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Morphological, Phylogenetic, and Ecological Analyses of the New Model Grass Species *Setaria viridis* (Poaceae) and its Close Relative *Setaria faberi*

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Abstract

Species limits as defined by herbarium taxonomists are nearly always based on intuitive morphological comparisons with little concrete data or statistical analysis. *Setaria viridis* (tribe Paniceae, subtribe Cenchrinae), an emerging model organism for the study of C_4 photosynthesis, is one such inadequately defined species. In order to evaluate its relationship with the morphological intergrading *Setaria faberi,* a "total data" approach was taken. Statistical morphology, cytology, molecular phylogenetics, and growth experimentation were employed to examine the putative species boundary in this group. Principal components analysis of 70 morphological characters in 85 individuals revealed consistent separation between the two species in morphospace, largely driven by spikelet characters. Flow cytometry demonstrated that *Setaria viridis* is consistently diploid, while *S. faberi* is consistently tetraploid. Phylogenetic analysis of the nuclear gene *knotted1* (*kn1*) showed that one of the two *kn1* paralogues in *S. faberi* is identical to its orthologue in *S. viridis,* while the other *S. faberi* paralogue is only slightly differentiated. This suggests either an autopolyploid origin, or an allopolyploid origin resulting from hybridization between *S. viridis* and an unsampled but closely related taxon. Finally, a controlled drought stress experiment showed that drought induces morphological effects on either species, but not to the extent that the species cannot be readily differentiated.

Introduction

The debate over the importance, definition, and validity of the species unit has raged more or less continuously since the earliest days of biological inquiry. Species definitions have included the typological unit of Linnaeus, the wholly arbitrary rank of Darwin, and nearly everything in between. Since the New Synthesis, however, it has generally been argued that a species represents a fundamental unit of biology, different from the inherently subjective higher ranks such as genus and family (Coyne and Orr, 2004). Nonetheless, how to consistently define a species remained contentious throughout the 20th century, with a profusion of species concepts in the past fifty years helping little to settle the debate (De Queiroz, 1998). The general lineage concept of de Queiroz (1998), which views the species explicitly as part of a process of lineage formation, as well as the recognition that most so-called species concepts are in fact merely high-flown species detection criteria (Hey, 2006), have greatly helped to clarify what is meant by 'species'. Nonetheless, the delimitation of species remains a practical problem. This is especially true in cases where hybridization, uniparental reproduction, or polyploidy are common (Coyne and Orr, 2004).

All of the above confounding factors are present in *Setaria,* a widespread genus of grasses. The genus is part of the "bristle clade" (tribe Paniceae, subtribe Cenchrinae), a genetically and morphologically defined group characterized by the presence of sterile branches on the inflorescence (Doust and Kellogg, 2002). *Setaria viridis* and *S. faberi* (hereafter collectively referred to

as the '*S. viridis* group') are two common and closely related members of the genus, and both are widely introduced weeds. *S. faberi* was most likely introduced to North America sometime in the early 20th century from East Asia, while *S. viridis* probably arrived shortly after colonization from Europe (Fairbrothers, 1959). A third species, *S. pumila,* often co-occurs with the other two, but appears to be of African origin.

S. viridis is quickly becoming a model species for the study of C_4 grasses (Brutnell et al., 2010; Li and Brutnell, 2011; Bennetzen et al., 2012). In contrast to maize (*Zea mays*), the predominant C_4 model at present, *S. viridis* has a relatively small genome, a short annual lifecycle, and is believed to be a diploid. From the standpoint of practicality, all of these are important traits for a model species. However, while the significant morphological diversity of this species has long been known (Rominger, 1962), it remains poorly characterized. Understanding the range of variation in morphology therefore remains a critical first step towards realizing the full potential of the species for future genetic work.

S. viridis, S. faberi, and *S. pumila* are all important weeds in the United States, where they cause significant crop yield losses and account for heavy herbicide expenditures (Holm et al., 1977). *S. faberi* and large forms of *S. viridis* negatively affect bean and corn crops in the Midwestern United States, while *S. pumila* is more often a pest of lawns (Rominger, 2003). Recently, herbicide resistant varieties have emerged, causing additional problems in chemical-dependent agriculture systems (Stoltenberg and Wiederholt, 1995; Wang and Dekker, 1995). Resistance to atrazine and acetyl CoA carboxylase inhibitors has

been found in *S. viridis* and *S. faberi*, while trifluralin and acetolactate synthase inhibitor resistance is known from *S. viridis* (Darmency, 2005).

In addition to its status as a pest, *Setaria viridis* has had a more ancient and positive relationship with humans. It was actively foraged by people for its large grains as early as 12,000 years ago as evidenced by its presence in numerous human archaeological sites across Eurasia (Hunt et al., 2008). It was later domesticated into one of the earliest crops by 8,000 years ago in northern China. The morphologically distinct domesticate, *S. italica* (foxtail millet), spread to all corners of Eurasia over several thousand years, with independent domestication possible in several regions (Hunt et al., 2008; Hirano et al., 2011). *S. italica* and *S. viridis* remain interfertile, and low levels of gene flow have probably occurred continuously since domestication (Darmency et al., 1987; Shi et al., 2008).

Polyploidy has likely been responsible for the formation of many new species in the genus *Setaria* (Hunt et al., 2008). While polyploidy can blur the boundaries of a species, it is paradoxically a primary force in the genesis of species richness in plants. Recent macroevolutionary genomic studies provide firm evidence that polyploidy is responsible for much of angiosperm diversity (Soltis et al., 2009). However, polyploidy is no less important in a microevolutionary context. Hybridization followed by genome doubling can result in immediate speciation due to chromosomal incompatibility with the parent species, as has been observed in the composite *Tragopogon* (Soltis et al., 1995) and the grass *Spartina* (Ayres and Strong, 2001). The same process is probably

responsible for a significant portion of crop diversity (Judd et al., 2008). Several species, including *S. faberi,* appear to have formed via polyploid events involving *S. viridis* (Clayton, 1980; Benabdelmouna et al., 2001).

Herrmann (1910) first described *S. faberi* as a new species, citing a single specimen from Szechuan, China. The description points out that the plant was imperfectly known and the specimen used was fragmentary. No direct comparison with *S. viridis* was made at that time, although the author's description is thorough and captures many of the distinguishing characters that would be cited by later authors such as larger spikelets and shorter upper glumes. Ohwi (1938) described the later synonym *S. autumnalis*, describing its distribution in Japan, Korea, and China, while also making explicit comparison with *S. viridis*. He writes that his species differs in phenology (fall), size (larger overall), and morphology (nodding spikes, with spikelets being more than 3 mm long). Rominger (1962) notes that the first collection of *S. faberi* in the U.S. was made in 1925. The species was then frequently confused with *S. viridis* for many years until Fernald (1944) compared the two. He used largely the same characters as Ohwi, but also mentioned the strigose pubescence on the leaves and the "distinctly cross-wrinkled" (vs. "slightly rugose") anthecium of *S. faberi*.

Fairbrothers (1959) performed the first objective comparison between the species using 150 specimens from 12 populations of *S. faberi* and 100 specimens from 9 populations of *S. viridis*. Both were collected only from within New Jersey. He found that spikelet lengths and the sterile lemma to upper glume ratio fell into two discrete groups. When plants were grown in common garden

conditions, panicle length also formed two non-overlapping groups, although only 32 plants were grown. Other characters, such as fertile lemma markings, leaf length, and culm length, failed to consistently separate the two. Pohl (1962) noted forms of *S. faberi* in Iowa with glabrous leaves and forms with both surfaces pubescent; he also found that the offspring of these types could have either kind of leaf. Spikelet lengths were reported from his study that fell in to the range of *S. viridis* as reported by Fairbrothers, but he claimed the short upper glume remained diagnostic.

Recent phylogenies of *Setaria* elucidate relationships within the genus. Doust et al. (2007) used the chloroplast marker *ndhF* and the nuclear marker *knotted1* to produce a well-resolved tree. Unfortunately the study did not include *S. faberi*, but it does demonstrate that three accessions of *S. viridis* plus two of *S. italica* form a clade. Southern blots were also performed on single accessions of *S. viridis* and *S. italica*, showing that *knotted1* had only one copy and thus demonstrating diploidy. Kellogg et al. (2009) produced a more heavily sampled tree based on only *ndhF,* which placed one accession of *S. faberi* at the base of a clade containing *S. viridis* and *S. italica* with good support. Assuming allotetraploidy in *S. faberi,* this suggests a *S. viridis* seed parent for this accession at some point in the past. In both phylogenies, *S. pumila* occurred in unrelated clades with good support.

Despite general agreement on the distinctiveness of *S. viridis* and *S. faberi,* some authors have previously questioned the validity of their separation. In addition to the morphological arguments cited above, their status as distinct

species is also supported by several cytological studies showing *S. viridis* to be diploid and *S. faberi* to be a tetraploid, with karyotypes suggesting an allopolyploid origin for the latter species (Li et al., 1942; Willweber-Kishimoto, 1962; Pohl, 1962). Genomic in situ hybridization also suggested that *S. faberi* is of allopolyploid origin (Benabdelmouna et al., 2001). Artificial crosses between the two usually result in sterile seed, but occasionally triploid offspring are produced (Li et al., 1942; Willweber-Kishimoto, 1962). Nonetheless, cytological examinations have been few considering the range of morphological variation observed and the geographic extent of the species. Existing morphological analyses (Fairbrothers 1959, Pohl 1962) have been too restricted to discount the possibility of a continuum of variation in the *S. viridis* group. As the species are commonly understood, *S. viridis* is highly variable in size, completely encompassing the size range of *S. faberi* (Rominger, 1962). Adding to the uncertainty are two tetraploid counts of plants identified as *S. viridis* (Saxena and Gupta, 1969; Mulligan, 1984), suggesting either misidentification or independent polyploidy in *Setaria viridis*. Because *S. viridis* is becoming an increasingly important target of study as a model organism (Brutnell et al., 2010), it is essential that these uncertainties be resolved in order to aid sampling in the field by geneticists. Although *S. pumila* occurs together with *S. viridis* and *S. faberi*, it is morphologically, phylogenetically, and karyotypically distinct (Benabdelmouna et al., 2001; Doust et al., 2007). Nonetheless, it remains possible that gene flow occurs or has occurred between *S. pumila* and the *S. viridis* group given their consistent proximity in the field.

This study presents a more thorough examination of morphology, ploidy, and phylogenetics in the *S. viridis* group in order to characterize the morphological variation of an emerging model organism and to assess the validity of the existing taxonomy. The complex problem of species definitions in the face of varying ploidy levels and intergrading morphology confounds taxonomy in many angiosperm groups, (e.g. *Galax urceolata* in Burton and Husband, 1999) and as such this study could also inform the study of similar systems. A morphometric analysis of *S. viridis* and *S. faberi* addresses the hypothesis that the two are discrete morphological entities. This complements an updated phylogeny and a flow cytometry-based ploidy analysis of the group. A drought tolerance experiment examines the possibility of an environmental influence on morphological convergence.

Methods

Field Collections

Approximately 220 specimens of *Setaria viridis, S. faberi,* and *S. pumila* were collected in the United States and Canada, primarily in the Midwest, by a variety of collectors between 2010 and 2012 (see Appendix II). Most collections were made along major interstate and state highways. Of the collections made, approximately 144 were *S. viridis,* 42 were *S. pumila,* and 37 were *S. faberi.* Initial determinations were made based on Rominger (2003).The differential sampling effort between species was due to an emphasis on sampling variation in general, the difficulty of distinguishing the species in the field, and the absence

of *S. faberi* from some of the collection routes. Each collection included a pressed voucher specimen and seeds from five individuals from within the same local population. As such, the seeds collected do not necessarily correspond to the exact genetics of the pressed voucher specimen. Geographic coordinates and occasionally photographs were also gathered with each collection.

Morphology

Fifty-five morphological characters (see Appendix I) were measured from eighty-five herbarium specimens. Fifty-six sheets of *S. viridis,* twenty-six sheets of *S. faberi,* and three sheets of *S. pumila* were examined. *S. pumila* was included as a control in order to address the formal hypothesis that *Setaria* species are indistinguishable from one another by morphological analysis. *S. pumila* often co-occurs with *S. viridis* and *S. faberi*, so its inclusion also served to assess potential morphological introgression. The differential sampling effort among species was due to the availability of field collections as noted above.

Characters were chosen by initially measuring a set of 77 characters. After examining 20 specimens and again after 35 specimens, characters were removed that were 1) invariant or difficult to measure consistently; 2) strongly and consistently co-variant with another character, or 3) as variable within specimens as between specimens. Co-variance was determined by creating pairwise scatter plots and calculating r^2 values and their significance values in R (version 2.15.1, www.r-project.org). Characters that were removed from the study are shown in Table 1. Lower lemma length had high covariance with spikelet length (r^2 .97), but it was nonetheless retained in order to calculate a ratio

between it and the upper glume, a common diagnostic character for the genus. Macro-characters were measured using Fowler Sylvac (Model S 235) digital calipers, while micro-characters were measured on a Wild Heerbrugg M8 stereoscope fitted with a calibrated reticule. Spikelets were soaked in room temperature water for ca. 20 minutes before measuring in order to loosen the tissues. The soaking process also caused the spikelets to expand slightly.

Univariate distributions were visualized as histograms for each character in R in order to identify gaps, potentially indicating distinctive species characters. PCA was used in order to reduce the dimensionality of the data and to capture multivariate variation without any prior assumptions regarding groups. It was performed either using the “principal()” function in R from the “psych” package with “nfactors” set to three and using a verimax rotation, or using JMP (version 10.0.0, www.jmp.com) with default settings. Various character sets were explored in the PCAs in order to understand which elements of morphology were capable of separating the species in morphospace (Appendix III). Separate analyses were also performed excluding immature specimens (i.e. those with little or no disarticulation of the spikelets) and with the *S. pumila* outgroup removed. Finally, *S. viridis* specimens that corresponded to a preliminary Structure (Pritchard et al., 2000) analysis using microsatellite data (Katrien Devos, pers. comm.) were included in a separate PCA in order to assess variation of genetic populations of that species. Because the specimens measured came from the same populations but were not the same individuals used for the microsatellite survey, specimens collected from mixed genetic

populations were removed from the analysis. This particular PCA was thus was meant to be preliminary due to the small sample size.

Phylogenetics

DNA was extracted from living tissue of six accessions grown from seed at the University of Missouri – St. Louis (see Appendix III). These included four individuals of *S. faberi*, one of *S. viridis*, and one of *S. pumila.* Because *S. viridis* and *S. pumila* had been sampled extensively in previous phylogenies (Doust et al., 2007; Kellogg et al., 2009), sampling here focused on *S. faberi.* Leaf tissue was frozen in liquid nitrogen followed by grinding with a mortar and pestle. DNA was then extracted from the ground tissue using a DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, California). PCR was then used to isolate two overlapping segments of the chloroplast gene *ndhF* totaling about 2040 bases, and about 630 bases of the nuclear gene *knotted1* (*kn1*) using protocols and primers described by Doust et al. (2007) and Kellogg et al. (2009).

PCR products of the genes *kn1* and *ndhF* were purified via gel extraction using a QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, California). The resulting products of the *ndhF* samples were then cycle sequenced directly using fluorescent Big Dye (Applied Biosystems, Foster City, California) and then sequenced on an ABI 3130 automated sequencer (Applied Biosystems, Foster City, California). The first fragment of *ndhF* was sequenced using varying combinations of the following primers: 5F, 33F, 445F, 536R, 536F, 866R, 972R, 972F, 1278R, and 1318R. The second fragment was sequenced with the following primers: 1278F,1318F, 1580F, 1630R, 1888F, 1888R, 2110R. All

primers are described in Doust et al. (2007) and Kellogg et al. (2009), except for 33F (5'-ATCCCTCTTCTCCCACTT-3'), 866R (3'-CAACCATAGTTGCTGCGTGT-3'), 1278F (5'-TCCACCTCTTGCTTCGTTCT-3'), 1278R (5'-AGAAGCAAGCAAGAGGTGGA-3'), 1888F (5'-GCAATTCTTGGTCTATTCATAGCA-3'), and 1888R (5'-TGCTATGAATAGACCAAGAATTGC-3'), which were designed for this study using Primaclade (Gadberry et al., 2005) from sequences of the *S. viridis* group produced by Kellogg et al. (2009). It should be noted that the numbers used to name these new primers only approximately indicate their position in the DNA sequence as it was unclear which scheme was used in order to number the older primers. In order to capture paralogous copies in the nuclear gene *kn1*, gel-extracted products were cloned into PGEM-R Easy Vector heat-competent *E. coli* (Promega Corp., Madison, Wisconsin) and the bacteria grown on selective media. Eight colonies were sequenced for each accession. Cloned products were sent to the Penn State University Nucleic Acid Facility for sequencing using the T7 and M13R primers.

Geneious version 5.6.5 (Biomatters Ltd., Auckland, New Zealand) was used for editing and contig assembly. Included sequences were double-stranded for a minimum of 80% of their length. The *ndhF* sequence of the *S. faberi* accession *Vela 70* did not meet this threshold after several re-sequencing attempts and was thus excluded. Geneious was also used to assess sequence quality; regions with a Phred quality value less than 50 were excluded unless they agreed with another sequencing strand of high quality. *Kn1* sequences were

of uniform high quality, so quality assessment was only an issue with the direct sequenced *ndhF* sequences. A small number of Geneious's chromatogram calls were corrected by hand when a peak unambiguously corresponded to a nucleotide different from what had been automatically assigned. *Kn1* sequences of the same copy were combined into consensus sequences. Edited sequences were then inserted into the existing alignments produced by Doust et al. (2007) for *kn1* and Kellogg et al. (2009) for *ndhF*. These alignments were obtained from TreeBASE (treebase.org) under the study numbers S1731 and S10018, respectively. Note that these study numbers differ from those reported in the original journal articles (given as S1695 and SN3906, respectively). In the course of reviewing the *ndhF* alignment from Kellogg et al. (2009), it was found that the sequence for *Setaria faberi* included in the published analyses was in fact chimeric, with the 3' half containing bases from a clearly distinct species. It was thus removed along with the pre-existing *Setaria viridis* sequence, which was deemed to be of low quality based on a number of base omissions and questionable bases in highly conserved regions of the alignment.

All data were analyzed with maximum likelihood (ML) and Bayesian analyses, with ML support assessed via bootstrapping. ML was performed in RAxML version 7.2.8 (Stamatakis, 2006; Stamatakis et al., 2008) using the BlackBox setting on the CIPRES Science Gateway version 3.1 (phylo.org). MrBayes version 3.1.2, also as hosted on CIPRES, was used for Bayesian inference (Ronquist and Huelsenbeck, 2003). Models of DNA evolution were determined for each of the two datasets using jModelTest (Posada, 2008). The

TPM1+G model (“Three-Parameter Model”, also known as K81; Kimura, 1981) was selected for the *kn1* dataset with a gamma shape of 0.6380 and the following relative basepair frequencies: AC = 1.0000, AG = 2.9700, AT = 1.1939, CG = 1.1939, CT = 2.9700, GT = 1.0000. This model was selected using both the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). The TIM3+I model (“transitional model”; Posada, 2003) was selected for the *ndhF* dataset using the AIC, but the TPM3uf+I model was preferred by the BIC. However, because the delta score between these two models using BIC was less than the significant threshold of two, TIM3+I was chosen. The parameters for the model included the proportion of invariant sites at 0.7280 and the following relative base pair frequencies: AC = 2.4031, AG = 5.6679, AT = 1.000, CG = 2.4031, CT = 5.1755, GT = 1.0000. The model parameters were entered into the “advanced parameters” menu of the MrBayes interface on CIPRES. For each dataset, four separate Bayesian analyses were run with four chains and 10 million generations. The first quarter of the sampled values were discarded as burn in for each analysis, after which all trees were combined. Because RAxML is optimized for the GTR substitution model, that model was used for the ML analyses of each dataset.

Flow Cytometry

Genome size and ploidy level were estimated with flow cytometry. Fresh leaf material was collected from young plants grown from seed. When more than one seed germinated in a pot, one plant was allowed to grow to maturity, one was used for flow cytometry, and the others were discarded. Leaf material was then wrapped in slightly moist paper towels, packed in plastic bags, and shipped

to the Flow Cytometry Core Lab at the Benaroya Research Institute at Virginia Mason in Seattle, Washington for processing. Five genome size estimations were averaged for each sample to estimate mean picograms of DNA per somatic cell. A total of 97 counts were made from 87 accessions. Of these, 58 accessions were *S. viridis*, 14 were *S. faberi*, and 15 were *S. pumila.*

Drought Experiment

Seven accessions of *S. viridis*, three of *S. faberi*, and three of *S. pumila* were subjected to a simple drought experiment. Plants were randomly assigned to one of two treatment groups, a normal group and a drought group, in order to test the influence of water availability on morphology. Each treatment group included four replicates of the same age, each grown from seed from the same mother plant, for a total of eight individuals per accession. The amount of water used for the treatments was chosen based on how much was required to soak a pot of soil just before water began to flow through the bottom. Prior experience suggested fully soaked pots would dry at the surface after two days. Therefore, this amount was used for the normal group, and half this amount was used for the drought group. Watering was simplified by bottom watering into a tray containing 18 plants such that the amount determined above was multiplied by 18. This amount was rounded to one l. Thus, the normal group received one l of water per flat of 18 plants every two days, while the low water group received the same amount every four days.

Individual plants were randomly distributed across flats within their group in order to reduce positional bias. The normal group was fertilized every fourth

watering, while the drought group was fertilized every second watering such that an equal amount of fertilizer was given to both groups every eight days. The fertilizer used was Peters Excel CalMag 15-5-15 at 150 ppm. MetroMix 360 was used as a potting soil, while the pots themselves were 3-inch square pots 3.5 inches deep. The experiment took place in a growth chamber with twelve hours of light and twelve hours of darkness. Temperature was set at 31 degrees C during the day and 21 degrees C at night, while humidity remained at 50-60% at all times. These environmental parameters were based on recommendations from colleagues with experience growing the species (Hui Jiang, pers. comm.)

Seeds for the experimental plants were sown on the seventh and eighth of July, except for the accession *Vela 78*, which was sown on the twelfth of July to replace an accession that showed no germination. About four seeds were sown directly in the pots where they would be grown. In a few cases, seeds failed to germinate in a given pot. In these cases, extra sprouts from other pots containing the same accession were transplanted into the empty pots. For the accessions *Kellogg 1213* and *Vela 70*, thirty selfed F1 seed of each accession were sown in addition to the field collected seed. Germination of field-collected seed was low, so the F1 seedlings were transplanted into pots for inclusion in the experiment. During the first week of growth pots were thinned to a single plant in cases where more than one seed had sprouted. All plants were well watered for about three weeks (until July 30) to allow for establishment. The watering regimes discussed above were then started and continued until September 16 (about 7 weeks) when vertical growth had ceased among the majority of accessions. The

following morphological characters were measured for all experimental plants: height, number of tillers (originating at or below the second node), number of inflorescences, inflorescence length, and flag leaf length.

These morphological data were analyzed in order to identify differences between the high water and low water groups, as well as between the accessions. Plants that died before attaining a height of 5 cm were removed from the analysis. This occurred sporadically in both the high water and low water groups and was therefore most likely not an effect of treatment. One-way analyses of variance (ANOVA) were performed using water as the explanatory variable. These were repeated for each morphological character on each species individually, as well as the three species together as an aggregate. In this latter case, species effect was also used in the ANOVA as an explanatory variable in addition to the treatment effect. Multivariate analyses of variance (MANOVA) were used in order to compress the effects on the five characters into an overall response variable. These were performed on each species individually, each possible pair of species, and all three species together. In cases where more than one species was included, species effect was also used in the MANOVA as an explanatory variable in addition to the treatment effect. ANOVAs were performed in R using the ‘aov()’ function, while MANOVAs were performed using the ‘manova()’ function with the default Pillai’s Trace used to approximate the F statistic.

Results

Morphology

Morphological analysis of *Setaria viridis, S. faberi, and S. pumila* revealed that the three species can be consistently distinguished based on a number of characters. *S. faberi* was distinct from *S. viridis* based on the former's laminae with sparse adaxial pubescence, spikelets larger than 2.5 mm, and with upper glumes not longer than 90% of the lower lemma length. *S. faberi* could also be nearly always be distinguished from *S. viridis* on the basis of the former having spikelets that become a uniform brown without mottling once hardened (whereas *S. viridis* is blackish with brown mottling when fully hardened). However, many specimens had immature spikelets, often with intermediate character states. It is thus unclear if these examples represent only a transitional phase during development or truly intergrading morphology. Finally, the upper anthecium of *S. faberi* appears consistently more deeply rugose than that of *S. viridis*, but this character was not quantified in this study. *S. pumila* can be easily distinguished from the other two species based on its sessile spikelets, sheaths lacking hairs at the margins, laminae with pubescence only at the adaxial base, peduncles with scabrosity only immediately below the inflorescence (versus on the upper tenth to quarter of its length in the other species), inflorescence axes with short prickles (as opposed to long pilose hairs in the other species), large spikelets with a deeply rugose upper anthecium maturing to a uniform brown or black, upper glumes less than half of the total spikelet length, and tan inflorescence bristles.

Other general observations of morphology were made during the study. While basal branching almost always occurs in *S. viridis* and *S. faberi*, occasional specimens have only a single shoot. Hairs always occurred along the entire length of the sheaths in *S. viridis* and *S. faberi*, but not at all in *S. pumila*. Roots are generally cream to light brown in color, but often show purple coloration, especially near the base. Purple also appears frequently in all species studied at the internodes (usually quite close to the nodes), on the nodes themselves, and on the leaf sheaths. However, purple was more likely to occur on these organs in basal portions of the plant only. Purple was less frequently observed on the leaves, inflorescence bristles, and on the glumes and outer lemma. Observations of many greenhouse-grown accessions suggest that purple coloration is at least in part genetically determined. Lower palea width varied significantly, with *S. pumila* and *S. faberi* usually having very wide and rounded lower paleas, while those of *S. viridis* were typically straight and narrow. All species often had anthers that were found inside the upper anthecium with a fully developed caryopsis, indicating they were probably never exserted.

Principal components analyses (PCA) consistently separated the species of interest in morphospace. A representative PCA using all specimens and all measured characters for *Setaria viridis* and *S. faberi* is a case in point (Figure 1). The first principal component axis explained 17% of the variance, the second 7%, and the remaining axes less than 5%. The first principal component is clearly a size axis, with high loadings for size of both floral and vegetative characters (Figure 2). Most bivariate characters had weaker loadings. The vectors for the

vegetative characters nearly all aggregate in the upper right quadrant of the graph. Among this group, most load positively on both principal components, but nonetheless load primarily on the first principal component axis. On the other hand, most spikelet characters, hair morphology, prickle morphology, and bivariate color characters, group along the second principal component axis. Some, such as spikelet length and adaxial hair per area, load positively on the both principal components. Others, such as the upper glume to lower lemma ratio, load negatively on the first principal component and negatively on the second principal component. Taken together, spikelet, hair, and prickle morphology are primarily responsible for separating the two species, while their vegetative size variance within their respective species is summarized along the first principal component. *S. pumila* was excluded from the PCA presented here in order to show that its inclusion does not artificially drive the analysis. However, when PCAs were performed with *S. pumila* with either other species, it was distinctly separated (Figure 3, discussed below).

In addition to the PCA presented above, forty subsets of the data were used to make as many PCAs in order to assess which subsets were sufficient to separate the species (Appendix III). Any combination of morphological characters that included hair and/or spikelets was able to separate *S. viridis* and *S. faberi* into distinct clusters. This rule did not hold, however, when only the bivariate spikelet characters were included. These consisted almost entirely of apparently uninformative color characters. The inflorescence character set (not including spikelet characters) on its own was nearly able to produce a separation of the

two species, but a small band of overlap remained. Adding vegetative characters to the inflorescence set removed all separation between the two, indicating the latter is a poor predictor of species identity. No difference was noted when repeated measures of characters on the same specimen were averaged or if maximum values were used instead.

When *S. pumila* was included in PCAs with the other two species, it usually grouped with one or the other species depending on the characters used. However, the full morphological dataset did separate it into its own distinct cluster when the third principal component axis was included (Figure 3). Additionally, PCAs were performed using only *S. pumila* and *S. viridis,* and also as only *S. pumila* and *S. faberi* (not shown). In both cases, the species were distinctly separated.

The PCA performed on *S. viridis* and *S. faberi* specimens corresponding to the preliminary results of a microsatellite-based Structure analysis gave hints of morphologically significant intraspecific variation (Figure 4). The data suggested correspondence between population genetics, morphology, and geography. The Canadian and northern U.S.A specimens, which are morphologically distinguishable from other accessions based on their large spikelets and generally small vegetative stature, appeared together in a distinct cluster. Specimens sharing microsatellite signatures from central to southern latitudes also grouped together. In this PCA they were distinguished by their smaller spikelets. The remaining two groups were represented only by single specimens. One was part of a group including specimens from China, central

latitudes of the U.S.A., and central Europe. The other was part of a group from China and the central latitudes of the U.S.A. that is characterized by much wider inflorescences due to longer primary branches. Sampling was insufficient for the PCA to definitely separate the Structure groups, however.

Phylogenetics

The phylogenetic analyses did not contradict the notion that *S. viridis* and *S. faberi* are distinct species. However, sampling was insufficient to convincingly demonstrate a phylogenetic species boundary. Because both the *knotted1* and *ndhF* phylogenetic analyses used existing alignments with only very minor additions, the backbones of both trees are identical to those of the previous studies and are not presented here. General results for these trees can be found in Doust et al. (2007) and Kellogg et al. (2009). However, the internal structure of the clades containing *S. viridis* were different given the addition of *S. faberi* to the *kn1* tree and the removal of poor *S. viridis* and *S. faberi* sequences from the *ndhF* tree.

A clade in the *ndhF* tree containing *S. viridis, S. faberi, S. italica,* and one accession of *S. verticillata* is strongly supported with a posterior probability of 100 and 100% bootstrap support (Figure 5). The same clade is recovered in the *kn1* analysis, albeit containing only one of the two paralogues of *Setaria faberi* (Figure 6). The *kn1* analysis suggests that the *S. verticillata* accession is sister to the rest of the clade, which is moderately supported with 86 percent bootstrap and 0.76 posterior probability. The *ndhF* analysis, on the other hand, suggested the same *S. verticillata* accession is part of a clade with *S. faberi*, which is in turn

sister to *S. viridis* and/or *S. italica*. This *S. faberi* plus *S. verticillata* clade has similar moderate support (69 bootstrap, 97 posterior probability). As mentioned above, the *kn1* analysis placed the "B copy" of *S. faberi* separately from the other sequences of the *S. viridis group* (Figure 6). Neither analysis was able to determine the immediate sister relationship of the "B copy" clade, however. The additional *S. pumila* accession fell as expected next to other accessions of that species (Figure 7).

Flow Cytometry

Flow cytometry strongly suggested that the three putative species of interest each have a unique genome size range, or ranges in the case of *S. pumila,* which in turn provides strong support for separation of the species. Genome size estimates for plants identified morphologically as *S. viridis* fell in the range of 0.92-1.29 pg/2C (n=55, of which 9 were counted twice; Appendix IV). For plants identified as *S. faberi,* estimates ranged from 2.08-2.83 pg/2C (n=12, of which two were counted twice). *S. pumila* yielded two ranges, one from 2.09-2.15 (n=10), and the other from 4.87-5.17 (n=5). Two estimates were outside of the above ranges: the *S. faberi* accession Kellogg *1213* (from F1 seed) had 3.97 pg/2C, and the *S. viridis* accession *Estep ME014* had 2.25 pg/2C. In the case of *Estep ME014*, a new estimate was obtained that yielded 1.00 pg/2C, and it was thus assumed that the first count was the result of an error. For *Kellogg 1213*, a previous estimate of the parent plant showed 2.77 pg/2C. In this case, the first estimate was taken to be accurate and the F1 count was assumed to be an error. In both cases, different plants from the same genetic seed stock were

used for the estimates, so the possibility of spontaneous autopolyploidy cannot be completely discounted. In two cases, *S. viridis* plants at the high end of the range, accessions *Estep ME028V* and *Ahart 17139*, were recounted, resulting in estimates closer to the median value. Again different plants from the same seed stock were used and thus genome size variation and cytometric error cannot be disentangled with the available data. A list of all genome size estimates from flow cytometry is given in Appendix IV

Drought Experiment

The drought experiment revealed that 1) *S. viridis* was only minimally affected by the drought treatment in terms of both morphology and mortality; 2) *S. pumila* showed a strongly significant reduction in height due to the drought treatment; 3) *S. faberi* accessions were significantly shorter with significantly fewer tillers and/or inflorescences when subjected to drought; 4) *S. faberi* was three times more likely to die prematurely due to the drought treatment than either other species; and 5) inflorescence length and leaf length were only significantly affected in *S. pumila*, and then only insofar as inflorescences and flag leaves failed to develop by the time the experiment ended. ANOVAs, using the treatment group as the explanatory variable with the five measured morphological characters as dependent variables, are summarized in Table 2.

S. faberi and *S. pumila* accessions were significantly ($p < 0.05$) shorter in the drought group compared to the normal group. The height of *Setaria viridis*, on the other hand, was not significantly affected by drought in our sample. The number of inflorescences produced was significantly lower in *S. faberi* and *S.*

viridis, but not in *S. pumila*. The treatment effect was much stronger in *S. faberi* than in *S. viridis* for this character. The number of tillers produced showed a similar pattern to inflorescence number. Inflorescence length and flag leaf length each were only affected by the drought treatment in *S. pumila.* However, this was the case because two of the three accessions of *S. pumila* failed to produce any inflorescences or flag leaves in the drought group by the time the experiment had ended. These measurements were thus entered as zero, greatly reducing the overall mean for the species.

MANOVAs (Table 3) showed that the drought treatment significantly affects the overall morphology of *S. faberi* and *S. pumila*, but not *S. viridis.* MANOVAs performed with pairs of species and with all three species together, that is when these species groups are considered in aggregate, showed that the drought treatment significantly affects overall morphology in all cases. They likewise showed that the species' morphologies are all significantly different from one another when the treatment groups are considered in aggregate.

Drought tolerance was also variable amongst the species in terms of mortality. Of the 12 *Setaria faberi* individuals within the three accessions in the low water group, eight died before fruiting (67%). This compares with five *Setaria viridis* out of 28 (18%) dying before fruiting in the low water group and two of twelve *Setaria pumila* (17%). Yet another effect of drought was observed in flowering times. Two of the three accessions of *S. pumila* responded to drought by failing to flower in the low water group. All three accessions flowered during the course of the experiment in the high water group.

Discussion

Morphology

This study was undertaken in part because of a perceived lack of clear species boundaries in field observations of the *Setaria viridis* group. While canonical *S. faberi* individuals are described as being much taller than their *S. viridis* cousins, we observed some very small specimens with various *S. faberi* characters such as laminal hair, drooping panicles, and short upper glumes. On the other hand, *S. viridis*-like individuals attaining heights of a meter and a half with slightly drooping panicles and very large inflorescences were also seen. Pohl (1962) reported *S. faberi* with glabrous leaves, which others before him had attributed exclusively to *S. viridis*. Li et al. (1942) and Willweber-Kishimoto (1962) both successfully crossed the two species to produce fertile seed, further adding to doubts about their consistent distinctiveness.

The morphological analyses presented here demonstrate beyond a reasonable doubt that the two entities are morphologically distinct. These results corroborate and expand on those of Fairbrothers (1959), who upon conducting a quantitative analysis of 150 specimens of *S. faberi* and 100 of *S. viridis,* concluded that spikelet length, the presence of hairs, and the lower lemma-upper glume ratio were sufficient to statistically separate the two. The fact that his samples were exclusively from New Jersey apparently did not hinder his ability to observe a sufficiently large swath of the variation, which was an initial concern when beginning this study.

Univariate distributions of single characters often did not detect separate groups as they did in Fairbrothers' study, however. For example, the range of spikelet length for our sample of *S. viridis* was 1.93-2.85 mm, while *S. faberi* ranged from 2.55-3.1 mm. Fairbrothers reported 1.8-2.2 mm and 2.5-2.9 mm, respectively, indicating a clear gap. This can be attributed to the larger geographic sampling in this study: the ten *S. viridis* specimens with spikelet lengths falling within the range of *S. faberi*, and indeed all *S. viridis* specimens sampled with spikelets larger than 2.3 mm, were from Manitoba and the extreme north Midwestern United States. For these northern *S. viridis* specimens, large spikelets were the rule and not the exception, although in other respects the plants conformed to *S. viridis*. We also observed three specimens of *S. viridis* from the southern end of our sampling that overlapped with *S. faberi* in terms of the upper glume-lower lemma ratio.

Fairbrothers (1959) noted that vegetative characters, with the exception of laminal hairs, could not consistently separate *S. viridis* and *S. faberi.* The PCAs conducted in this study were likewise unable to separate the species based on solely vegetative datasets, despite the inclusion of many more such characters. In fact, the dataset of vegetative characters, excluding pubescence characters, showed near complete overlap between the two species.

The results of this study differed markedly from those of Fairbrothers (1959) with respect to inflorescence characters. In his study, inflorescence width ranged from 3-7 cm in *S. viridis* and 5-9 cm in *S. faberi.* In our study, 4-15 cm was the range for the former and 6-11 cm for the latter. Specimens of both

species with long primary inflorescence branches resembling those of *S. italica* were responsible for the upper range of both species. Similarly, Fairbrothers reported inflorescence length at 20-95 cm for *S. viridis* and 89-142 cm for *S. faberi*, whereas in this study those ranges were 28-178 cm and 53-180 cm, respectively. While PCAs using only inflorescence characters did provide some degree of separation between species in this study, there was significant overlap between the two. Bristle length was the primary character providing some separation in these PCAs, a character Fairbrothers (1959) also noted to be overlapping but significantly different in its distribution between the two species.

One character that was initially thought to be promising in separating *S. faberi* and *S. viridis* was the length of the minute marginal prickle hairs along the laminae. Because these hairs are composed of a single cell, it was initially thought that those of diploid *S. viridis* would be about half the size of those of tetraploid *S. faberi.* While the vast majority of *S. viridis* specimens had prickles less than 0.15 mm in length and most of those of *S. faberi* were greater than 0.2 mm, one *S. viridis* accession (*Estep ME013*) had prickles 0.21 mm long, one *S. faberi* accession (*Layton 153)* was 0.12 mm long, and nine specimens including both species had prickles that fell between 0.15 mm and 0.2 mm. The *S. viridis* accessions with the largest prickle hairs tended to be large plants with long primary inflorescences, while the *S. faberi* plants with short prickles tended to be small or immature. Flow cytometry of both *Estep ME013* and *Layton 153* showed that their unusual prickle lengths were not due to ploidy differences.

In *S. viridis,* extensive tillering is the norm, especially with greenhouse-grown plants. *S. faberi* also tillers in most cases, albeit to a lesser degree. Certain accessions of both species occasionally show little or no tillering, however, indicating a possible relationship with *S. italica,* which was selectively bred not to tiller. It remains unclear if non-tillering genes were donated from *S. italica* to these accessions via gene flow, or if *S. italica* was simply selected from non-tillering varieties of *S. viridis.*

The study also serves as a quantitative survey of the variation in *S. viridis*. The species is in the early stages of becoming a model organism, primarily for the study of C_4 photosynthesis (Brutnell et al., 2010; Li and Brutnell, 2011). The genome of *S. italica*, a domesticate of *S. viridis*, has been recently published (Bennetzen et al., 2012), providing genetic resources for molecular studies. The highly detailed morphological analysis of the 55 *S. viridis* individuals presented here makes it quite plain that variation in this species is exceptionally broad. Several traits in particular, such as primary inflorescence branch length and peduncle length (ranging from 52-465 cm), show high amounts of variability. This variation will hopefully provide much fodder for the researchers adopting *S. viridis* as a study system.

Phylogenetics and Ploidy Estimates

The phylogenetic portion of this study was intended to place the two genomes of the tetraploid *S. faberi* on a nuclear gene tree. *S. faberi* clearly carries two paralogs for the *kn1* gene, each of which bears a unique history. One

paralog is essentially identical to *kn1* from *S. viridis,* indicating that that species was a likely parent. The other paralog differs from *S. viridis* at only about 10 bases. However, phylogenetic analyses failed to recover well-supported clades containing both paralogs as immediate sisters, which would have provided strong evidence for autopolyploidy. On the other hand, these paralogs do not group with any other species in the existing sample from Doust et al. (2007), which would have implicated allotetraploidy. Both forms of polyploidy, or some intermediate form, remain possible from a phylogenetic standpoint.

Benabdelmouna et al. (2001) argued for the allopolyploid origin of *S. viridis* based on genomic in-situ hybridization (GISH). Probes from *S. viridis* successfully hybridized with the "A genome" of *S. faberi,* while sequences from a diploid plant the authors called *S. adhaerens* hybridized with the "B genome". Taxonomically, this name is usually considered a synonym of *S. verticillata* (e.g. Veldkamp, 1994), which is known from multiple ploidy levels and has a complex and probably polyphyletic evolutionary history (Kellogg et al., 2009). Wang et al. (2009) suggest that diploid *S. verticillata* should be called *S. adhaerens,* but provide little direct evidence for this assertion. Both Doust et al. (2007) and Kellogg et al. (2009) found that specimens identified as *S. verticillata* fell in two clades, but it remains unclear if this reflects polyploidy or simply polyphyly of the "species". Although the GISH study suggests a connection between *S. faberi* and *S. adhaerens*/*S. verticillata,* the *kn1* tree presented here fails to group the two. It is possible that incomplete lineage sorting could be responsible for this result because only one gene is being used to reconstruct the nuclear phylogeny.

Regardless, because one accession of *S. verticillata* clusters with the *S. viridis* group in both the *kn1* and *ndhF* phylogenies, it seems that further investigation into the *S. verticillata* complex may help to better clarify the evolutionary history of the *S. viridis* group.

Flow cytometry in this study was intended to evaluate the consistency of ploidy in plants identified morphologically as *S. viridis* or *S. faberi*. A hypothesis at the outset of the study was that *S. viridis* may include polyploids, in which case it would potentially be interfertile with polyploid *S. faberi.* Our data do not support such a hypothesis. Consistency between morphology and ploidy level was essentially 100%. The two exceptions discussed above were repeated and found to be consistent the second time around. The genome sizes in *S. faberi* were slightly more than double those of *S. viridis.* This could perhaps indicate that the non-*S. viridis* parent of *S, faberi* had a larger genome. Alternatively, this could be interpreted as an expansion in genome size following polyploidy due to a release of transposable elements. Without further information, however, both of these hypotheses remain speculative. The fifteen *S. pumila* samples fell into two distinct ploidy groups, although without observing chromosomes these ploidy groups cannot be confidently identified from genome size estimates. At any rate, this finding is unsurprising given that the species has been reported in forms from diploid to octoploid and everything in between (Rominger, 2003).

Drought Experiment

After a sizeable number of specimens had been measured for the morphological portion of the study, it became apparent that many of the *S. viridis*-like *S. faberi* specimens were in fact simply immature or had been mowed or cut. This led to the hypothesis that given sufficient environmental stress, *S. faberi* would intergrade with *S. viridis* morphologically. Given water's fundamental importance to growth and development, a drought experiment was devised in order to test effect of this particular ecological factor on morphology. The data did not support this hypothesis. Instead, the MANOVAs performed on *S. viridis* and *S. faberi* as a group showed convincingly that their morphologies were significantly different from one another when both treatment groups were considered together, indicating that no such intergradation occurs in mature plants under controlled conditions.

The drought experiment suggested that *S. viridis* and *S. pumila* are more drought tolerant than *S. faberi.* The mortality rate of *S. faberi* was more than three times greater than either *S. viridis* or *S. pumila.* A potential caveat to these finding is that the *S. viridis* accessions used in this study were smaller than either of the other species. They therefore presumably had lower rates of transpiration and were thus stressed less than their larger relatives by the drought treatment. Nonetheless, the *S. pumila* accessions were by far the largest of all, yet these exhibited rates of mortality similar to *S. viridis.* Drought tolerance also varied within *S. viridis*. The accession *Yang 8054* was the tallest *S. viridis* accession included, yet t-tests of mean measurements showed no statistically significant change in morphology based on treatment group (data not shown). Despite this,

much smaller accessions such as *Estep ME051V* were significantly affected in terms of inflorescence number. Taken together, this indicates that the drought experiment was successful in demonstrating the effect of drought on morphology.

Later onset of flowering was observed in *S. pumila,* but not in the other two species. However, the study was designed such that data was gathered only at the end of the experiment. As such, it may be possible that drought stress slows flowering in the other species as well, but it was not captured by this experimental design.

This study shows that *S. viridis* and *S. faberi* are indeed morphologically and cytologically distinct entities. Phylogenetic analyses of the nuclear gene *knotted1* and the chloroplast gene *ndhF* are also consistent with such a separation, although further sampling could provide stronger evidence in this regard. While observations in the field initially suggested continuous morphological variation between the two species, it was found that this was often due to immature *S. faberi* specimens being compared with fully mature plants of *S. viridis*, as well as due to a general over reliance on vegetative characters and size.

The study found a wide range of morphological variation within *S. viridis*, especially in plant size, inflorescence branch length, and spikelet size. The data suggest this variation may be associated with latitude as well as population genetics (Katrien Devos pers. comm.), but more work is needed in this regard.

Several characters can consistently separate the species. *S. viridis* always has glabrous leaves, while *S. faberi* has pubescent adaxial laminae (with

glabrous leaves being reported as very rare in the literature, but not encountered in this study). The relatively large gap between the apices of the upper glume and lower lemma compared to the very small or non-existent gap of *S. viridis* also serves to separate the two consistently. The drought experiment suggests a potential ecological distinction with *S. faberi* apparently being much more sensitive to low water situations.

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TABLES & FIGURES

Table 1: Characters removed from the morphological analysis after examining 35 specimens and the reason they were removed. For further details, see Appendix I.

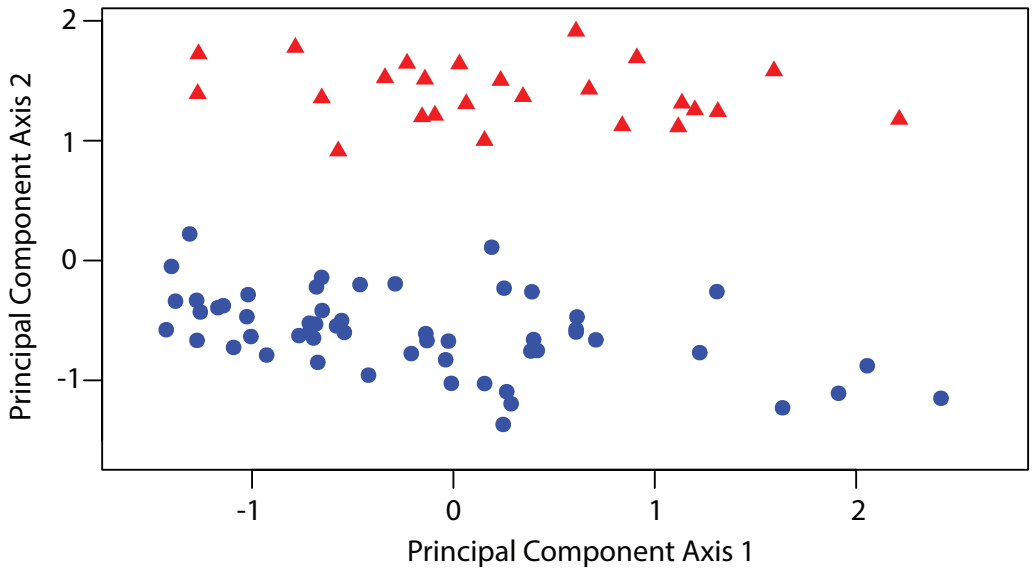
Character removed	Reason Removed
Low node rooting	Invariant
Node compression	Invariant
Internode Prickles	Invariant
Sheath Margin Pubescence	Invariant
Ligule Color	Invariant
Scabrosity in top 25% of adaxial lamina	Invariant
Number of white veins in adaxial midrib	Subjective
Peduncle scabrosity	Invariant; Subjective
Rachis hair type	Invariant; Subjective
Bristles per spikelet	Subjective
Lower glume vein number	Invariant
Upper glume width	r^2 0.86 lower glume length; Subjective
Lower lemma width	r^2 0.92 spikelet width; Subjective
Lower palea length	r^2 0.89 spikelet length; Difficult
Lower palea width	r^2 0.90 lower palea length; Subjective; Difficult
Upper anthecium length	r^2 0.96 spikelet length
Upper anthecium width	r^2 0.91 spikelet width; Subjective
Lodicule length	Subjective; Difficult
Style length	Subjective; Difficult
Caryopsis length	r^2 0.89 spikelet length; Difficult
Caryopsis width	r^2 0.86 spikelet width; Difficult
Hilum visibility	Subjective; Difficult
Hilum length	r^2 0.81 spikelet length; Subjective; Difficult
Hilum width	r^2 0.79 caryopsis width; Subjective; Difficult
Embryo length	r^2 0.86 spikelet length; Subjective; Difficult

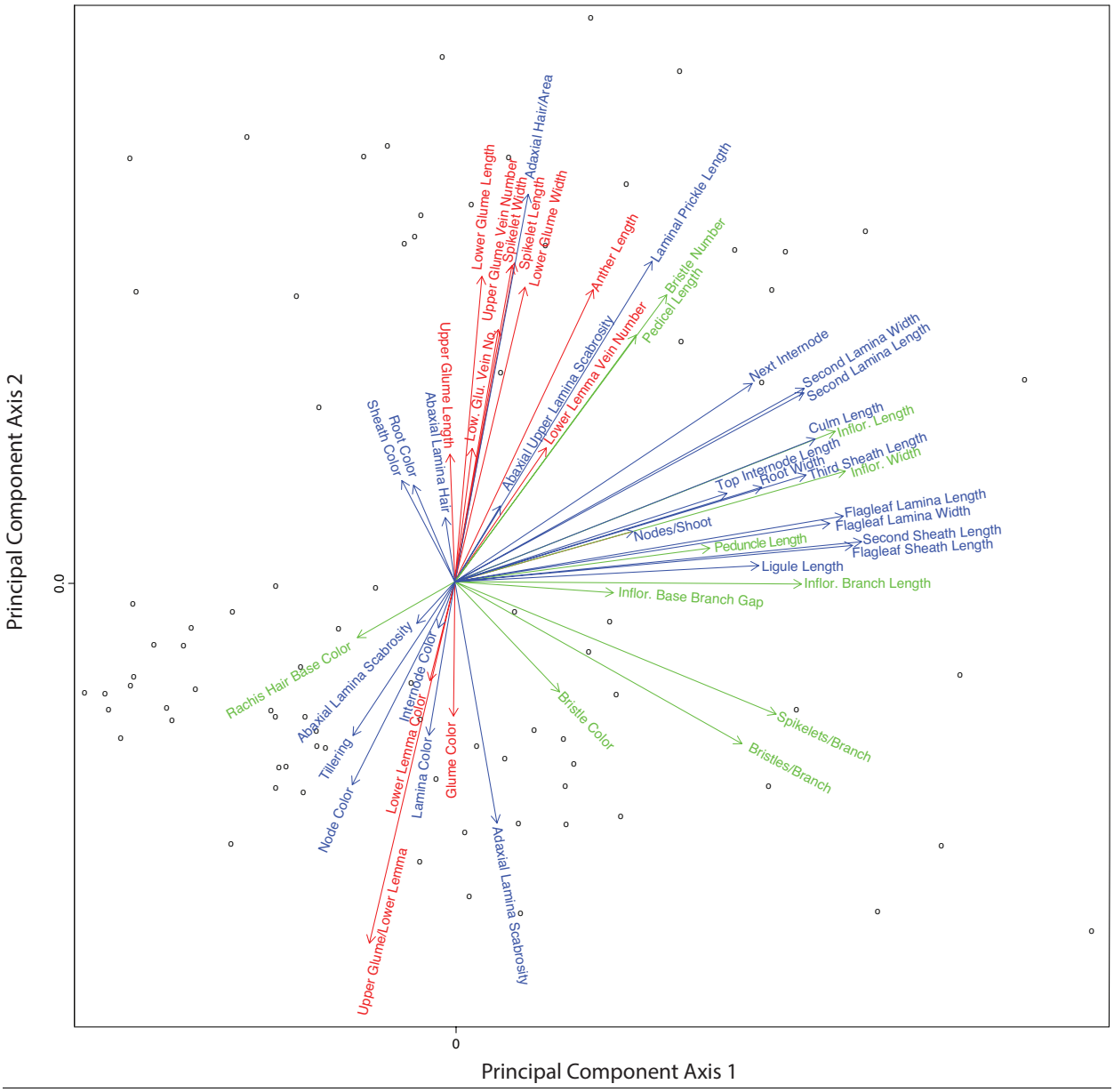
Table 2: Results of ANOVAs using water treatment as the explanatory variable. Each row is a separate ANOVA on a single morphological character of a single species. Bold P values indicate a statistically significant difference.

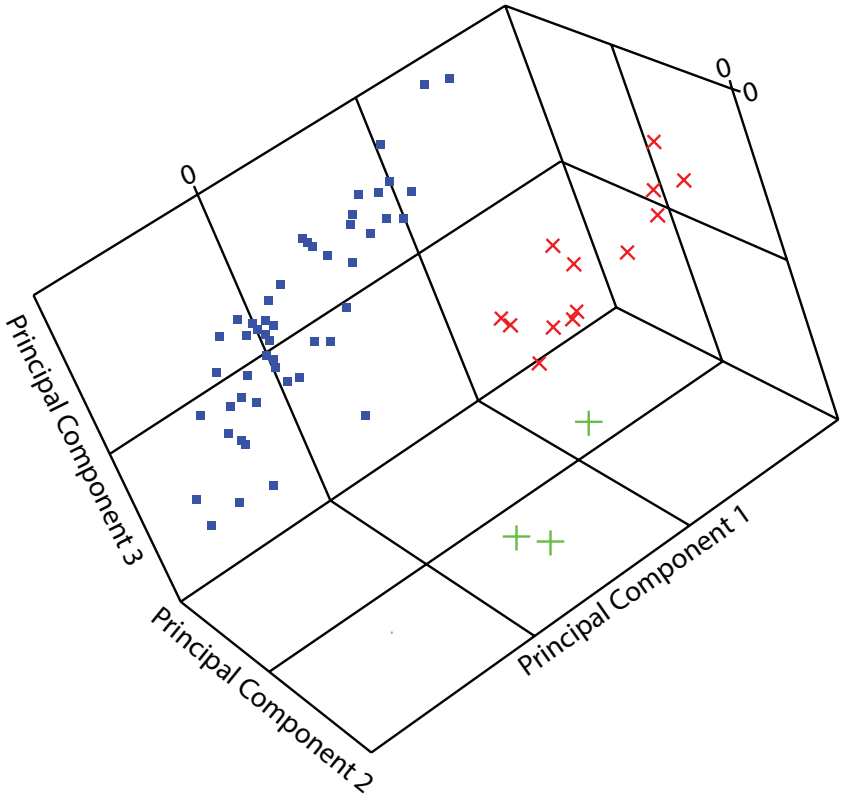
Species	Character	Deg. Free.	Sum Sq	Mean Sq	F	P	Res. Deg. Free.	Res. Sum Sq.	Res. Mean Sq.
S. viridis	Height	1	137	136.9	0.774	0.383	53	9371	176.8
	No. of Tillers	1	298	298.5	4.193	**0.0456**	53	3773	71.9
	No. of Inflorescences	1	721	721.4	4.784	**0.0332**	53	7992	150.8
	Inflorescence Length	1	0.281	0.2805	0.473	0.495	53	31.429	0.593
	Flag Leaf Length	1	0.18	0.182	0.059	0.809	53	163.35	3.082
S. faberi	Height	1	1361	1361.4	22.08	**0.000122**	21	1295	61.6
	No. of Tillers	1	76.52	76.52	11.21	**0.00304**	21	143.3	6.82
	No. of Inflorescences	1	190.8	190.8	6.67	**0.0174**	21	600.6	28.6
	Inflorescence Length	1	0.258	0.2582	0.405	0.531	21	13.394	0.6378
	Flag Leaf Length	1	5.48	5.481	0.94	0.343	21	122.43	5.83
S. pumila	Height	1	13631	13631	26.1	**4.64E-05**	21	10967	522
	No. of Tillers	1	1.1568	1.568	1.082	0.31	21	30.432	1.449
	No. of Inflorescences	1	42	41.98	2.498	0.129	21	353	16.81
	Inflorescence Length	1	177.5	177.46	17.59	**0.000408**	21	211.8	10.09
	Flag Leaf Length	1	844.3	844.3	8.805	**0.00735**	21	2013.6	95.9

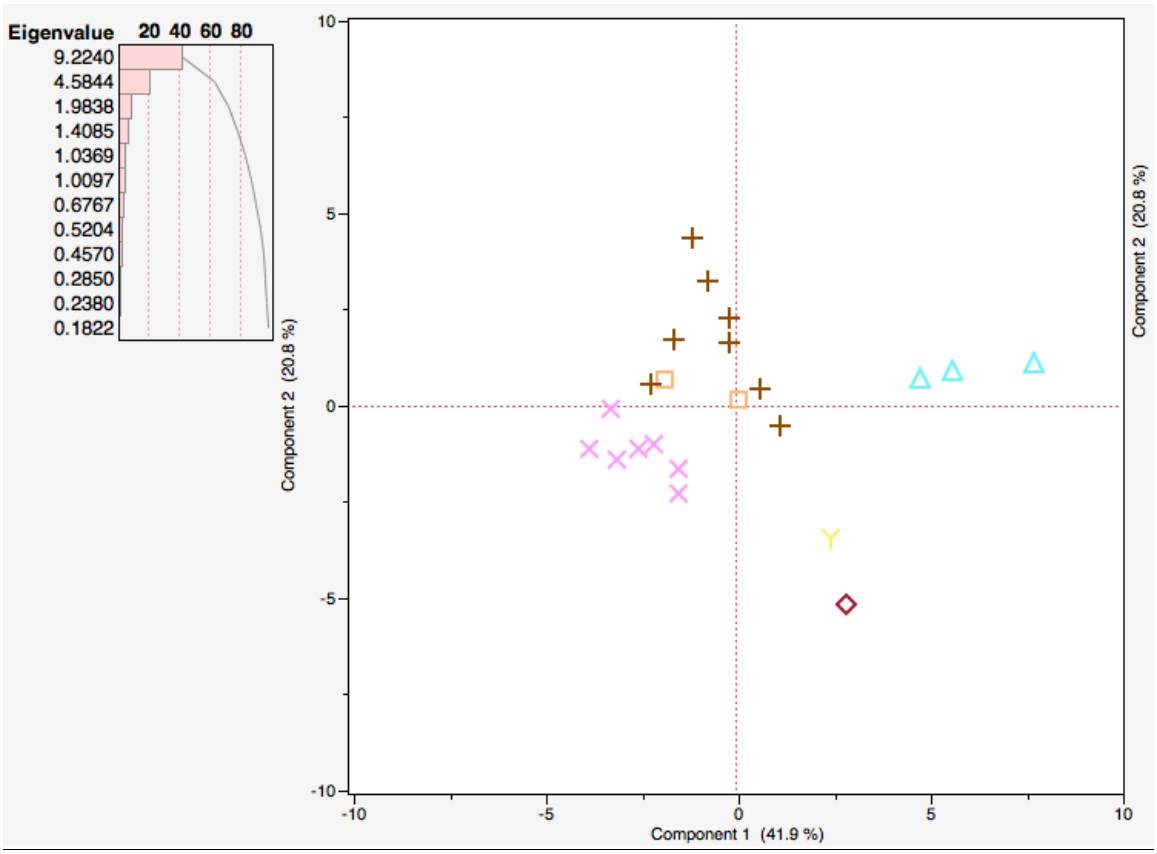
Table 3: Results of MANOVAs performed on each species separately, all possible pairs of species, and all pairs together. In cases where two or more species were used, both treatment and species were treated as independent variables.

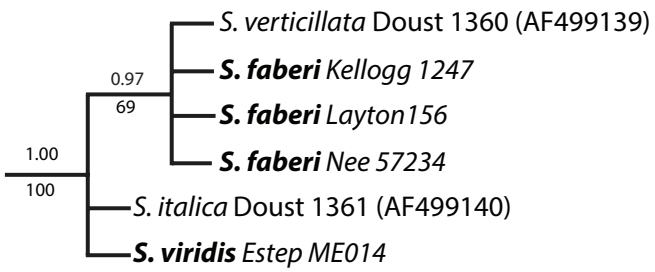
Species	Independent Variable	D.F.	Pillai	F	Num. D.F.	Den. D.F.	P	Res. D.F.
S. faberi	treatment	1	0.68664	7.4503	5	17	0.0007274	21
S. viridis	treatment	1	0.090319	0.97301	5	49	0.4436	53
S. pumila	treatment	1	0.82202	15.704	5	17	7.49E-06	21
S. viridis - S. faberi	treatment	1	0.1573	2.651	5	71	0.02968	75
	species	1	0.86784	93.242	5	71	2.00E-16	
S. viridis - S. pumila	treatment	1	0.22746	4.181	5	71	0.002187	75
	species	1	0.66649	28.378	5	71	1.09E-15	
S. faberi - S pumila	treatment	1	0.66146	15.24	5	39	2.67E-08	43
	species	1	0.45569	6.53	5	39	0.0001683	
S faberi - S. pumila - S. viridis	treatment	1	0.26316	6.6429	5	93	2.49E-05	97
	species	2	0.8027	12.6041	10	188	2.20E-16	

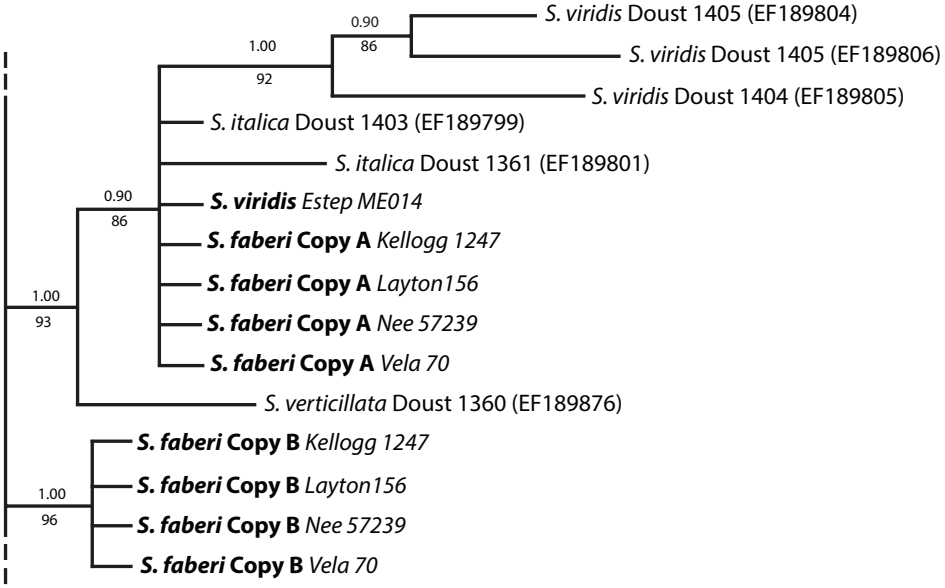

2
1
0
-1
Principal Component Axis 2
-1
0
1
2
Principal Component Axis 1

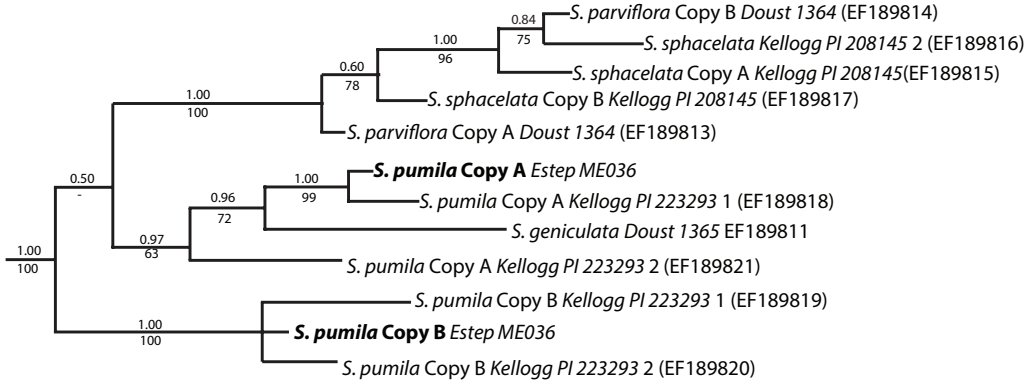

Principal Component Axis 2
0.0
0
Principal Component Axis 1
Lower Glume Length
Upper Glume Vein Number
Spikelet Width
Adaxial Hair/Area
Spikelet Length
Lower Glume Width
Anther Length
Laminal Prickle Length
Bristle Number
Pedicel Length
Lower Lemma Vein Number
Abaxial Upper Lamina Scabrosity
Upper Glume Length
Low. Glu. Vein No.
Abaxial Lamina Hair
Root Color
Sheath Color
Next Internode
Second Lamina Width
Second Lamina Length
Culm Length
Inflor. Length
Top Internode Length
Root Width
Third Sheath Length
Inflor. Width
Nodes/Shoot
Flagleaf Lamina Length
Flagleaf Lamina Width
Second Sheath Length
Flagleaf Sheath Length
Peduncle Length
Ligule Length
Inflor. Branch Length
Inflor. Base Branch Gap
Rachis Hair Base Color
Abaxial Lamina Scabrosity
Internode Color
Lower Lemma Color
Tillering
Lamina Color
Node Color
Glume Color
Bristle Color
Spikelets/Branch
Bristles/Branch
Adaxial Lamina Scabrosity
Upper Glume/Lower Lemma

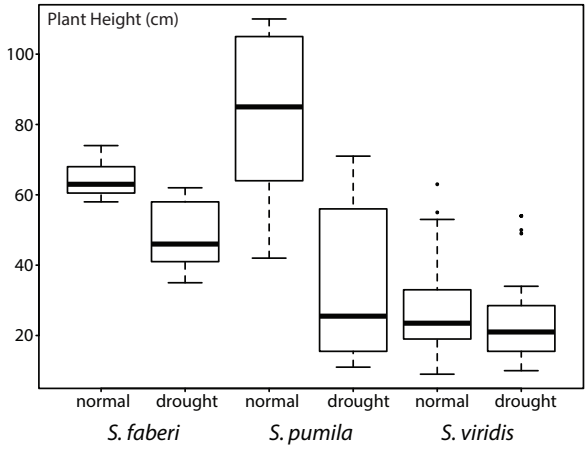

0
0
0
Principal Component 3
Principal Component 2
Principal Component 1

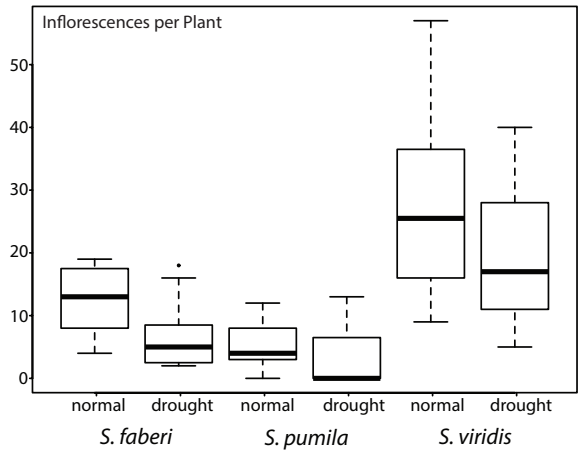

Eigenvalue
20 40 60 80
9.2240
4.5844
1.9838
1.4085
1.0369
1.0097
0.6767
0.5204
0.4570
0.2850
0.2380
0.1822
10
5
0
-5
-10
-10
-5
0
5
10
Component 2 (20.8 %)
Component 1 (41.9 %)
Component 2 (20.8 %)

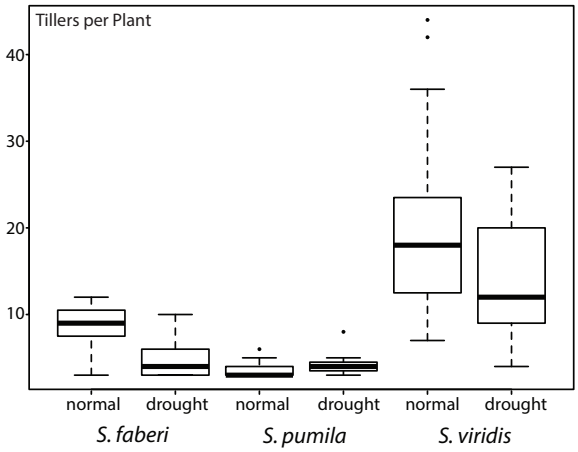

S. verticillata Doust 1360 (AF499139)
S. faberi Kellogg 1247
S. faberi Layton156
S. faberi Nee 57234
S. italica Doust 1361 (AF499140)
S. viridis Estep ME014
0.97
69
1.00
100

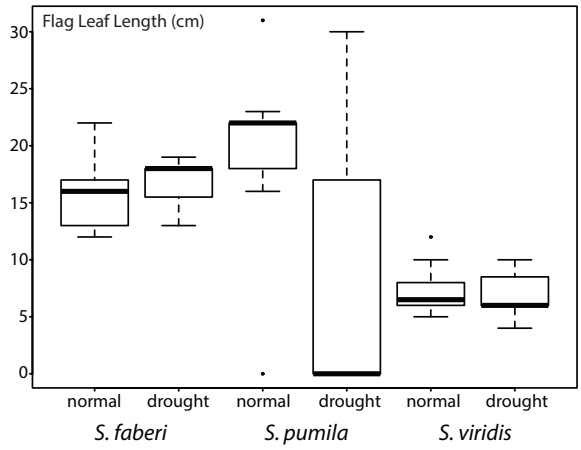

0.90
86
1.00
92
S. viridis Doust 1405 (EF189804)
S. viridis Doust 1405 (EF189806)
S. viridis Doust 1404 (EF189805)
S. italica Doust 1403 (EF189799)
S. italica Doust 1361 (EF189801)
0.90
86
S. viridis Estep ME014
S. faberi Copy A Kellogg 1247
1.00
93
S. faberi Copy A Layton156
S. faberi Copy A Nee 57239
S. faberi Copy A Vela 70
S. verticillata Doust 1360 (EF189876)
S. faberi Copy B Kellogg 1247
1.00
96
S. faberi Copy B Layton156
S. faberi Copy B Nee 57239
S. faberi Copy B Vela 70

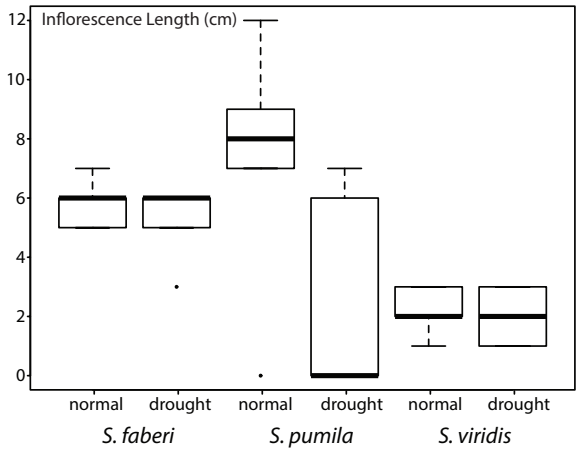

0.84
75
S. parviflora Copy B Doust 1364 (EF189814)
1.00
96
S. sphacelata Kellogg PI 208145 2 (EF189816)
0.60
78
S. sphacelata Copy A Kellogg PI 208145(EF189815)
1.00
100
S. sphacelata Copy B Kellogg PI 208145 (EF189817)
S. parviflora Copy A Doust 1364 (EF189813)
0.50
-
1.00
99
S. pumila Copy A Estep ME036
0.96
72
S. pumila Copy A Kellogg PI 223293 1 (EF189818)
S. geniculata Doust 1365 EF189811
1.00
100
0.97
63
S. pumila Copy A Kellogg PI 223293 2 (EF189821)
S. pumila Copy B Kellogg PI 223293 1 (EF189819)
1.00
100
S. pumila Copy B Estep ME036
S. pumila Copy B Kellogg PI 223293 2 (EF189820)


Plant Height (cm)
100
80
60
40
20
normal
drought
normal
drought
normal
drought
S. faberi
S. pumila
S. viridis


Inflorescences per Plant
50
40
30
20
10
0
normal
drought
normal
drought
normal
drought
S. faberi
S. pumila
S. viridis


Tillers per Plant
40
30
20
10
normal
drought
normal
drought
normal
drought
S. faberi
S. pumila
S. viridis


Flag Leaf Length (cm)
30
25
20
15
10
5
0
normal
drought
normal
drought
normal
drought
S. faberi
S. pumila
S. viridis


Inflorescence Length (cm)
12
10
8
6
4
2
0
normal
drought
normal
drought
normal
drought
S. faberi
S. pumila
S. viridis

CAPTIONS

Figure 1: Specimen scores from a PCA of the full morphological data set using averaged values when more than one measurement was made on a single character. Blue diamonds are *Setaria viridis;* red circles are *Setaria faberi*. *Setaria pumila* was not included in this analysis.

Figure 2: Character loading plot showing the contribution of morphological characters to the first two principal component axes.

Figure 3: PCA including *S. viridis* (squares), *S. faberi* (circles), and *S. pumila* (X's) using the full morphological dataset.

Figure 4: Specimen scores from a PCA of accessions included in a preliminary microsatellite analysis. Triangles = *S. faberi;* Crosses = *S. viridis* from Canada and northern U.S.A.; Squares = *S. viridis* from Canada and northern U.S.A.; 'X's = *S. viridis* from the Near East, and central and southern latitudes of the U.S.A.; 'Y' = *S. viridis* from China, central latitudes of the U.S.A, and Germany; diamonds = *S. viridis* from China and central latitudes of the U.S.A with wide inflorescences.

Figure 5: Excerpt of majority rule Bayesian tree for the chloroplast gene *ndhF* showing the position of the *Setaria viridis* group plus one accession of *Setaria verticillata*. Bootstrap values from the ML analysis are shown below branches, with Bayesian posterior probability above. Bold text indicates accessions added in this study.

Figure 6: Excerpt of the maximum likelihood tree for the nuclear gene *kn1* show the monophly of the *Setaria viridis* group as well as the unplaced "B copies" of *Setaria faberi.* Bootstrap values from the ML analysis are shown below branches, with Bayesian posterior probability above. Bold text indicates accessions added in this study.

Figure 7: Excerpt of a majority rule Bayesian tree of the nuclear gene *kn1* showing the position of the two *Setaria pumila* paralogs. Bootstrap values from the Maximum Likelihood analysis are shown below branches, while the Bayesian posterior probabilities are above branches. Bold text indicates newly added sequences.

Figure 8: Box plots of showing the effect of normal water versus drought on measurements of five morphological characters in *S. viridis, S. faberi,* and *S. pumila.*

APPENDICES

Appendix I: Character Descriptions

Up to three measurements were taken per specimen for each morphological character depending on the quantity of material available. In cases where more than three replicates of a character were available on a single plant, the largest possible three measurements were taken. Characters listed in italics were not included in the final analyses.

1) Culm length (mm) – The single longest culm is measured from the root crown to the tip of the inflorescence.

2) Branching at base (1/0) – Branching may (state 1) or may not (state 0) occur at or near the base of the culm.

3) *Rooting at low nodes* (1/0) – Self-explanatory. After initial measurements, this character was no longer recorded because roots were always present on the lowest nodes.

4) Root width (mm) – The widest root is measured near where it emerges from the base.

5) Root color (purple present/not present) – If any purple was detected on the roots, this character was scored as “purple present”.

6) Internode length below flag leaf (mm) – The uppermost internode, below the flag leaf, was measured from the center of the node at each end.

7) Internode length second below flag leaf (mm) – The next-to-uppermost internode (that second below the flag leaf) was measured from the center of the node at each end.

8) Internode color (purple present/purple absent) – If any purple was detected, this character was scored as “purple present”. During the measuring process, “purple present” was further broken into “purple present only on basal internodes” and “purple present on all internodes”, but these were not used due to complications with more than two character states.

9) *Internode prickles* (1/0) – This character was mentioned in a description of the genus *Setaria* species was thus initially included. However, the character was never encountered and was removed after initial measurements.

10) Nodes per shoot (meristic) – The longest shoot(s) were used. The base of the plant itself was considered a node.

11) Node color (purple present/purple absent) - Self-explanatory. If any purple nodes were detected, the plant was scored as “purple present”. During the

measuring process, “purple present” was further broken into “purple present only on basal nodes” and “purple present on all nodes”, but these were not used due to complications with more than two character states.

12) *Node compression* (1/0)– This character was mentioned in a description of the genus *Setaria* species and was initially included. Nodes were always compressed and the character was thus removed.

13) Sheath length of flag leaf (mm) – Length of the flag leaf’s sheath from the collar to the top of the node.

14) Sheath length of second highest leaf on culm (mm) - Length of the second highest (second down from the inflorescence) leaf’s sheath from the collar to the top of the node.

15) Sheath length of third highest leaf on culm (mm) - Length of the third highest (third down from the inflorescence) leaf’s sheath from the collar to the top of the node.

16) Sheath color (purple present/purple absent) – If any purple sheaths were detected, the plant was scored as “purple present”. During the measuring process, “purple present” was further broken into “purple present only on basal

sheaths" and "purple present on all sheaths", but these were not used due to complications with more than two character states.

17) *Hair coverage on sheath margin* (percentage) – Long acicular hairs are found along the margin of the leaf sheath. The character was invariant and also difficult to measure without cracking the sheath because the hairs are often tucked inside it. Older specimens tended to have sparse hairs, possibly due to mechanical removal with age.

18) Ligule length on flag leaf (mm)– The length of the ligule, from the point of attachment at the lamina-sheath junction to the tallest hair, was always measured on the flag leaf.

19) *Ligule color* – All ligules were translucent and the character was thus removed.

20) Lamina length of flag leaf (mm) – Length of the flag leaf's lamina from the tip to the base. If a portion of the leaf was torn off, a leaf from another culm was substituted when possible. If this was not possible, an estimate was made if only a relatively small portion was absent. If a substantial portion of the leaf was missing, the measurement was left as missing data.

21) Lamina length of leaf below flag leaf (mm) – Length of the lamina of the leaf below the flag leaf from the tip to the base. If a portion of the leaf was torn off, a leaf from another culm was substituted when possible. If this was not possible, an estimate was made if only a relatively small portion was missing. If a substantial portion of the leaf was missing, the measurement was left as missing data.

22) Lamina width of flag leaf (mm) – Width of the flag leaf's lamina at the widest point. Because leaves often crumple significantly after drying, the nearest whole number was used to make up for uncertainty.

23) Lamina width of leaf below flag leaf (mm) – Width of the leaf below the flag leaf's lamina at the widest point. Because leaves often crumple significantly after drying, the nearest whole number was used to make up for uncertainty

24) Lamina color (purple present/purple absent) - If any purple laminae were detected, the plant was scored as "purple present".

25) Lamina pubescence adaxially (1/0) – Self-explanatory. In *Setaria pumila*, hairs are only found at the base of the lamina, but they are scored simply as 'present' for this character

26) Lamina pubescence abaxially (1/0) - Self-explanatory.

27) Lamina adaxial pubescence density (number of hairs per 5 sq. mm) – This measurement was taken by aligning the reticule bar with the midrib at roughly the center of the lamina, maintaining a 2.5 mm section of the bar fixed in one spot, and counting the hairs in a box formed by the scale bar and 2 mm from the midrib.

28*) Scabrosity in upper 25% of adaxial lamina* (1/0) – All plants had this character and it was thus removed.

29) Scabrosity in lower 25% of adaxial lamina (1/0) –Prickles were scored as absent when 200x magnification did not reveal any prickles on brief inspection. Some specimens were especially scabrous, but this was not reflected in the character scoring because of the subjectivity involved.

30) Scabrosity in upper 25% of abaxial lamina (1/0) - Prickles were scored as absent when 200x magnification did not reveal any prickles on brief inspection.

31) Scabrosity in lower 25% of abaxial lamina (1/0) – Prickles were scored as absent was scored when 200x magnification did not reveal any prickles on brief inspection.

32) Marginal prickle length on lamina of flag leaf (mm) – 500x magnification was used to measure the marginal prickles in the basal half of the same three laminae used for the lamina length and width measurements. The longest prickle from each leaf was found and recorded. Prickles were measured from base to tip parallel to the margin.

33) *White adaxial midrib vein number* (whole number) – The number of white veins on the adaxial lamina midrib was counted. It was difficult to consistently score this character because minor and major veins often appeared to be overlapping, or the whiteness faded gradually towards the margins, making it difficult to draw exact boundaries. This character was removed for these reasons.

34) Peduncle length (mm) – The longest and second longest peduncles were measured. If a peduncle was clearly not fully expanded as evidenced by a poorly developed inflorescence, it was not measured.

35) *Peduncle scabrosity* (percentage of coverage) – This character was measured imprecisely and was largely invariant and was thus removed.

36) Inflorescence length (mm) – The longest and second longest inflorescences were measured. If an inflorescence was clearly not fully expanded, it was not measured.

37) Inflorescence width (mm) – The widest and second widest inflorescences were measured. If an inflorescence was clearly not fully expanded, it was not measured. The measurement ignored bristles and was rounded to the nearest whole number because of the flexibility of the primary branches.

38) Gap between lowest primary branch and second lowest primary branch (mm) – Some specimens showed a large gap between the lowest primary branch and the second lowest primary branch. Where the gap was very small, measurement became difficult.

39) *Rachis hair* (short/long/stiff) – Hairs were categorized into three categories based on visual inspection. After observing 35 specimens, the character was invariant in *S. viridis* and *S. faberi*, at least without measuring individual hairs, and it was thus removed.

40) Rachis hair base color (white/purple) – The bases of the pilose hairs on the inflorescence rachis were usually a translucent white, but were occasionally purple. Any plant showing a purple hair base was scored as “purple” for this character.

41) Primary inflorescence branch length (mm) – One primary branch was removed with forceps from the lower half of each of the same three inflorescences and measured for length and width. When less than three

inflorescences were available, more than one branch was taken from a single inflorescence. These were placed under a dissecting scope at 200x and measured from the base of the branch to the base of the uppermost pedicel.

42) Spikelets per branch – Because spikelets had often fallen off, pedicels were used as a proxy for spikelets. Sometimes spikelets are not fully developed on mature inflorescences, but were nonetheless included in this count. On some inflorescences with more than 15-20 spikelets per branch, counts were difficult to make with complete accuracy. 200x magnification was used to make the counts

43) Pedicel length (mm) – Three pedicels, the longest from each primary branch removed, were measured at 200x magnification. The pedicel was measured from its point of attachment with a branch to the top of the cupule subtending the spikelet.

44) Bristles per branch – The number of bristles on each of the three primary branches removed was counted.

45) *Bristles per spikelet* – This character was included because it is often given in descriptions of *Setaria*. However, because bristles are sterile branches that do not subtend spikelets per se, it is impossible to say which bristles belong to which spikelets and which do not. Furthermore, many bristles “subtend” the fertile branches themselves and not any particular spikelet, so simply dividing the

number of bristles per primary branch by the number of spikelets per primary branch does not yield bristles per spikelet. The character was removed because of its subjectivity.

46) Bristle length (mm) – The longest bristle from each of three primary branches removed was measured at 100x magnification.

47) Bristle color (purple/cream/tawny) – Self-explanatory.

48) Spikelet disarticulation (much disarticulation, disarticulation, no disarticulation) – This character was intended to estimate plant maturity and was not used directly as a character in PCAs. Instead, some PCAs were performed that excluded immature specimens on the basis of this character. Plants with no disarticulation almost always have undeveloped spikelets and thus may be unreliable for many measurements. The categories are subjective, but relatively easy to assess. “Much disarticulation” was chosen when the newspaper containing the specimen was littered with spikelets or when the inflorescences had few spikelets still attached. “No disarticulation” was chosen when no spikelets had detached, all were green, and none were on the newspaper. For anything intermediate, “disarticulation” was chosen.

Characters 49-77. For all spikelet measurements, three of the most well developed spikelets were selected from each specimen. This was determined

based on size, color (darker colors or dark mottling were taken as indicators of maturity), dryness of leafy floral parts (with less green taken as more mature), and hardness (soft spikelets were taken to be immature). All spikelets were soaked for at least 20 minutes in tepid water to increase pliability. 200x magnification was used in all cases.

49) Spikelet length (mm) – This character was measured from the lowest point of the spikelet, not including any remnants of the pedicel, to the apex of the spikelet.

50) Spikelet width (mm) – Width was measured at the widest point. Occasionally the glumes had come loose and hung over a single side, distorting the width. In these cases, the hanging glume was ignored in the measurement and the next intact bract was used as the starting point for the measurement.

51) Lower glume length (mm) – Length was measured from the lowest to highest point. Occasionally the lower glume had folded such that the bottom of the glume hung below the spikelet several millimeters. In these cases, forceps were used to hold the glume flush to the spikelet while a measurement was made.

52) Lower glume width at center (mm) – The width measurement was taken at roughly the lengthwise midpoint of the lower glume. Because the lower glume is

curved, reflecting the shape of the spikelet, this measurement was somewhat difficult to make consistently.

53) *Lower glume vein number* (whole number) – Lower glume vein number was essentially fixed at three, although four and five were observed very occasionally. This character was removed.

54) Upper glume length (mm) – This character was measured from the lowest visible point of the upper glume to its apex. In cases where the upper glume extended beyond the upper anthecium, this measurement was always equal to spikelet length.

55) *Upper glume width* (mm) – This character was measured at the widest point of the upper glume. Upper glume width was essentially identical to spikelet width as measured. The upper glume curves around the spikelet so its true width is probably not identical to spikelet width. However, in order to accurately measure this “true width”, the glume would need to be cut longitudinally and flattened in order to reduce its three dimensionality, which was not practical for this study. As such this character was removed.

56) Upper glume vein number – This ranged between five and nine, with five and seven being most common. When more than five veins were observed, the additional veins tended to extend only about halfway to the base of the glume,

although seven veins all running the full length of the spikelet were rarely observed. Nine veins were only observed once, and eight veins were very rare as well.

57) Glume color (not purple/purple) – This character was scored as purple when any amount of purple was observed on the glumes. Purple coloration always occurred in patches and never covered the entire surface of the glume.

58) Lower lemma length (mm) – This character was measured after the glumes had been removed so that the base of the lemma was visible.

59) *Lower lemma width* (mm) – This character was measured at the widest point of the lower lemma. It was removed for the same reasons as upper glume width discussed above.

60) Lower lemma vein number – The number of veins on the lower lemma ranged from five to seven. Eight and nine were never observed as on the upper glume, despite the fact that the lower lemma was more likely to have seven veins than the upper glume.

61) Lower lemma color (green/purple) – This character was evaluated in the same way as glume color above.

62) *Lower palea length* (mm) – The lower palea is small and hyaline, and was difficult to observe without damaging it. Sometimes it detached along with the lower lemma, while other times it remained attached to the upper anthecium after the lower lemma had been removed. In both cases its length was measured from the point of attachment at the base to its tip. It was nearly invisible when wet, but quickly shriveled when dry. Clefts were often observed at the lower palea apex, although the presence or absence of these was not consistent. Due to the high covariance with other characters as well as the difficulty in measuring this character, it was removed.

63) *Lower palea width* (mm) – This character was even harder to measure than lower palea length because of its tendency to roll in on itself. Slight rips would also make the measurement impossible. The character was removed due to covariance and difficulty of measurement.

64) Presence of lower flower (absent/present) – The lower flower was absent for all specimens examined except for two specimens of *S. faberi*. In one case *(Kellogg 1203)* only anthers were present, but on the other *(Kellogg 1230)* the gynoecium was present as well.

65) *Upper anthecium length* (mm)– This character was measured after all other floral bracts were removed. It was nearly the same measurement as spikelet length, and was removed because of covariance.

66) *Upper anthecium width* (mm) – This character was measured after all other floral bracts were removed. It was nearly the same measurement as spikelet width, and was removed because of covariance.

67) Upper anthecium rugosity (rugose/faintly rugose) – This character was difficult to break into two categories because of the range of variation. Despite ambiguity, it was retained because of its importance in previous accounts of diagnostic characters.

68) Upper anthecium mottling at maturity (mottled/not mottled) – This character was also difficult to break in to two categories because of the range of variation. Upper anthecia were considered mottled if at least several clear spots of black on a lighter background were present, and were considered not mottled if they were uniformly one color or otherwise clearly not spotted. This character was problematic on immature spikelets because mottling appears to occur only with maturity. In some cases mottling was so heavy as to make the spikelet appear of a solid color.

69) Lodicule length (mm) – Lodicules were difficult to observe after breaking open the upper anthecium. Their size also seemed to vary significantly with the development stage of the plant.

70) Anther length (mm) – One to three dry, dehisced anthers were measured from end to end. Dry anthers often curled up, so an effort was made to include only straight anthers when possible. Immature anthers were not measured.

71) *Style/stigma length* (mm) – This character was wildly variable depending on the developmental stage of the plant and was therefore removed.

72) *Caryopsis length* (mm) – Mature caryopses were dissected from the upper anthecium using fine forceps, which took a significant amount of time. Because of the relatively high covariance with spikelet length in addition to the above reason, the character was removed from the study.

73) *Caryopsis width* (mm) – This character was removed for the same reasons as the above.

74) *Hilum visibility* (visible/not visible) – Some specimens had a highly distinct, deep hilum, while others were only slightly discernable from the other parts of the surface. The character is possibly related to the level of maturity. It was removed along with other caryopsis characters due to the difficulty of dissection.

75) *Hilum length* (mm) – This character was measured from the base of the hilum to the top of the uppermost ridge of the hilum. It was removed along with other caryopsis characters due to the difficulty of dissection.

76) *Hilum width* (mm) – This character was measured at the widest point of the hilum. It was often hard to discern the exact edges of the hilum, especially for specimens with an already indistinct hilum. Compounding this difficulty was the fact that the caryopsis surface is curved. It was removed along with other caryopsis characters for these reasons and due to the difficulty of dissection.

77) *Embryo length* (mm) – This character was measured from the lowest visible point of the embryo to its apex. It was removed along with other caryopsis characters due to the difficulty of dissection and strong covariance.

Appendix II: Field Collection Data

All specimens are deposited at the herbarium of the Missouri Botanical Garden (MO). Specimens shown in bold were used to generate the phylogeny. Specimens underlined were used in the drought experiment.

Species	Collection Number	Collector	Collection Date	Latitude	Longitude	Elevation (m.)
viridis	*101*	*D.Vela & J.Baker*	19-Aug-11	40.391820°	-82.467210°	1177
viridis	*102*	*D.Vela & J.Baker*	19-Aug-11	40.813880°	-81.892670°	1066
faberi	*103*	*D.Vela & J.Baker*	19-Aug-11	40.813880°	-81.892640°	1071
faberi	*104*	*D.Vela & J.Baker*	19-Aug-11	41.020460°	-81.370510°	1152
faberi	*105*	*D.Vela & J.Baker*	19-Aug-11	41.023410°	-80.690250°	999
faberi	*106*	*D.Vela & J.Baker*	19-Aug-11	41.023420°	-80.690320°	1009
faberi	*107*	*D.Vela & J.Baker*	19-Aug-11	40.433800°	-80.780950°	1234
faberi	*108*	*D.Vela & J.Baker*	19-Aug-11	40.391900°	-81.388170°	847
faberi	*109*	*D.Vela & J.Baker*	20-Aug-11	39.767940°	-82.542920°	1050
pumila	*110*	*D.Vela & J.Baker*	20-Aug-11	40.015420°	-82.117740°	747
faberi	*111*	*D.Vela & J.Baker*	20-Aug-11	40.015420°	-82.117740°	747
faberi	*65*	*D.Vela & J.Baker*	2-Sep-11	40.602730°	-83.073100°	667
pumila	*66*	*D.Vela & J.Baker*	2-Sep-11	41.063197°	-83.661080°	757
viridis	*67*	*D.Vela & J.Baker*	2-Sep-11	41.109300°	-83.649490°	811
viridis	*68*	*D.Vela & J.Baker*	3-Sep-11	41.712930°	-83.685720°	593
viridis	*69*	*D.Vela & J.Baker*	3-Sep-11	41.712930°	-83.685720°	593
faberi	***70***	***D.Vela & J.Baker***	**3-Sep-11**	**41.712930°**	**-83.685720°**	**923**
pumila	*71*	*D.Vela & J.Baker*	3-Sep-11	41.712930°	-83.685720°	923
faberi	*72*	*D.Vela & J.Baker*	3-Sep-11	41.712930°	-83.685720°	923
viridis	*73*	*D.Vela & J.Baker*	3-Sep-11	42.379240°	-83.754830°	908
pumila	*74*	*D.Vela & J.Baker*	3-Sep-11	42.379240°	-83.754830°	908
viridis	*75*	*D.Vela & J.Baker*	3-Sep-11	43.119530°	-83.760210°	737

faberi	*76*	*D.Vela & J.Baker*	3-Sep-11	43.119530°	-83.760210°	737
viridis	*77*	*D.Vela & J.Baker*	3-Sep-11	43.853720°	-84.013200°	607
viridis	*78*	*D.Vela & J.Baker*	3-Sep-11	43.853720°	-84.013200°	607
viridis	*79*	*D.Vela & J.Baker*	3-Sep-11	44.293670°	-83.441490°	585
viridis	*80*	*D.Vela & J.Baker*	3-Sep-11	44.726920°	-83.820330°	933
viridis	*81*	*D.Vela & J.Baker*	3-Sep-11	45.108550°	-84.166440°	902
viridis	*82*	*D.Vela & J.Baker*	3-Sep-11	45.108550°	-84.166440°	902
viridis	*83*	*D.Vela & J.Baker*	3-Sep-11	45.774220°	-84.733410°	596
viridis	*84*	*D.Vela & J.Baker*	4-Sep-11	45.181510°	-84.914210°	701
viridis	*85*	*D.Vela & J.Baker*	4-Sep-11	44.566340°	-85.302920°	1079
viridis	*86*	*D.Vela & J.Baker*	4-Sep-11	44.555650°	-85.303210°	1105
viridis	*87*	*D.Vela & J.Baker*	4-Sep-11	43.888110°	-85.530820°	1069
viridis	*88*	*D.Vela & J.Baker*	4-Sep-11	43.888110°	-85.530820°	1069
viridis	*89*	*D.Vela & J.Baker*	4-Sep-11	43.039900°	-85.663580°	610
viridis	*90*	*D.Vela & J.Baker*	4-Sep-11	43.039900°	-85.663580°	610
viridis	*91*	*D.Vela & J.Baker*	5-Sep-11	42.261540°	-85.196200°	954
viridis	*92*	*D.Vela & J.Baker*	5-Sep-11	42.261540°	-85.196200°	954
viridis	*93*	*D.Vela & J.Baker*	5-Sep-11	41.707560°	-85.005750°	1016
viridis	*94*	*D.Vela & J.Baker*	5-Sep-11	41.027730°	-85.263480°	112
faberi	*95*	*D.Vela & J.Baker*	5-Sep-11	40.783590°	-85.919590°	673
pumila	*96*	*D.Vela & J.Baker*	5-Sep-11	40.783590°	-85.919590°	673
faberi	*97*	*D.Vela & J.Baker*	5-Sep-11	40.783590°	-85.919590°	673
viridis	*98*	*D.Vela & J.Baker*	5-Sep-11	40.657680°	-84.949150°	844
viridis	*99*	*D.Vela & J.Baker*	5-Sep-11	40.556390°	-84.064940°	1056
viridis	*100*	*D.Vela & J.Baker*	5-Sep-11	40.247970°	-83.307460°	1065
viridis	*ME005*	*M.Estep*	9-Sep-11	39.33408°	-91.18223	887
pumila	*ME006*	*M.Estep*	9-Sep-11	39.91700°	-91.52341	511
pumila	*ME007*	*M.Estep*	9-Sep-11	40.71172°	-91.5643	703
viridis	*ME008*	*M.Estep*	9-Sep-11	41.55582°	-91.54157	588

pumila	*ME008P*	*M.Estep*	9-Sep-11	41.55582°	-91.54157	588
viridis	*ME009*	*M.Estep*	9-Sep-11	42.45927°	-92.29887	888
viridis	*ME010*	*M.Estep*	9-Sep-11	43.03940°	-92.67078	1063
viridis	*ME011*	*M.Estep*	9-Sep-11	43.44701°	-93.35497	1302
viridis	*ME012*	*M.Estep*	9-Sep-11	44.28409°	-93.29373	996
viridis	*ME013*	*M.Estep*	10-Sep-11	45.55244°	-94.22726	1095
viridis	***ME014***	***M.Estep***	**10-Sep-11**	**46.03728°**	**-95.83433**	**1281**
viridis	*ME015*	*M.Estep*	10-Sep-11	46.66004°	-96.39323	1055
viridis	*ME016*	*M.Estep*	10-Sep-11	47.24074°	-97.01051	929
viridis	*ME017*	*M.Estep*	10-Sep-11	49.19226°	-98.47718	1243
viridis	*ME018*	*M.Estep*	10-Sep-11	49.38815°	-99.31496	1433
viridis	*ME019*	*M.Estep*	10-Sep-11	49.40199°	-98.83965	1474
viridis	*ME020*	*M.Estep*	11-Sep-11	49.59538°	-98.86997	1267
viridis	*ME021*	*M.Estep*	11-Sep-11	49.59117°	-98.8241	1328
viridis	*ME022*	*M.Estep*	11-Sep-11	49.59578°	-98.80132	1370
viridis	*ME023V*	*M.Estep*	11-Sep-11	49.62839°	-98.72547	1235
pumila	*ME023P*	*M.Estep*	11-Sep-11	49.62839°	-98.72547	1235
viridis	*ME024V*	*M.Estep*	11-Sep-11	49.62346°	-98.76953	1270
pumila	*ME024P*	*M.Estep*	11-Sep-11	49.62346°	-98.76953	1270
viridis	*ME025V*	*M.Estep*	11-Sep-11	49.63163°	-98.80143	1242
pumila	*ME025P*	*M.Estep*	11-Sep-11	49.63163°	-98.80143	1242
viridis	*ME026*	*M.Estep*	11-Sep-11	49.65886°	-98.80159	1227
pumila	*ME027*	*M.Estep*	11-Sep-11	49.68103°	-98.80172	1216
viridis	*ME028V*	*M.Estep*	11-Sep-11	49.69464°	-98.79431	1207
pumila	*ME028P*	*M.Estep*	11-Sep-11	49.69464°	-98.79431	1207
viridis	*ME029V*	*M.Estep*	11-Sep-11	49.69462°	-98.76737	1247
pumila	*ME029P*	*M.Estep*	11-Sep-11	49.69462°	-98.76737	1247
pumila	*ME030*	*M.Estep*	11-Sep-11	49.65054°	-98.73884	1271
pumila	*ME031*	*M.Estep*	11-Sep-11	49.63558°	-98.76704	1212

viridis	*ME032V*	*M.Estep*	11-Sep-11	49.65183°	-98.71057	1205
pumila	*ME032P*	*M.Estep*	11-Sep-11	49.65183°	-98.71057	1205
viridis	*ME033V*	*M.Estep*	11-Sep-11	49.68554°	-98.61968	1032
pumila	*ME033P*	*M.Estep*	11-Sep-11	49.68554°	-98.61968	1032
viridis	*ME034V*	*M.Estep*	11-Sep-11	49.64306°	-98.61948	1110
pumila	*ME034P*	*M.Estep*	11-Sep-11	49.64306°	-98.61948	1110
viridis	*ME035*	*M.Estep*	11-Sep-11	49.53270°	-98.68748	1506
pumila	***ME036***	***M.Estep***	**11-Sep-11**	**49.50261°**	**-97.93767**	**794**
pumila	*ME037*	*M.Estep*	11-Sep-11	49.50251°	-97.82132	842
pumila	*ME038*	*M.Estep*	11-Sep-11	49.67209°	-97.44172	784
pumila	*ME039*	*M.Estep*	12-Sep-11	49.45824°	-97.41842	789
viridis	*ME040*	*M.Estep*	12-Sep-11	49.48732°	-97.48177	851
viridis	*ME041*	*M.Estep*	12-Sep-11	49.50261°	-97.64426	784
viridis	*ME042*	*M.Estep*	12-Sep-11	49.50090°	-98.02792	865
viridis	*ME043*	*M.Estep*	12-Sep-11	49.49886°	-98.03411	850
viridis	*ME044*	*M.Estep*	12-Sep-11	49.49835°	-98.04111	880
pumila	*ME045*	*M.Estep*	12-Sep-11	49.49413°	-98.04089	883
viridis	*ME046*	*M.Estep*	12-Sep-11	49.50081°	-98.02994	894
pumila	*ME047*	*M.Estep*	13-Sep-11	46.74592°	-96.84202	978
viridis	*ME048V*	*M.Estep*	13-Sep-11	45.96494°	-96.83842	1067
pumila	*ME048P*	*M.Estep*	13-Sep-11	45.96494°	-96.83842	1067
viridis	*ME049V*	*M.Estep*	13-Sep-11	45.40278°	-97.02318	1795
pumila	*ME049P*	*M.Estep*	13-Sep-11	45.40278°	-97.02318	1795
viridis	*ME050V*	*M.Estep*	13-Sep-11	44.66000°	-96.8174	1930
pumila	*ME050P*	*M.Estep*	13-Sep-11	44.66000°	-96.8174	1930
viridis	*ME051V*	*M.Estep*	13-Sep-11	43.90858°	-96.75929	
pumila	*ME051P*	*M.Estep*	13-Sep-11	43.90858°	-96.75929	
viridis	*ME052V*	*M.Estep*	13-Sep-11	43.08312°	-96.79729	1459
pumila	*ME052P*	*M.Estep*	13-Sep-11	43.08312°	-96.79729	1459

viridis	*ME053V*	*M.Estep*	13-Sep-11	42.37566°	-96.355	1113
pumila	*ME053P*	*M.Estep*	13-Sep-11	42.37566°	-96.355	1113
viridis	*ME054V*	*M.Estep*	13-Sep-11	41.71054°	-96.02542	1020
pumila	*ME054P*	*M.Estep*	13-Sep-11	41.71054°	-96.02542	1020
viridis	*ME055V*	*M.Estep*	14-Sep-11	41.08266°	-95.82869	998
pumila	*ME055P*	*M.Estep*	14-Sep-11	41.08266°	-95.82869	998
faberi	*ME056V*	*M.Estep*	14-Sep-11	40.73140°	-95.00751	998
pumila	*ME056P*	*M.Estep*	14-Sep-11	40.73140°	-95.00751	998
viridis	*ME057V*	*M.Estep*	14-Sep-11	40.04024°	-94.8802	1021
pumila	*ME057P*	*M.Estep*	14-Sep-11	40.04024°	-94.8802	1021
viridis	*ME057BV*	*M.Estep*	14-Sep-11	39.38572°	-94.79032	808
pumila	*ME057BP*	*M.Estep*	14-Sep-11	39.38572°	-94.79032	808
viridis	*ME058V*	*M.Estep*	14-Sep-11	39.01105°	-94.07388	881
pumila	*ME058P*	*M.Estep*	14-Sep-11	39.01105°	-94.07388	881
faberi	*ME059V*	*M.Estep*	14-Sep-11	38.95258°	-93.08917	789
pumila	*ME059P*	*M.Estep*	14-Sep-11	38.95258°	-93.08917	789
faberi	*ME060V*	*M.Estep*	14-Sep-11	38.95324°	-92.12699	902
pumila	*ME060P*	*M.Estep*	14-Sep-11	38.95324°	-92.12699	902
viridis	*ME061V*	*M.Estep*	14-Sep-11	38.82778°	-91.03855	737
pumila	*ME061P*	*M.Estep*	14-Sep-11	38.82778°	-91.03855	737
viridis	*ME062*	*M.Estep & D.Vela*	26-Sep-11	38.82356	-90.18184	131
viridis	*ME063*	*M.Estep & D.Vela*	26-Sep-11	38.82356	-90.18184	131
viridis	*ME064*	*M.Estep & D.Vela*	26-Sep-11	38.82356	-90.18184	131
viridis	*ME065*	*M.Estep & D.Vela*	26-Sep-11	38.82356	-90.18184	131
viridis	*ME066*	*M.Estep & D.Vela*	26-Sep-11	38.82356	-90.18184	131
viridis	*ME067*	*M.Estep & D.Vela*	26-Sep-11	38.82356	-90.18184	131
viridis	*ME068*	*M.Estep & D.Vela*	26-Sep-11	38.82356	-90.18184	131
faberi	*ME069*	*M.Estep & D.Vela*	26-Sep-11	38.82356	-90.18184	131
viridis	*ME070*	*M.Estep & D.Vela*	26-Sep-11	38.82356	-90.18184	131

viridis	*ME071*	*M.Estep & D.Vela*	26-Sep-11	38.82356	-90.18184	131
viridis	*ME072*	*M.Estep & D.Vela*	26-Sep-11	38.82851	-90.18047	131
viridis	*ME073*	*M.Estep & D.Vela*	26-Sep-11	38.82851	-90.18047	131
faberi	*1241*	*E. Kellogg*	9-Sep-11	39.0215	-89.76494	
faberi	*1242*	*E. Kellogg*	9-Sep-11	39.0215	-89.76494	
viridis	*1243*	*E. Kellogg*	9-Sep-11	40.09238	-89.42172	
viridis	*1243*	*E. Kellogg*	9-Sep-11	40.09238	-89.42172	
viridis	*1244*	*E. Kellogg*	9-Sep-11	40.09238	-89.42172	
pumila	*1245*	*E. Kellogg*	9-Sep-11	40.09238	-89.42172	
viridis	*1246*	*E. Kellogg*	9-Sep-11	40.75617	-88.71358	
faberi	***1247***	***E. Kellogg***	**9-Sep-11**	**40.75617**	**-88.71358**	
viridis	*1248*	*E. Kellogg*	11-Sep-11	41.64819	-88.07844	
viridis	*1249*	*E. Kellogg*	11-Sep-11	41.64819	-88.07844	
faberi	*1250*	*E. Kellogg*	11-Sep-11	41.64819	-88.07844	
viridis	*1251*	*E. Kellogg*	11-Sep-11	39.80136	-89.60564	
faberi	*1180*	*E. Kellogg*	17-Jul-10	38.61733333	-90.26513889	
viridis	*1181*	*E. Kellogg*	17-Jul-10	38.61733333	-90.26513889	
viridis	*1186*	*E. Kellogg*	22-Jul-10	38.64672222	-90.25205556	163
viridis	*1192*	*E. Kellogg*	22-Jul-10	38.85680556	-89.40369444	
faberi	*1193*	*E. Kellogg*	22-Jul-10	38.13852778	-88.44452778	
viridis	*1194*	*E. Kellogg*	30-Jul-10	39.40472222	-87.65325	
viridis	*1195*	*E. Kellogg*	30-Jul-10	39.56491667	-86.62216667	
viridis	*1196*	*E. Kellogg*	30-Jul-10	39.79491667	-85.53222222	
faberi	*1197*	*E. Kellogg*	31-Jul-10	39.88363889	-84.27325	
viridis	*1198*	*E. Kellogg*	31-Jul-10	39.89052777	-84.19994444	
viridis	*1199*	*E. Kellogg*	31-Jul-10	39.96711111	-83.3620555	
viridis	*1201*	*E. Kellogg*	31-Jul-10	39.96711111	-83.3620555	
faberi	*1202*	*E. Kellogg*	31-Jul-10	39.96711111	-83.3620555	
faberi	*1203*	*E. Kellogg*	31-Jul-10	39.96444444	-83.35855555	

viridis	*1204*	*E. Kellogg*	31-Jul-10	39.96444444	-83.35855555	
viridis	*1205*	*E. Kellogg*	31-Jul-10	39.94066666	-82.4791666	
viridis	*1206*	*E. Kellogg*	31-Jul-10	40.03088889	-82.44852778	
viridis	*1207*	*E. Kellogg*	31-Jul-10	40.06611111	-80.56966666	384
viridis	*1208*	*E. Kellogg*	31-Jul-10	40.20788889	-79.67652778	
viridis	*1209*	*E. Kellogg*	1-Aug-10	40.68397222	-75.15036111	
viridis	*1210*	*E. Kellogg*	4-Aug-10	41.92019444	-71.35716667	
viridis	*1211*	*E. Kellogg*	4-Aug-10	41.92019444	-71.35716667	
viridis	*1211*	*E. Kellogg*	4-Aug-10	41.92019444	-71.35716667	
viridis	*1212*	*E. Kellogg*	4-Aug-10	42.89125	-70.87152778	33
faberi	*1213*	*E. Kellogg*	6-Aug-10	42.56986111	-71.42122222	
faberi	*1213*	*E. Kellogg*	6-Aug-10	42.56986111	-71.42122222	
viridis	*1214*	*E. Kellogg*	6-Aug-10	42.08344444	-72.09852778	
viridis	*1216*	*E. Kellogg*	7-Aug-10	39.57991667	-75.58869444	6
viridis	*1218*	*E. Kellogg*	8-Aug-10	39.31333333	-76.95063889	172
faberi	*1219*	*E. Kellogg*	8-Aug-10	39.31333333	-76.95063889	172
viridis	*1220*	*E. Kellogg*	8-Aug-10	39.61513889	-77.69763889	167
viridis	*1221*	*E. Kellogg*	8-Aug-10	39.64891667	-78.76072222	192
viridis	*1222*	*E. Kellogg*	8-Aug-10	39.66022222	-79.777	669
viridis	*1223*	*E. Kellogg*	8-Aug-10	39.66022222	-79.777	669
faberi	*1224*	*E. Kellogg*	8-Aug-10	39.28977778	-80.49772222	324
viridis	*1225*	*E. Kellogg*	8-Aug-10	39.25175	-81.554833	
viridis	*1226*	*E. Kellogg*	9-Aug-10	39.1925	-82.26936111	242
faberi	*1227*	*E. Kellogg*	9-Aug-10	39.1925	-82.26936111	242
viridis	*1229*	*E. Kellogg*	9-Aug-10	39.04363889	-83.16447222	179
faberi	*1230*	*E. Kellogg*	9-Aug-10	39.04363889	-83.16447222	179
viridis	*1231*	*E. Kellogg*	9-Aug-10	39.04363889	-83.16447222	179
pumila	*1232*	*E. Kellogg*	9-Aug-10	39.04363889	-83.16447222	179
viridis	*1233*	*E. Kellogg*	26-Aug-10	38.64852778	-90.31563889	171

viridis	*1235*	*E. Kellogg*	8-Sep-10	38.87622222	-90.19041667	
pumila	*1236*	*E. Kellogg*	8-Sep-10	38.87622222	-90.19041667	
viridis	*1237*	*E. Kellogg*	8-Sep-10	38.87622222	-90.19041667	
viridis	*1238*	*E. Kellogg*	8-Sep-10	38.90208333	-90.20775	140
viridis	*17139*	*L. Ahart*	31-Aug-10	41.17225	-121.0237778	1277
viridis	*17140*	*L. Ahart*	1-Sep-10	40.03678056	-122.1122528	64
viridis	*10106*	*C. Roché*	11-Sep-10	42.29444444	-123.7816666	610
viridis	*P1*	*J. Penagos*	15-Sep-10	35.85597222	-86.29008333	199
viridis	*P2*	*J. Penagos*	16-Sep-10	36.08930556	-86.37091667	
viridis	*P3*	*J. Penagos*	16-Sep-10	36.03488889	-86.30625	212
viridis	*P4*	*J. Penagos*	16-Sep-10	36.02544444	-86.34844444	230
viridis	*P5*	*J. Penagos*	16-Sep-10	36.07691667	-86.39636111	189
viridis	*P6*	*J. Penagos*	18-Sep-10	36.07472222	-86.5108333	
viridis	*P7*	*J. Penagos*	18-Sep-10	36.06644444	-86.60033333	164
viridis	*P8*	*J. Penagos*	14-Oct-10	39.02519444	-90.91727778	170
viridis	*s.n.*	*Gall*	14-Sep-10			
viridis	*s.n.*	*Gall*	20-Sep-10	41.1222	-73.3166	
faberi	***57239***	***M. Nee***				
viridis	*2035*	*P. Sweeney*	19-Sep-10	41.274617	-72.817634	
viridis	*2033*	*P. Sweeney*	16-Sep-10	41.27346	-72.80977	
viridis	*s.n.*	*K. Waselkov*	17-Sep-10	40.3229	-84.61955	
viridis	*s.n.*	*K. Waselkov*	17-Sep-10	40.84973333	-84.58045	
viridis	*s.n.*	*K. Waselkov*	25-Sep-10	38.96068333	-89.08911666	
viridis	*s.n.*	*K. Waselkov*	26-Sep-10	41.78606667	-89.68251666	
faberi	*s.n.*	*K. Waselkov*	26-Sep-10	41.1620333	-89.66153333	
viridis	*s.n.*	*K. Waselkov*	15-Oct-10	34.61215	-86.9785	
viridis	*s.n.*	*S. Thompson*	20-Sep-10	42.01228333	-93.91575	
faberi	***156***	***D. Layton***	**15-Oct-11**	**38.712886**	**-90.30617**	

Appendix III: Dataset Combinations in PCAs

Character Sets Included						**Data Types Included**			**Data Sets Used**				**Result**
Vegetative	Prickle	Hair	Inflor.	Spikelet	Spikelet Veins	Continuous	Bivariate	Ratios	Maximum Measure	Average Measure	Pumila Included	Immature Excluded	Two Groups?
X	X	X	X	X	X	X	X	X	X		X		Yes
X	X	X	X	X	X	X	X	X	X				Yes
X	X	X				X	X		X				Yes
X	X		X			X	X	X	X				Overlap
X	X		X	X		X		X	X				Yes barely
X	X					X	X		X				No
				X		X		X	X				Yes
				X	X	X		X	X				Yes
				X	X	X	X	X	X				Yes
X	X	X	X	X			X		X				Yes
X	X		X	X			X		X				No
			X			X	X		X				Overlap
			X			X	X	X	X				Small Overlap
X	X		X			X	X	X	X				Small Overlap
X			X			X	X	X	X				No
X	X	X	X	X	X	X	X	X	X			X	Yes
X			X			X	X	X	X			X	No
X	X	X	X			X	X	X	X			X	Yes
X	X					X	X	X	X			X	No
				X	X	X	X	X	X			X	Yes
X			X			X	X	X	X			X	No

X	X	X	X	X	X	X	X	X		X	X		Yes
X	X	X	X			X	X	X		X	X		Yes; pumila with viridis
				X	X	X	X	X		X	X		Yes; pumila with faberi
				X	X	X		X		X	X		Yes
X	X					X	X			X	X		No
X	X	X				X	X			X	X		Yes; pumila with faberi
			X			X	X	X		X	X		Overlap
X	X					X				X	X		No
	X	X				X	X			X	X		Yes; pumila with viridis
	X	X				X				X	X		Yes; pumila with viridis
X	X	X	X	X	X	X	X	X		X			Yes
X	X	X	X			X		X		X			Yes
				X	X	X		X		X			Yes
			X			X	X	X		X			Small Overlap
X	X	X	X			X	X	X		X			Yes barely
	X	X				X	X			X			Yes
X		X	X	X			X			X			Overlap
X			X			X	X			X			No
X	X					X	X			X			No

Appendix IV: Accessions Used for Drought Experiment

Where seeds from more than one individual per population were available, a packet number is given indicating which individual the seeds came from. “Sheet” indicates that seeds were removed directly from the associated herbarium specimen, while “F1” indicates the plants were grown with seeds acquired from cultivated plants grown from the original field collected seed. Collections with the prefix “ISE” and “Yang” were obtained from Katrien Devos’ lab.

Species	**Accession**	**Packet Number**
Setaria viridis	*Yang 1108*	N/A
Setaria viridis	*Yang 8054*	N/A
Setaria viridis	*Estep ME051V*	3
Setaria viridis	*Estep ME013*	1
Setaria viridis	*Thompson s.n.*	1
Setaria viridis	*Vela 78*	3
Setaria viridis	*Kellogg 1211*	Sheet F1
Setaria faberi	*Nee 57234*	1
Setaria faberi	*Kellogg 1213*	Sheet F1
Setaria faberi	*Vela 70*	1 F1
Setaria pumila	*Estep ME057P*	1
Setaria pumila	*Estep ME045*	3
Setaria pumila	*ISe 1430*	N/A

Appendix V: Flow Cytometry Estimates

The following table lists all flow cytometry estimates made during the course of this study. Some samples were used more than once, either to ensure consistency or to double check dubious results. Where seeds from more than one individual per population were available, a packet number is given indicating which individual the seeds came from. “Sheet” indicates that seeds were removed directly from the associated herbarium specimen, while “F1” indicates the plants were grown with seeds acquired from cultivated plants grown from the original field collected seed. Collections with the prefix “ISE” and “Yang”, as well as “FUBerlin”, were obtained from Katrien Devos’ lab.

Species	Accession	Packet Number	Mean DNA content (pg/2C)	St. Dev (±)
Setaria viridis	Ahart 17139	1	0.96	0.001
Setaria viridis	Ahart 17139	1	1.28	0.002
Setaria viridis	FUBerlin	2	0.95	0.006
Setaria viridis	ISE1282	N/A	0.99	0.002
Setaria viridis	Kellogg 1181	2	0.94	0.008
Setaria viridis	Kellogg 1209	1	0.98	0.015
Setaria viridis	Kellogg 1211	Sheet F1	1.05	0.003
Setaria viridis	Kellogg 1211	Sheet	1.09	0.009
Setaria viridis	Kellogg 1231	1	0.98	0.005
Setaria viridis	Kellogg 1235	Sheet	0.97	0.005
Setaria viridis	Kellogg 1237	1	0.98	0.009
Setaria viridis	Kellogg 1243	3	0.96	0.004
Setaria viridis	Kellogg 1243	2	0.97	0.008
Setaria viridis	Estep ME013	1	0.97	0.015
Setaria viridis	Estep ME014	1	1	0.012
Setaria viridis	Estep ME014	1	2.25	0.02
Setaria viridis	Estep ME028V	1	1.03	0.007
Setaria viridis	Estep ME033V	3	1.01	0.001
Setaria viridis	Estep ME035	1	0.99	0.006

Setaria viridis	Estep ME042	2	0.98	0.003
Setaria viridis	Estep ME043	2	0.98	0.022
Setaria viridis	Estep ME046	1	0.96	0.006
Setaria viridis	Estep ME048V	1	1.02	0.01
Setaria viridis	Estep ME065	2	0.98	0.006
Setaria viridis	Estep ME066	2	0.97	0.002
Setaria viridis	Estep ME070	2	0.94	0.016
Setaria viridis	Estep ME074	1	0.95	0.007
Setaria viridis	Estep ME17 (plant 1)	2	1.14	0.025
Setaria viridis	Estep ME17 (plant 3)	2	1.07	0.016
Setaria viridis	Estep ME22	1	1.29	0.03
Setaria viridis	Estep ME25	1	1.1	0.017
Setaria viridis	Estep ME28	1	1.27	0.012
Setaria viridis	Estep ME40	1	1.07	0.018
Setaria viridis	Estep ME46	1	1.14	0.014
Setaria viridis	Estep ME51	3	1.29	0.017
Setaria viridis	Estep ME52	1	1.06	0.016
Setaria viridis	Estep ME54	1	1.19	0.039
Setaria viridis	Estep ME66	2	1.13	0.007
Setaria viridis	Penagos P5	1	1	0.012
Setaria viridis	Penagos P7	4	1	0.008
Setaria viridis	PI204625	N/A	1.01	0.011
Setaria viridis	PI204727	N/A	0.99	0.01
Setaria viridis	PI212625	N/A	1	0.022
Setaria viridis	PI223677	N/A	0.95	0.003
Setaria viridis	PI230134	N/A	0.97	0.011
Setaria viridis	Roche 10106	2	0.97	0.01
Setaria viridis	Sweeney 2033	2	0.95	0.013
Setaria viridis	Thompson s.n.; PI324509	1	1.06	0
Setaria viridis	Vela 78	3	0.94	0.009
Setaria viridis	Vela 80	1	1.06	0.015
Setaria viridis	Vela 86	3	1	0.003
Setaria viridis	Vela 86	3	1.04	0.014
Setaria viridis	Vela 87	2	1.11	0.008
Setaria viridis	Waselkov-Momence	1	0.98	0.007
Setaria viridis	Waselkov-Momence	4	1.02	0.011
Setaria viridis	Waselkov-Rock Falls	1	0.93	0.003
Setaria viridis	Waselkov-Rock Falls	3	0.95	0.007
Setaria viridis	Waselkov-Vandalia	1	0.92	0.013
Setaria viridis	Yang 1108	N/A	0.94	0.014
Setaria viridis	Yang 8008	N/A	1	0.017
Setaria viridis	Yang 8019	N/A	0.99	0.018

Setaria viridis	Yang 8032	N/A	0.94	0.015
Setaria viridis	Yang 8043	N/A	0.97	0.007
Setaria viridis	Yang 8054	N/A	0.95	0.012
Setaria verticilliformis	ISE1713	N/A	2.68	0.019
Setaria pumila?	ISE1432	N/A	2.67	0.038
Setaria pumila?	ISE1442	N/A	2.63	0.011
Setaria pumila	Estep ME007	5	2.12	0.01
Setaria pumila	Estep ME023P	1	2.15	0.01
Setaria pumila	Estep ME025P	1	2.1	0.043
Setaria pumila	Estep ME029P	1	2.14	0.02
Setaria pumila	Estep ME033P	N/A	2.1	0.004
Setaria pumila	Estep ME034P	N/A	2.09	0.016
Setaria pumila	Estep ME036	1	2.13	0.018
Setaria pumila	Estep ME039P	1	2.1	0.013
Setaria pumila	Estep ME047V	3	2.1	0.006
Setaria pumila	Estep ME048P	1	2.12	0.027
Setaria pumila	Estep ME054P	1	4.89	0.044
Setaria pumila	Estep ME059P	4	4.87	0.038
Setaria pumila	Estep ME061P	1	4.97	0.043
Setaria pumila	Estep ME57P	1	5.17	0.008
Setaria pumila	Vela 102	4	5.1	0.134
Setaria faberi	Kellogg 1197	1	2.59	0.015
Setaria faberi	Kellogg 1203	2	2.43	0.003
Setaria faberi	Kellogg 1213	Sheet	2.77	0.065
Setaria faberi	Kellogg 1213	Sheet F1	3.97	0.035
Setaria faberi	Kellogg 1230	2	2.36	0.009
Setaria faberi	Kellogg 1247	5	2.29	0.016
Setaria faberi	Layton 156	N/A	2.32	0.018
Setaria faberi	Estep ME059V	5	2.24	0.011
Setaria faberi	Nee 57234	1	2.28	0.008
Setaria faberi	Vela 104	3	2.08	0.014
Setaria faberi	Vela 105	2	2.17	0.013
Setaria faberi	Vela 70	1 F1	2.43	0.012
Setaria faberi	Vela 70	5	2.83	0.026
Setaria faberi	Vela 97	Sheet	2.08	0.012