraploids. Fig. 4.5c presents the 1C values by cytotype and Fig. 4.5d presents them by species. The x=6 group displayed the greatest variance with a standard deviation of 0.620, followed by 0.395 in the x=7 diploids, and 0.282 in the x=7 tetraploids. The oneway ANOVA showed significant difference among groups (P=0.0049). When analyzing the 1C values, the Tukey-Kramer HSD only found a significant difference between the x=6 diploid/x=7 diploid group (P=0.0037).

Self-incompatibility

Phalaris canariensis, P. paradoxa, and *P. coerulescens* did not yield viable seed material when bagged. In the case of *P. angusta, P. appendiculata, P. brachystachys,* and *P. platensis,* less than half the inflorescences yielded viable seed material when bagged (Table 4.2). Exact counts of the number of viable caryopses vs. sterile florets

were conducted for a subset of the inflorescences and can be found in Table 4.3 where it is presented as the percent of viable caryopses out of the total number of spikelets.

Table 4.2. Percent of bagged and unbagged (control) *Phalaris* inflorescences and the production of caryopses. Production of caryopses in bagged specimens indicates the plant was able to fertilize itself. A lack of caryopses in a bagged specimen indicates self-incompatibility.

Species	Bags collected	Percent with caryopses (bagged)	(control)
P. angusta	12	57	80
P. appendiculata	21	43	100
P. brachystachys	16	29	100
P. californica	5	0	100
P. canariensis	9	0	100
P. paradoxa	5	0	100
P. platensis	96	17	100
P. truncata	9	0	0

Table 4.3. Percent of spikelets producing caryopses for bagged and unbagged inflorescences in *Phalaris*.

Species	Percent spikelets/caryopses (bagged)	(control)
P. angusta	41, 85	0, 62, 68, 82, 83
P. appendiculata	0	98
P. brachystachys	0, 0, 0.4, 43, 56	60, 71, 75, 80, 85
P. californica	0, 0	16, 54, 57
P. canariensis	0, 0, 5.2, 19	70
P. paradoxa	0	9.3, 95
P. platensis	0, 0, 0	62, 67, 85
P. truncata	0, 0, 0, 0	0, 0, 0, 0, 0.7

Artificial polyploids

Flow cytometry data indicated that the five plants which survived exposure to the colchicine treatment remained diploids and did not experience genome doubling. The two accessions of *P. coerulescens* treated with colchicine yielded C values of 1.59 pg and 1.34 pg which are comparable to the diploid accessions with C values of 1.69 pg and 1.34 pg of DNA. The C value for the treated accession of *P. truncata* was 3.52 pg which falls between the C values of the diploids (2.85 pg and 5.03 pg). The treated accession of *P. paradoxa* had a genome size of 1.68 pg which was identical to the diploid accession. *Phalaris appendiculata* had a C value lower than the diploids (1.59 pg vs 2.52 pg), but the plant material was in poor condition and may have caused the anomalous result.

Phalaris hybrids

Table 4.4 shows all the seed material which was generated during the hybridization experiment and the rate of germination success. DNA was isolated from the ITS region was sequenced for each potential hybrid. Comparison of the DNA sequences to the parental species revealed that the species were not hybrids. Fig. 4.6 shows the alignment of the potential hybrids with the parental species. With the exception of *P. canariensis* X *P. brachystachys*, there are numerous mutations between the two parental species. If hybrids had been generated, double peaks would be visible and ambiguity codes would be present in the alignment. All sequences match the maternal source as expected.

Table 4.4. Attempted crosses to generate Phalaris hybrids and success rate of germination. A "0" indicates that no seeds germinated.

Maternal species x paternal species	seeds germinated	seeds produced
P. paradoxa 253579(5) x *P. coerulescens* 236530(2)	2	2
P. canariensis 163357(6) x *P. appendiculata* 331404(5)	3	3
P. canariensis 163357(3) x *P. truncata* 535562	2	3
P. canariensis 251274(5) x *P. truncata* 535562	2	3
P. paradoxa 253579(4) x *P. brachystachys* 202678(5)	1	3
P. canariensis 251274(4) x *P. appendiculata* 331404(2)	2	2
P. brachystachys 202678(1) x *P. canariensis* 163357(2)	2	3
P. canariensis 163357(7) x *P. truncata* 535562	0	2
P. canariensis 163357(4) x *P. appendiculata* 331404(2)	2	2
P. brachystachys 202678(4) x *P. appendiculata* 331404(2)	1	1
P. brachystachys 202678(3) x *P. paradoxa* SP165(6)	1	1
P. canariensis 251274(5) x *P. coerulescens* 239340(3)	0	3
P. brachystachys 202678(6) x *P. coerulescens* 236530(3)	0	3
P. brachystachys 202678(6) x *P. coerulescens* 239340(3)	0	3
P. brachystachys 239820(5) x *P. coerulescens* 236530(7)	2	2
P. coerulescens 236530(2) x *P. brachystachys* 202678(1)	0	3

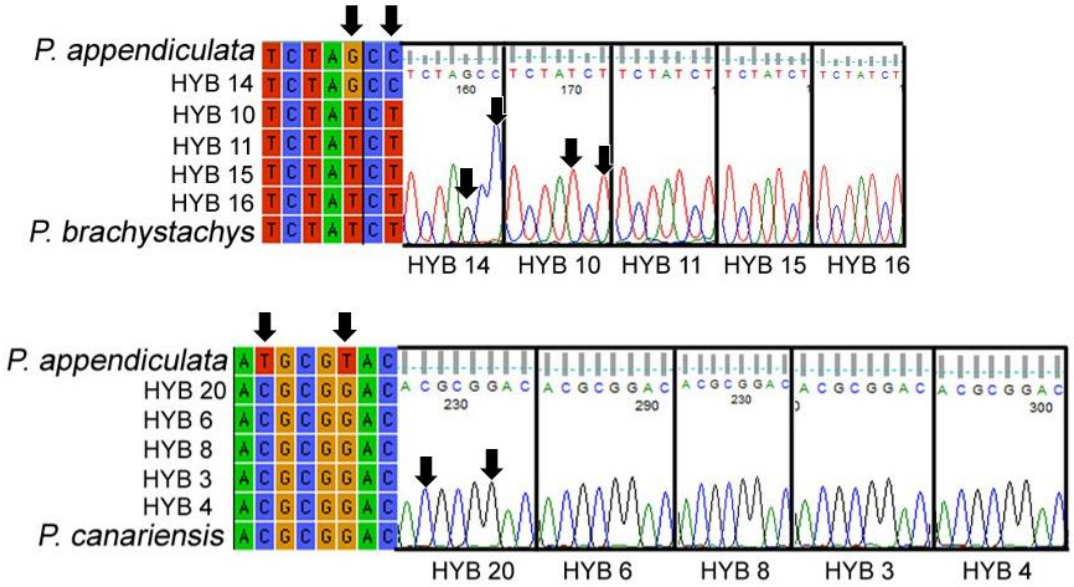


Fig. 4.6. DNA alignment of bi-paternally inherited ITS region of potential hybrids with parent species. Arrows point to mutation sites in the alignment and the corresponding peaks in the chromatogram.

DISCUSSION

Floret weights and biogeography

The data from the floret morphology assessment clearly shows that the type 1 floret of the x=6 species is significantly larger than type 3 and 4 florets of the x=7 diploids and Arundinacea species in both their dimensions and weight. Voshell & Hilu (2014) proposed that the structure of the type 3 and 4 florets played a role in their dispersal throughout the New World from their point of origin in the Mediterranean Basin. Floret size is known to play an important role in the dispersal of grasses (Harper & al. 1970). The significantly smaller size of the florets in the New World species (types 3 and 4) further supports the hypothesis that floret structure and weight is related to

expansion. Lighter weight florets have been shown to be better suited to animal transport (Romermann & al. 2005; Tackenberg & al. 2005; Cheplick, 2008; Cousens & al., 2008). Mean floret weight, length and width has been mapped on a phylogenetic summary tree (Fig. 4.7).

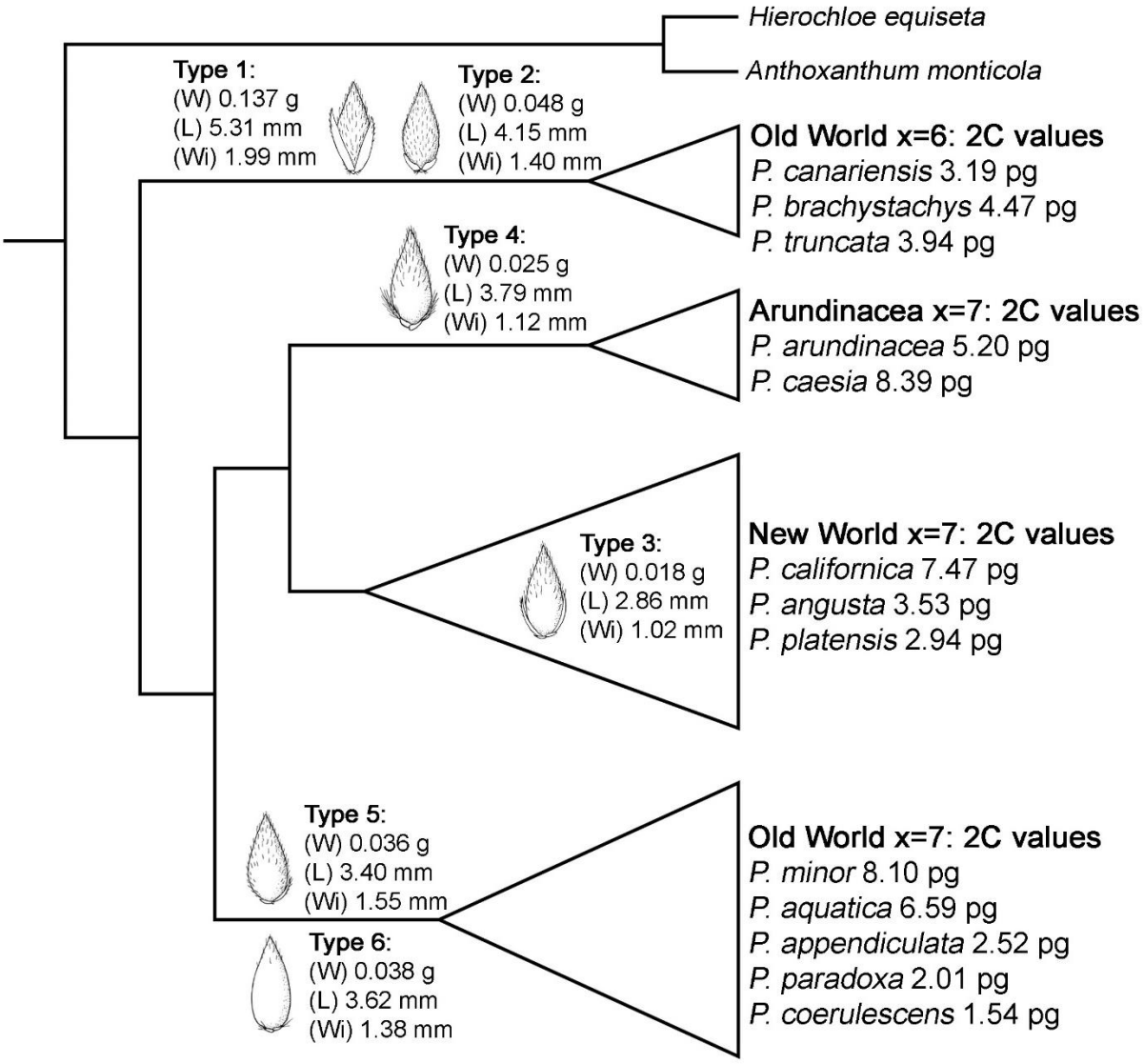


Fig. 4.7. Floret type, weight, length, width, and average genome sizes mapped on a summary tree based on combined ITS and *trnT-F* data (Chapter 3).

The x=6 species are able to survive in the New World when introduced and are even commonly cultivated as bird seed (Anderson, 1961; Baldini, 1995). This indicates that their natural inability to expand beyond the Mediterranean Basin is likely related to dispersal success rather than an environmental restriction.

The floret weight, length and width of hexaploid *P. caesia* was greater than tetraploid *P. arundinacea*, but the difference was not significant. It is intriguing that the diploid x=6 species display significantly larger dimensions and weight than the x=7 diploids or polyploids since polyploid plants are typically larger than diploid progenitors or relatives (Kondorosi & al., 2000).

A C value paradox in the x=6 cytotype

The C values obtained through flow cytometry (Table 4.1) were lower than those published by Kadir (1976; Table 4.1), but this is likely the result of different techniques being used. A fascinating pattern emerges when the genome sizes are compared across cytotypes (Fig. 4.7). The x=6 diploids have a larger total genome than the x=7 diploids, a comparison that remains the same after calculating the 1C scores (Fig. 4.5a,c). The 2C values were higher in the polyploid species before being adjusted to account for the increased number of chromosomes. The result that stands out the most is the size of the haploid x=6 genome when compared to the other groups before and after being adjusted for ploidy level (Fig. 4.5a, c). The x=6 species have one less chromosome, yet their genome is larger than that of the x=7 diploids (P=0.0037). Furthermore, the genome size

is not significantly different than the x=7 polyploid species for the 1C value. These findings suggest that the x=6 vs x=7 cytotype is not the result of a chromosome loss or simple rearrangement. Since the divergence of the x=6 and x=7 species, either the x=7 diploids have experienced genome downsizing or the x=6 diploids and x=7 polyploids have gained a significant amount of noncoding DNA. The gain or loss of noncoding DNA has been suggested as the cause of C value differences (Schmidt & Heslop-Harrison, 1998). The smaller 1C value obtained for the hexaploid is not surprising since genome downsizing has been reported for higher ploidy levels (Leitch & Bennett, 2004). All species were run through the flow cytometer during the same run, ruling out calibration errors between days or sessions. Metaphase chromosome counts did not reveal aneuploids or polyploids; all x=6 species were true diploids.

The same trends observed in *Phalaris* are also found when comparing basic chromosome numbers and genome sizes for other members of the Poaceae such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), and corn (*Zea mays*). Hilu (2004) noted a reductionary trend in basic chromosome number from x=12 in rice, to x=7 in wheat, and x=5 in corn. A 1C value of 0.5 pg has been reported for rice (Bennett & Smith, 1991), 5.76 pg for wheat (Bennett & Smith, 1976), and 2.73 pg for corn (Bennett & Smith, 1976). It is interesting to see the same trend of increasing genome size correlated with decreasing chromosome number at both the family and genus level.

Intraspecific variation in the x=6 cytotype

Another unusual result for the x=6 species was the variance in C values within the same species. The standard deviation of the x=6 species were by far the highest in the

data set. Intraspecific C value variation has been reported in prior studies, but has neither been confirmed as a true phenomenon nor disproven to be a technical error. Several studies have presented results supporting significant intraspecific variation of C values (Bennett & Thomas, 1991; Reeves & al., 1998; Hall & al., 2000; Moscone & al., 2003), while others found no evidence of the phenomenon being anything other than the result of methodology issues (Miksche, 1971; Dhir and Miksche, 1974; Greilhuber, 1998, 2005). In the absence of conclusive information, a conservative approach would view this intraspecific variation as the limit of methodological accuracy; however, if C value variation within a species is a true phenomenon it would be an important factor influencing the usefulness of this parameter in phylogenetic and evolutionary studies. Variation among species would be useful for population level studies to examine phylogenetic relationships, but it could warrant caution for broader studies. Greater sample sizes may be needed to examine the true diversity of C values in a species before comparing the variation between genera. Leitch and Bennett (2004) revealed that genome downsizing commonly occurs after polyploidization in angiosperms. Future studies examining the causes for genome size changes in the absence of polyploidization could reveal important information about the evolution of genome sizes and conditions which invoke changes. Murray (2003) suggests that intraspecific C value variation may be a useful indicator of taxonomic heterogeneity. Voshell & al. (2011) and Voshell & Hilu (2014) found little to no intraspecific variation in the DNA sequences of the three x=6 species making their C value variation even more intriguing.

Breeding systems

Self-incompatibility promotes genetic variation witin a species and is often linked with interspecific hybridization (Allen & Hiscock, 2008). *Phalaris coerulescens* was found to be self-incompatible, this is congruent with the findings of Hayman (1956), Li & al. (1994), and Bian & al. (2004) who used the plant to examine the S-Z allele system responsible for self-incompatibility in grasses. *Phalaris paradoxa* was also found to be self-incompatible which would increase the likelihood of outcrossing for these species. *Phalaris paradoxa*, *P. appendiculata*, and *P. coerulescens* are the likely diploid progenitors of tetraploids *P. minor*, *P. aquatica*, and *P. maderensis* based on their position in the phylogeny (Voshell & al., 2011; Chapter 1). The self-incompatibility of these Old World diploids may partially explain why polyploidy played a greater role in speciation in the Old World than the New World. New World *P. angusta* has a high rate of self-fertilization compared to the other *Phalaris* species (57%, Table 4.2), yet closely related *P. platensis* had a low rate (17%, Table 4.2). Baldini (1995) reported instances of hybridization among the New World species suggesting that they are closely related. The close relationships and lack of self-incompatibility in New World species may explain why speciation through diploidy has remained prominent. Among the x=6 species, only 29% of *P. brachystachys* inflorescences produced seeds when bagged and *P. canariensis* only had trace amount of seeds. The x=6 species appear to be mostly self-incompatible, but capable of some self-fertilization. *Phalaris truncata* appeared to truly be self-incompatible. The inflorescences all bloomed at different times so neither the bagged or control plants set seed. The lack of polyploidization in the x=6 species remains

intriguing given that they are mostly self-incompatible and one would expect to see the same trend of polyploid speciation found in the Old World x=7 species.

Artificial hybrids

Comparison of the ITS region of the potential hybrids to the parent species revealed that the hybridization trials were not successful. Many of the florets from the trial did not yield viable seeds and therefore never germinated. The florets which did produce viable seeds were pollinated by the same species. Several species were shown to be highly to partially self-incompatible (*P. canariensis, P. brachystachys, P. truncata, P. coerulescens, P. paradoxa*; Tables 4.2-4.3) indicating that it is possible that pollen from the same species, but different individuals loose in the air contaminated the experiment.

This study offers new insights and empirical data supporting several trends seen in the variable features of *Phalaris*. The significantly smaller size and dimensions of the florets of the New World and Arundinacea species likely gave them an advantage for dispersal outside the Mediterranean Basin. The polyploids in the genus are significantly smaller than the diploid x=6 species in terms of floret size and weight. The *Phalaris* genus has its own C value paradox with the species possessing fewer chromosomes possessing a larger genome size. This finding indicates a complex history of chromosome rearrangement rather than a simple event such as a loss or merger between two chromosomes. The breeding system of *Phalaris* varies with some species being self-incompatible and others capable of self-fertilization.

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Appendix C. Summary of floret morphology data. Means, standard deviation, and standard error are presented for floret weight, length and width by floret type, cytotype and species.

Floret Weight (g) – 20 florets

Category	mean	standard deviation	standard error
Type 1 florets	0.137	0.013	0.006
Type 2 florets	0.048	0.018	0.005
Type 3 florets	0.018	0.011	0.004
Type 4 florets	0.025	0.007	0.005
Type 5 florets	0.036	0.005	0.005
Type 6 florets	0.038	0.009	0.005
X=6 diploids	0.086	0.049	0.010
X=7 diploids	0.026	0.014	0.008
X=7 tetraploids	0.032	0.010	0.009
X=7 hexaploids	0.030	0.003	0.015
P. angusta	0.012	0.002	0.004
P. aquatica	0.034	0.002	0.005
P. arundinacea	0.017	0.002	0.005
P. brachystachys	0.064	0.006	0.005

P. caesia	0.030	0.003	0.004
P. californica	0.040	--	0.007
P. canariensis	0.137	0.013	0.004
P. coerulescens	0.042	0.005	0.004
P. minor	0.038	0.007	0.004
P. paradoxa	0.031	0.012	0.005
P. platensis	0.017	0.004	0.005
P. truncata	0.033	0.003	0.005

Floret Length (mm)

Category	mean	standard deviation	standard error
Type 1 florets	5.31	0.254	0.289
Type 2 florets	4.15	0.514	0.250
Type 3 florets	2.86	0.748	0.204
Type 4 florets	3.79	0.356	0.224
Type 5 florets	3.40	0.375	0.224
Type 6 florets	3.62	0.422	0.224
X=6 diploids	4.65	0.733	0.222
X=7 diploids	3.09	0.635	0.186
X=7 tetraploids	3.53	0.437	0.208
X=7 hexaploids	4.01	0.244	0.340
P. angusta	2.45	0.090	0.146
P. aquatica	3.74	0.114	0.179
P. arundinacea	3.45	0.062	0.179

P. brachystachys	4.44	0.631	0.179
P. caesia	4.01	0.244	0.146
P. californica	4.35	--	0.254
P. canariensis	5.31	0.254	0.146
P. coerulescens	3.90	0.225	0.146
P. minor	3.18	0.284	0.146
P. paradoxa	3.19	0.026	0.179
P. platensis	2.73	0.173	0.179
P. truncata	3.86	0.238	0.179

Floret Width (mm)

Category	mean	standard deviation	standard error
Type 1 florets	1.99	0.038	0.090
Type 2 florets	1.40	0.205	0.078
Type 3 florets	1.02	0.211	0.064
Type 4 florets	1.12	0.111	0.070
Type 5 florets	1.55	0.145	0.070
Type 6 florets	1.38	0.107	0.070
X=6 diploids	1.65	0.348	0.103
X=7 diploids	1.16	0.249	0.086
X=7 tetraploids	1.40	0.263	0.096
X=7 hexaploids	1.19	0.056	0.157
P. angusta	0.90	0.044	0.051
P. aquatica	1.42	0.019	0.063

P. arundinacea	1.03	0.119	0.063
P. brachystachys	1.56	0.138	0.063
P. caesia	1.19	0.056	0.051
P. californica	1.43	--	0.089
P. canariensis	1.99	0.038	0.051
P. coerulescens	1.38	0.054	0.051
P. minor	1.64	0.113	0.051
P. paradoxa	1.37	0.200	0.063
P. platensis	0.99	0.082	0.063
P. truncata	1.24	0.041	0.063

Chapter 5

Canary grasses (Phalaris, Poaceae): cytology, genome size and origins of aneuploidy

Stephanie Voshell, Khidir Hilu

ABSTRACT

The canary grass genus is ideal for a case study examining chromosome evolution. The phylogenetic relationship of species and biogeographical history of the genus have been thoroughly examined using molecular techniques. The genus contains four distinct cytotypes with biogeographic affinities (x=6 diploids, x=7 diploids, x=7 tetraploids, and x=7 hexaploids). Species are thought to have arisen both through allo- and autopolyploidy and the New World species have experienced a surprising degree of speciation success while remaining diploids. Representatives from each of the major groups were examined by staining with acetocarmine and DAPI to assess chromosome morphology and the presence of NORs (nucleolar organizer regions). NORs have been shown to be a useful sites to assess cytogenetic relationships in other members of the Aveneae tribe, but have not been examined in *Phalaris*. Secondary constriction sites indicative of NORs were not found in the New World species or *P. arundinacea*. The x=6 cytotype contained at least one visible NOR, as did the Old World x=7 species. Data from C values and chromosome morphology indicate that the x=6 cytotype was not the result of a simple rearrangement or loss of chromosome. Phylogenetic relationships presented in Voshell & al. (2011) and Voshell & Hilu (2014) were supported by comparison of chromosome morphologies.

INTRODUCTION

The canary grass genus (*Phalaris*, Poaceae) is relatively small with 20 species, yet it possess great variability in many features including cytotype, genome size, morphology, distribution, habit, and invasiveness, rendering the genus as an excellent group for evolutionary studies. Traditionally, *Phalaris* has been placed in the Aveneae tribe (Clayton & Renvoize, 1986). Molecular phylogenetics studies have revealed that the Aveneae and Poeae tribes form a complex rather than being two discreet clades, but *Phalaris* was recently reconstructed in a lineage of mostly Aveneae species (Döring & al., 2007; Quintanar & al., 2007, Schneider & al., 2009). The majority of *Phalaris* species possess a basic chromosome number of x=7, a common cytotype in the Aveneae tribe. Phylogenetic reconstruction of the genus placed the three species with a basic chromosome number of x=6 (*P. canariensis*, *P. brachystachys*, and *P. truncata*) in a single lineage as sister to all other members of the genus (Voshell & al., 2011/Chapter 1, Fig. 1.3; Voshell & Hilu, 2014/Chapter 2, Fig. 2.2.). Within the Aveneae tribe, the x=6 cytotype is confined to *Phalaris*, although other genera possess basic chromosome numbers of x=2, 4, 5, 8, and 9 (Hilu, 2004). The early split between the x=6 and x=7 species in *Phalaris* suggests an aneuoploidy event was part of the early evolutionary history of the genus (Voshell & al., 2011; Chapter 1, Fig. 1.3). A molecular dating study of the genus indicated that the divergence between the two cytotypes likely occurred ~17.1 MYA (Voshell & Hilu, 2014; Chapter 2, Fig. 2.2).

The genus possesses four major clades which have been reconstructed with strong to maximum support (Voshell & al., 2011; Voshell & Hilu, 2014; Chapter 3, Figs. 3.2-

3.3). Species with the x=6 cytotype form one clade, while species with the x=7 cytotype are divided into three clades: the Old World species, the New World species, and the Arundinacea species. Members of each clade share unique floret types (Voshell & al., 2011; Chapter 1, Figs. 1.3-1.5), possess strong biogeographical affinities (Voshell & Hilu, 2014; Chapter 2, Fig. 2.2), and display differential genome sizes compared with other clades (Chapter 4, Fig. 4.4, Table 4.1). A study formally analyzing karyotypes from each clade and placing the results in context with current phylogenetic knowledge of the genus is lacking.

While the early appearance of aneuploidy in the genus is fascinating from an evolutionary standpoint, it appears to have impacted the success of the x=6 species. *Phalaris canariensis, P. brachystachys, and P. truncata* have remained isolated to the Mediterranean Basin and not experienced the amount of speciation seen in the x=7 cytotype. The x=6 species have also remained diploids, unlike their x=7 relatives which have tetraploid and hexaploid cytotypes (Parthasarathy, 1938; Ambastha, 1956; Anderson, 1961; McWilliam & Neal-Smith, 1962; Stebbins, 1971; Kadir, 1976; Hunziker & Stebbins, 1987; Baldini, 1993, 1995). It has been suggested that polyploidy is responsible for much of the diversification in the grass family (Levy & Feldman, 2002). Hilu (2006) revealed that an increase in the number of species per genus among the grasses is correlated with the presence of polyploid and perennial habit. The majority of species in Poaceae are of polyploid origin (~80%) making it a more common feature than diploidy (Stebbins, 1985; Hunziker & Stebbins, 1987; Levy & Feldman, 2002; Hilu, 2006). The effects of polyploidy extend past mere "success" in some cases; *P. arundinacea* is a highly invasive pest in North America and polyploidy and genome size

have been cited as contributing factors for this species as well as other invasive species (Stebbins, 1985; Lavergne &Molofsky, 2004, 2007; Lavergne & al., 2010; Jakubowski & al., 2013).

Cytological investigations in Phalaris have been sporadic over the years, often focusing on a single species or pair of related species and usually aimed at obtaining counts with nondifferential staining techniques rather than making comparisons in an evolutionary context. Jenkin & Sethi (1932) carried out cytological studies of artificial crosses they generated between *P. arundinacea* and *P. aquatica*. Parthasarathy (1938) conducted a descriptive study of a few members of *Phalaris* and related genera leading to broad conclusions without a phylogenetic context. Ambastha (1955) continued cytological investigations of *Phalaris* citing the wide variation of chromosome numbers reported for the same species and generated a crude phylogeny based on chromosome number and centromere position. Anderson (1961) reported chromosome counts for several species and included this information in his hypothesis of phyletic relationships in the genus based on observed characteristics of the species. McWilliam (1962) followed up the work of Jenkin & Sethi (1932) by crossing the hexaploid *P. caesia* with *P. aquatica* producing F_1 hybrids with forage crop qualities that are superior to those of the parent species.

Chromosome banding with differential DNA staining has been used to assess relationships and chromosome evolution among groups of Aveneae species (Grebenstein, 1992; Röser & al., 2001; Irigoyen & al., 2002; Mitchell & al., 2003; Winterfeld & Röser, 2007; Winterfeld & al. 2009). NORs (nucleolar-organizing regions), sites which host the 45S rRNA genes, have been demonstrated as highly conserved and useful regions in

chromosome studies (Ritossa & Spiegelman, 1965; Winterfeld & Röser, 2007). These regions are typically very GC rich (King & al., 1993) and will not be stained by compounds that bind to AT rich DNA such as DAPI (Schweizer, 1976). Stains such as chromomycin will selectively stain GC rich regions of DNA, such as active or inactive NORs (Schweizer, 1976; Deumling & Greilhuber, 1982). NORs are usually observed at secondary constriction sites which can be detected without fluorescent staining.

The goals of this study were to produce karyotypes of representatives from each of the major clades in the genus (Old World x=6 diploids, Old world x=7 species, New World x=7 species, and the Arundinacea complex). *Phalaris canariensis, P. coerulescens/P. paradoxa/P. minor, P. angusta/P. californica,* and *P. arundinacea* were chosen. A comparison of the x=6 and x=7 diploids was conducted to search for an explanation for the loss or gain of a chromosome. Potential sites for NORs were located for the genus. Cytological investigations of *Phalaris* have not been placed in context with a molecular phylogeny for the genus. All karyotypes were assessed in terms of the phylogenetic relationships reconstructed by Voshell & al. (2011; Chapter 1, Figs. 1.3-1.5) and past studies were revisited in light of recent understand of the evolutionary history of the genus.

MATERIALS AND METHODS

Plant material

Plants used in the chromosomal studies were obtained from USDA seed accessions and field collection. Plants were grown in a glass greenhouse in standard potting soil. Plants were allowed to grow until the roots reached the edges of the pots. The plants were carefully removed from the pots to expose roots which were excised 1-2 cm from the root tip. Herbarium vouchers were prepared and stored in the Massey Herbarium at Virginia Tech.

Pretreatment

All root tips were placed in wet paper towels in petri dishes and stored on ice until they could be placed in the 4°C refrigerator overnight to shorten the chromosomes and halt the cell cycle at metaphase. Root tips were fixed in Carnoy's solution (3:1 ethanol:acetic acid) at 10:30 AM (11:30 AM during daylight savings time) and stored at 4°C until they were used in the cytology study. Initially, root tips from recently germinated seedlings were used to obtain metaphase chromosomes, but roots from mature plants were found to yield more metaphase spreads. Using roots from adult plants had the added advantage of not destroying the specimen, therefore allowing subsequent root harvests as well as herbarium voucher creation.

Acetocarmine staining

Root tips were macerated in 1:1 ethanol:HCl to break down the middle lamella so metaphase cells could be separated on the slide. The ideal maceration time for *Phalaris* was 3 minutes. Root tips were soaked in a 45% acetic acid solution containing 1% carmine for 2-3 minutes prior to be squashed under a cover slip. The slides were heated with an ethanol flame and pressed flat. Permanent slides were made by removing the cover with liquid nitrogen, dehydrating the slide in ethanol and mounting a cover slip with clear nail varnish. Chromosomes were viewed using an Olympus CH-2 light microscope and photographed under the 100X objective with an iPod touch camera.

Fluorescent staining

Root tips were macerated in 1:1 ethanol:HCl for 3 minutes at room temperature and squashed in either 45% proprionic acid or 45% acetic acid. Both treatments yielded similar results. Slides were examined with an Olympus BH-2 phase contrast microscope to confirm the presence and quality of chromosomes. Slides were frozen in liquid nitrogen to remove the cover slips and a serial dehydration was carried out by placing the slides in 70%, 90% and 100% ethanol for 10 minutes each. After the final ethanol bath, the slides were allowed to dry at room temperature. Slides were either stored at -20°C or stained the same day.

Slides were incubated in Chromomycin A3 at a concentration of 1mg/9mL PBS with 10 mM $MgCl_2$ at room temperature. After rinsing, a drop of DAPI stain was placed on the slides, cover slips were added, and the slides were left to incubate in the dark at

room temperature overnight. All slides were imaged the next day with an Olympus BX61 fluorescent microscope using Bio view Duet software.

Chromosome analysis

Chromosome photographs were edited in Adobe Photoshop Elements 7 and the karyotypes were assembled and described following Levan & al. (1964). Chromosomes with secondary constriction sites were placed first in the karyotype. The largest chromosomes were presented next followed by progressively smaller chromosomes. The staining and imaging for *P. minor*, *P. paradoxa*, and *P. coerulescens* were provided by the Röser lab (Martin-Luther-Universität Halle-Wittenberg, Germany) The raw images of the metaphase spreads were used in preparation of Figure 5.2.

Chromosome counts

Some chromosomes preparations were destroyed during cover slip removal or failed to be found again after DAPI staining. The number of chromosomes seen during these preparations were recorded.

RESULTS

Chromosome counts

Chromosome counts were recorded as slides were being prepared for staining. Accessions of *P. canariensis, P. brachystachys,* and *P. truncata* grown from USDA seed collections were observed to have a total of 12 chromosomes as previously reported. The locally collected *P. arundinacea* was a tetraploid with 2n = 28 total chromosomes. An accession of *P. arundinacea* from the USDA seed bank (PI 284179) was observed to be a hexaploid with 42 chromosomes. Subsequent examination of the seed material and plant indicate the accession is actually *P. caesia*. Material grown from a donated seed collection of *P. californica* revealed it to be a tetraploid with 28 chromosomes as expected. Examination of *P. angusta, P. appendiculata, P. platensis, P. paradoxa,* and *P. coerulescens* revealed diploid cytotypes with 14 total chromosomes as previously reported for those species. Metaphase chromosomes in *P. minor* and *P. aquatica* were not well spread so exact counts could not be taken, but they appeared to be tetraploids. No B chromosomes were detected. B chromosomes have only been reported in 10-15% of the angiosperms (Jones, 1995), but have been found in oats (Winterfeld & Röser, 2007) which are in the Aveneae tribe with *Phalaris*.

The x=6 cytotype

Phalaris canariensis was revealed to have one set of very long submetacentric chromosomes with an acrocentric secondary constriction site. Neither the primary or secondary constriction site was stained by acetocarmine (Fig. 5.1a) or DAPI (Fig. 5.1b) on this pair (pair 1 in Fig. 5.1a). A second set of slightly shorter set of chromosomes

appeared to have secondary constriction, though it was stained by both acetocarmine and DAPI indicating the DNA was not GC rich.

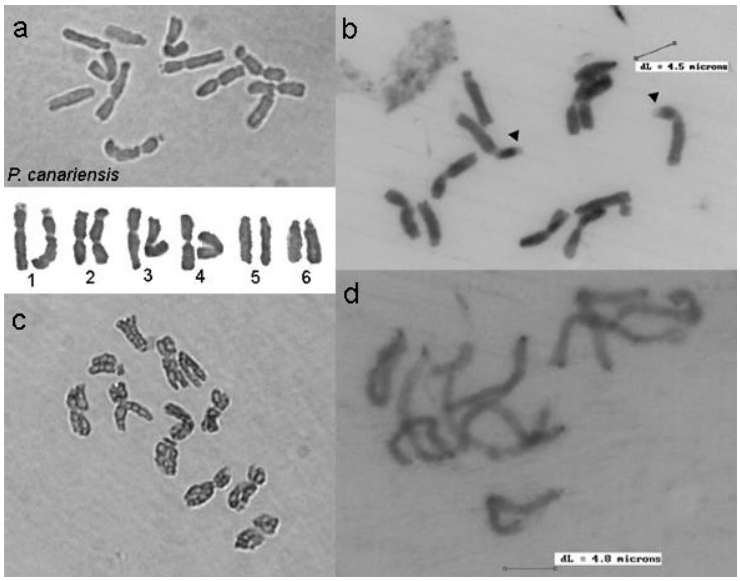

Fig. 5.1. Metaphase chromosome spreads and karyotype for diploid x=6 species, *P. canariensis*. a) Karyotype generated from acetocarmine stained metaphase chromsomes. b) Metaphase chromosomes stained with DAPI. Arrow head indicates secondary constriction site with reduced signal indicative of a NOR. c) Metaphase spread showing chromosome structure. d) Prometaphase chromosomes with increased DAPI signal on telomeres.

The rest of the karyotype was comprised of two metacentric and two acrocentric chromosomes. The chromosomes ranged from 4.5 – 10 microns in length. Both the acetocarmine and DAPI stains were less prominent near the centromeres, but no other banding was observed. Prometaphase chromosomes were stained with DAPI and a stronger signal was detected at the telomere region on several chromosomes (Fig. 5.1d).

The Old World x=7 cytotype

The karyotypes for *P. paradoxa* (Fig. 5.2b) and *P. coerulescens* (Fig. 5.2c) are very similar. Both have a nearly metacentric chromosome with a subterminal secondary constriction site indicating the presence of a NOR. The rest of the karyotype is comprised of two metacentric chromosome pairs which are larger than the others, three smaller metacentric chromosome pairs and a smaller acrocentric pair.

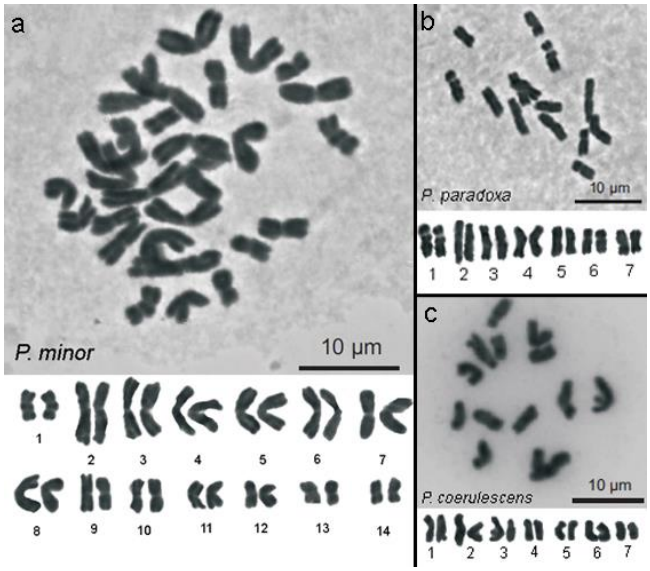

Fig. 5.2. DAPI-stained metaphase spreads for Old World x=7 species. Images of metaphase spreads were provided by the Röser laboratory. a) Karyotype for *P. minor*. b) Karyotype for *P. paradoxa*. c) Karyotype for *P. coerulescens*.

The acrocentric chromosome pair of *P. coerulescens* is slightly smaller than the one in *P. paradoxa*. The karyotype of *P. minor* contains a pair of chromosomes with a NOR with similar structure to the sets found in the two diploids (Fig. 5.2a). There are six sets of very long, nearly metacentric chromosomes, one submetacentric set of medium length, two smaller metacentric sets, and four small submetacentric sets.

The New World x=7 cytotype

The karyotype of the New World diploid, *P. angusta*, is very distinct from those of the Old World species. No secondary constriction sites were observed (Fig. 5.3c,d). The longest set of chromosomes were metacentric, followed by a pair of submetacentric chromosomes of similar length and four small sets of acrocentric chromosomes of similar length.

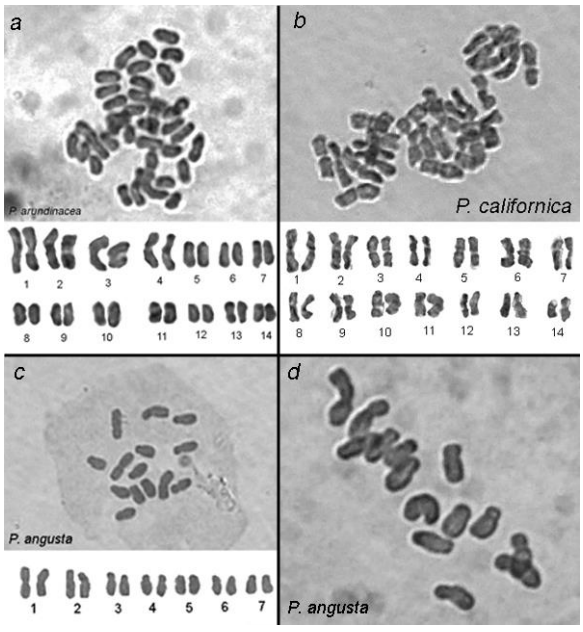

Fig. 5.3. Acetocarmine-stained metaphase chromosome spreads for New World species and *P. arundinacea.* a) Karyotype for *P. arundinacea*. b) Karotype for *P. californica*. c) Karyotype for *P. angusta*. d) Additional metaphase spread of *P. angusta*.

The karyotype of *P. californica*, the only known New World tetraploid, did not have any apparent secondary constriction sites. There were two pairs of longer submetacentric chromosome sets followed by progressively smaller sets of metacentric and submetacentric chromosome pairs, but the karyotype did not contain the small acrocentric chromosomes seen in *P. angusta*.

The Arundinacea cytotype

The karyotype for *P. arundinacea* illustrates four pairs of metacentric chromosomes that stand out as larger than the other sets (Fig. 5.3a). No secondary constriction sites were detected. The remaining pairs of chromosomes were of similar size. Six pairs were metacentric to submetacentric while the remaining four sets were subtelocentric to acrocentric.

DISCUSSION

The x=6 species (Old World)

Examination of *P. canariensis*, an x=6 diploid originating in the Mediterranean Basin revealed the presence of a secondary constriction site which resisted DAPI and acetocarmin stain indicating it is likely a GC rich site characteristic of a NOR (Fig. 5.1). A secondary constriction site was seen on both the acetocarmine slide (Fig. 5.1a) and the DAPI slide (Fig. 5.1b), but the the gap from the staining is not as apparent raising questions about whether the species has two sets of NORs. The karyotype of *P.*

canariensis is very distinct from the other species (Figs. 5.2-5.3). Differences in chromosome morphologies between major lineages have been mapped on a phylogenetic summary tree (Fig. 5.4). Examination of prometaphase chromosomes using DAPI revealed a stronger signal on the telomeres of certain chromosomes (Fig. 5.1d). This could either be the result of overlapping chromosomes giving a false signal, or indicate a region of greater AT richness which could serve as a marker to compare chromosomes of different species.

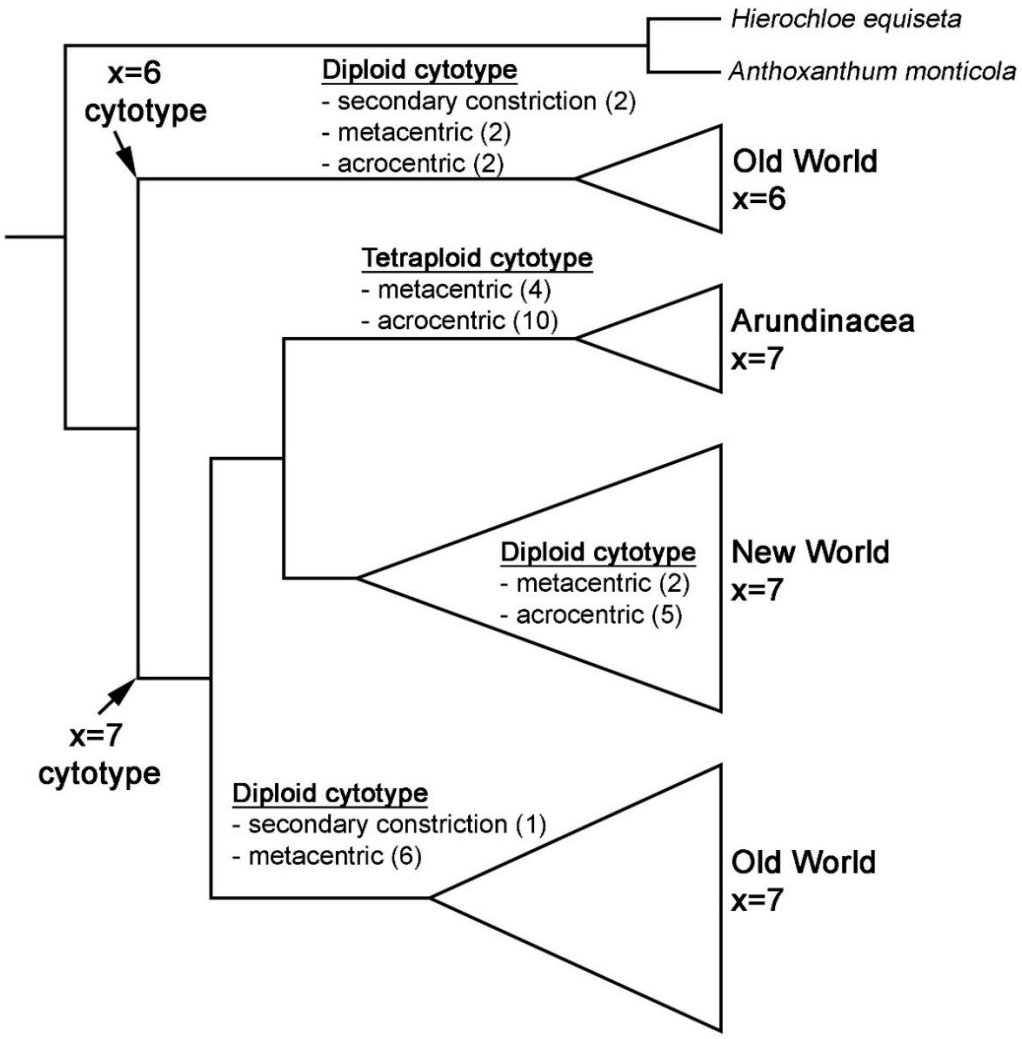

Fig. 5.4. Chromosome morphology and cytotypes mapped on a summary tree based on combined ITS and *trnT-F* data (Chapter 3). The divergence of the x=6 and x=7 cytotypes is noted as well as the presence of visible secondary constriction and the number of metacentric and acrocentric chromosome pairs.

Arundinacea species (x=7; Old World)

It is interesting to note that many citations exist for *P. arundinacea* displaying a chromosome count ranging from 2n = 14, 27, 28, 29, 30, 31, 35, all the way to 42 (Abastha, 1956; Anderson, 1961). While *P. arundinacea* may exhibit natural cases of anueoploidy, several factors probably influence this range of reported cytotypes. The reports of 2n = 14, 28, and 42 are easily explained since the three cytotypes were not recognized as distinct species when most of the studies were conducted. *Phalaris caesia* (2n = 42) and *P. rotgesii* (2n = 14) were treated as subspecies and often simply cited as *P. arundinacea* until *P. caesia* was elevated to the species level by Baldini & Jarvis (1991) and *P. rotgesii* was recognized by Baldini (1993). The aneuploids cited could be the result of the concentrations of colchicine used to prevent spindle fiber attachment in the early studies since the compound is known to produce spontaneous aneuploids (Metz, 1988). Refrigeration was used instead of colchicine to obtain metaphase plates in this study.

The even numbers of similar chromosome sets in *P. arundinacea* suggest the species could be of autopolyploid origin. The phylogenetic placement of *P. rotgesii* (diploid), *P. arundinacea* (tetraploid), and *P. caesia* (hexaploid) in a well-supported clade and similarities in both the maternal trnT-F and bipaternal ITS DNA sequences were indicative of possible autopolyploid origin (Voshell & al., 2011).

The New World species (x=7)

Examination of *P. angusta*, a New World diploid revealed a high number of small acrocentric chromosomes, a feature not found in the Old World diploids. The karyotype of *P. arundinacea* does contain medium sized metacentric chromosomes and small acrocentric chromosomes, indicating a closer relation between the two species than to the Old World diploids (Fig. 5.4). Voshell & Hilu (2014) suggested that a diploid progenitor of *P. arundinacea* likely traveled across the Bering Land Bridge giving rise to the New World species. The similarities between the chromosomes of *P. angusta* and *P. arundinacea* provide further support for the hypothesis since the New World diploids do not share close simililarities with the x=6 or Old World x=7 diploids (Fig. 5.4). The karyotypic similarities suggest that *P. angusta* and *P. arundinacea* are derived from the same common ancestor which presumably dispersed to the New World.

Phylogenetic reconstruction of the genus places *P. californica* as sister to the New World clade in the bipaternal ITS tree and as sister to all other members of the genus in the maternal *trnT-F* tree (Voshell & al., 2011; Chapter 1, Fig 1.4). Morphologically, *P. californica* is similar to the other New World species in having a type 3 floret with long, narrow sterile lemmas and is found in the same geographic range as *P. angusta* (Voshell & al., 2011; Chapter 1, Fig. 1.2). Unlike all other New World species it is a tetraploid. Voshell & al. (2011; Chapter 1) and Voshell & Hilu (2014; Chapter 2) revealed that in the maternal *trnT-F* data set *P. californica* shares indels and mutations with the outgroup species not found in other *Phalaris* species. These observations led to the hypothesis that *P. californica* may be an intergeneric hybrid. The karyotype of *P. californica* displays similarities to that of *P. arundinacea*, primarily the large to medium metacentric

chromosomes, but lacks the high number of small acrocentric chromosomes. Since the acrocentric chromosomes are a distinguishing feature in the *P. angusta* karyotype, these findings suggest that *P. californica* is not simply the result of genome duplication in *P. angusta*. *Phalaris arundinacea* and *P. californica* appear to share a common ancestor and the results do not confirm or refute the possibility of intergeneric hybridization.

The Old World species (x=7)

Phalaris coerulescens and *P. paradoxa* have similar chromosome structure and appear more closely related to each other than the other species studied (Fig. 5.2). This provides further support for their placement in the same clade in the phylogenetic tree (Voshell & al., 2011; Chapter 1, Fig. 1.3-1.5). The pair of chromosomes carrying the NOR are very apparent in *P. minor* indicating the tetraploid species is the descendant of diploid *P. paradoxa* or *P. coerulescens*. It will be difficult to determine whether *P. coerulescens*, *P. paradoxa*, or both have contributed to the genome of *P. minor* without further investigation since both karyotypes are so similar and do not display distinguishing markers in regard to banding patterns or the NOR location. The presence of only one pair of chromosomes with the NOR in the karyotype of *P. minor* and the number of long chromsomes is intriguing. It could supports the hypothesis that it is an allopolyploid (Fig. 5.2a) since one would expect to see two sets of secondary constriction sites if either *P. paradoxa* or *P. coerulescens* had undergone polyploidization. Furthermore, the presence of numerous long metacentric chromosomes suggest one of the progenitors is a species other than *P. paradoxa* or *P. coerulescens*.

The origin of the x=6 cytotype

The smaller size of the x=7 diploid chromosomes compared with the x=6 chromosomes has been observed in this study and cited in others (Parthasarathy, 1939; Ambastha, 1956; Anderson, 1961). Size discrepancies can be the result of differences in preparations, but it is interesting to note that the C values obtained for *Phalaris* support this observation. The mean 1C and 2C value for the x=7 diploids was significantly lower than that of the x=6 indicating that the x=6 species do have a larger amount of DNA content. The loss of a chromosome or merger between two from the x=7 cytotype would not lead to such an increase in genome size. It is of course possible that this happened and the genome increased later through subsequent rearrangements of the accumulation of repetitive DNA. Voshell & Hilu (2014) placed the divergence of the cytotypes ~17.1 MYA leaving a long period of time for complex rearrangements to have occurred.

CONCLUSIONS

It is clear that *Phalaris* species do not possess as many NORs as other members of the Aveneae tribe (Winterfeld & Röser, 2007) and, like many grasses, do not display distinctive banding patterns when stained for DAPI indicating heterogeneous AT base pair content. The results of this study support the relationships presented in the phylogenetic reconstruction of the genus (Fig. 5.4; Voshell & al., 2011), namely the sister relationship of the x=6 species to all x=7 species, the close relationship between *P. coerulescens* and *P. paradoxa*, and the possibility of one being the diploid progenitor of *P. minor*. Data from both Chapter 4 and this study indicate that the origin of the x=6

cytotype is not the result of a simple loss or rearrangement in the x=7 cytotype. The hypothesis presented by Voshell & Hilu (2014) regarding the migration of the diploid progenitor of *P. arundinacea* to the New World is supported by the similarities seen between it and *P. angusta*. *Phalaris californica* remains an enigma, but the karyotype suggests a closer relationship with *P. arundinacea* than *P. angusta* and does not negate the possibility of intergeneric hybridization.

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