Annals of Botany **116**: 291–299, 2015
doi:10.1093/aob/mcv097, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

Phylogenetic reconstruction using four low-copy nuclear loci strongly supports a polyphyletic origin of the genus *Sorghum*

Jennifer S. Hawkins*, Dhanushya Ramachandran, Ashley Henderson, Jasmine Freeman, Michael Carlise, Alex Harris and Zachary Willison-Headley

Department of Biology, West Virginia University, Morgantown, WV 26506, USA
** For correspondence. E-mail Jennifer.hawkins@mail.wvu.edu*

Received: 20 February 2015 Returned for revision: 13 April 2015 Accepted: 14 May 2015 Published electronically: 2 July 2015

• **Background and Aims** *Sorghum* is an essential grain crop whose evolutionary placement within the Andropogoneae has been the subject of scrutiny for decades. Early studies using cytogenetic and morphological data point to a poly- or paraphyletic origin of the genus; however, acceptance of poly- or paraphyly has been met with resistance. This study aimed to address the species relationships within *Sorghum*, in addition to the placement of *Sorghum* within the tribe, using a phylogenetic approach and employing broad taxon sampling.
• **Methods** From 16 diverse *Sorghum* species, eight low-copy nuclear loci were sequenced that are known to play a role in morphological diversity and have been previously used to study evolutionary relationships in grasses. Further, the data for four of these loci were combined with those from 57 members of the Andropogoneae in order to determine the placement of *Sorghum* within the tribe. Both maximum likelihood and Bayesian analyses were performed on multilocus concatenated data matrices.
• **Key Results** The *Sorghum*-specific topology provides strong support for two major lineages, in alignment with earlier studies employing chloroplast and internal transcribed spacer (ITS) markers. Clade I is composed of the *Eu-*, *Chaeto-* and *Heterosorghum*, while clade II contains the *Stipo-* and *Parasorghum*. When combined with data from the Andropogoneae, Clade II resolves as sister to a clade containing *Miscanthus* and *Saccharum* with high posterior probability and bootstrap support, and to the exclusion of Clade I.
• **Conclusions** The results provide compelling evidence for a two-lineage polyphyletic ancestry of *Sorghum* within the larger Andropogoneae, i.e. the derivation of the two major *Sorghum* clades from a unique common ancestor. Rejection of monophyly in previous molecular studies is probably due to limited taxon sampling outside of the genus. The clade consisting of *Para-* and *Stiposorghum* resolves as sister to *Miscanthus* and *Saccharum* with strong node support.

Key words: *Sorghum*, phylogeny, Andropogoneae, Poaceae, low-copy nuclear loci, polyphyly.

INTRODUCTION

Sorghum L. Moench consists of approx. 25 species of C_4 subtropical grasses that are widely distributed throughout the Americas, Africa, Asia and Australia (Garber, 1950). Given that *Sorghum* is an agronomically important crop used for food, fibre and fuel, it is not surprising that a large part of the taxonomic literature focuses on the domestication of wild African *Sorghum* and its subsequent migration to the Americas during the early 20th century (Snowden, 1935, 1936; de Wet and Huckabay, 1967; de Wet and Harlan, 1971; Dillon *et al*., 2007). The cytology and morphology of *Sorghum* species endemic to Australia received little attention until much later (Lazarides *et al*., 1991; Spangler, 2003). Current classification divides *Sorghum* into five sub-sections: *Eusorghum*, *Chaetosorghum*, *Heterosorghum*, *Parasorghum* and *Stiposorghum* (Garber, 1950; Dahlberg, 2000). Ambiguous relationships between these sub-sections, however, have been disputed for over half a century (Garber, 1950; Celarier, 1959). Early work by Garber (1950) distinguished *Eu-*, *Chaeto-* and *Heterosorghum* from *Stipo-* and *Parasorghum* based on the presence/absence of bearded culm nodes and the prominence of awns. The sub-sections were further distinguished by panicle branching, callus morphology, the presence of pedicellate or sessile spikelets and several other morphological, geographic and cytological characteristics. More recent herbarium-based specimen analyses by Spangler (2003) provided support for the use of bearded culm nodes, spikelet morphology and lemma awn characteristics for classification, yet many traits were variable within sub-sections. Such difficulties in taxonomic distinction are probably caused by environmentally malleable morphology that contributes to ambiguities not only in *Sorghum* classification, but also throughout the larger Andropogoneae (Mathews *et al*., 2002).

Before the widespread use of molecular markers, several studies examined the relationships within *Sorghum* via cytological methods. *Sorghum* species contain a variable range (from ten to 40) of diploid chromosomes (Doggett, 1988; Lazarides *et al*., 1991). An early observation showed that species of *Sorghum* display different mean chromosome sizes, with a subset of species displaying larger mean chromosome sizes (Magoon and Shambulingappa, 1961). This observation has

© The Author 2015. Published by Oxford University Press on behalf of the Annals of Botany Company.
All rights reserved. For Permissions, please email: journals.permissions@oup.com

since been verified genus wide (Price *et al.*, 2005). Furthermore, there was no correlation between geographical distribution and mean chromosome size. The authors found that *Sorghum* species clustered into two distinct lineages, one consisting of the chromosomes of larger size of $n = 5$ and their polyploid derivatives (sub-sections *Parasorghum* and *Stiposorghum*) and the other of the smaller sized chromosomes of $n = 10$ and their polyploid derivatives (sub-sections *Eusorghum*, *Chaetosorghum* and *Heterosorghum*). As with molecular markers, these cytological studies suggest a polyphyletic origin of *Sorghum* (i.e. a different ancestor for each mean chromosome size). To date, this work may best explain the two distinct lineages of *Sorghum*; however, the study did not include a large sampling of sister taxa to test this hypothesis.

Early studies employing molecular markers suggested that *Sorghum* contains two major clades; one exclusive to *Stiposorghum* and *Parasorghum*, and the other composed of the remaining sub-sections (Duvall and Doebley, 1990; Sun *et al.*, 1994). Both of these studies included the sister taxon *Cleistachne sorghoides*, and both suggested that *C. sorghoides* is more closely related to one of the *Sorghum* clades than the two *Sorghum* clades are to each other, indicating that the genus may be para- or polyphyletic. A subsequent study, using the same gene sequences from a larger taxonomic sampling, affirmed the ambiguous relationships among *Sorghum* sub-sections (Dillon *et al.*, 2001). This study supported the previous evidence that *Sorghum* is divided into two major lineages, with the *Eu*-, *Chaeto*- and *Heterosorghum* sections making up one lineage and the *Para*- and *Stiposorghum* sections making up the second. Additionally, *Saccharum officinarum* and *C. sorghoides* failed to resolve outside the *Sorghum* genus, again indicating para- or polyphyletic relationships.

Suggestions of paraphyly are not confined to *Sorghum*, but found throughout the Andropogoneae. While molecular phylogenetic data support the monophyly of the tribe, the relationships between genera have been difficult to resolve (Spangler *et al.*, 1999; Mathews *et al.*, 2002). Spangler *et al.* suggested that rapid radiation and the presence of continuous morphological characters are proving problematic in delineating relationships between genera. The authors used chloroplast *ndhF* sequences from 13 *Sorghum* species, and observed that *Sorghum* is split into three lineages most easily explained by geographical distribution. Again, these results indicated para- or polyphyly of *Sorghum*.

Despite the consistency of data suggesting *Sorghum* is para- or polyphyletic, several studies have argued the contrary. One found that *S. officinarum* resolved outside of *Sorghum*; however, as with the earlier studies based on internal transcribed spacer (ITS) sequence, *C. sorghoides* again resolved within the *Para*- and *Stiposorghum* lineage (Dillon *et al.*, 2004). The authors argued for monophyly of the genus, but they supported a reduction of the sub-sections from five to three. The second study resolved *Sorghum* as one distinct genus composed of two major lineages; however, the authors determined that *C. sorghoides* resolved within their *Eu*-, *Chaeoto*- and *Heterosorghum* clade (Dillon *et al.*, 2007). *Cleistachne sorghoides* has previously been reported as $2n = 36$ chromosomes (Celarier, 1959), which is incongruent with $2n = 20$ chromosomes of *Eusorghum*, and thus its inclusion within *Sorghum* may not be the most parsimonious explanation. Both studies included a limited number of Andropogoneae taxa outside of *Sorghum* and may, therefore, lack the power to evaluate monophyly of the genus. Further, all of these studies employed the ITS gene, which has been suggested to be problematic for resolving genus-level relationships due to rapid duplication and gene conversion events (Alvarez and Wendel, 2003). Recently, several low-copy nuclear gene sequences have proven to be phylogenetically informative in the Panicoideae (Estep *et al.*, 2012). These sequences were used to reconstruct evolutionary relationships within the Andropogoneae in an effort to resolve genera-level relationships within the tribe (Estep *et al.*, 2014). Here, we use the same low-copy nuclear genes from several *Sorghum* species, and combine these data with those of Estep *et al.* to test explicitly the generic limits of *Sorghum*.

MATERIALS AND METHODS

Plant material

We included 16 *Sorghum* species representative of all five sub-sections: *Eusorghum*, *Heterosorghum*, *Chaetosorghum*, *Stiposorghum* and *Parasorghum* (Table 1). *Sorghum propinquum* seed for accession PI653737 was obtained from the USDA Agricultural Research Service Plant Genetic Resources Conservation Unit (Griffin, GA, USA), and seed for the unnamed *S. propinquum* accessions was provided courtesy of Dr Bill Rooney (Texas A&M University, College Station, TX, USA). All Australian accessions were obtained from the Australian Tropical Grains Germplasm Centre (Biloela, Central Queensland, Australia). Verification of ploidy in *S. halepense* was determined via flow cytometry, performed in triplicate, in the Flow Cytometry Core Lab at the Benaroya Research Institute at Virginia Mason (Seattle, WA, USA). The genome sizes of all other accessions are reported in Price *et al.* (2005). Plants were grown in the WVU Life Sciences greenhouse under normal greenhouse conditions. Leaves were flash frozen in liquid nitrogen and stored at –80 °C.

Molecular techniques

Frozen leaf tissue was ground with a mortar and pestle, and DNA was extracted using either the Promega Wizard Genomic DNA Purification Kit (Madison, WI, USA) or the QIAGEN DNeasy Plant Mini Kit (Germantown, MD, USA) following the manufacturer's protocol. Eight low-copy nuclear gene sequences were employed in this study and are as follows: *Erect Panicle 2* (*EP2_ex7 and EP2_ex8*), *Liguleless 1* (*LIG1*), *Vanishing Tassel 2* (*VT2*), *Ramosa 1* (*RA1*), *Ramosa 2* (*RA2*), *Dwarf 8* (*D8*) and *Aberrant Panicle Organization 1* (*APO1*). The PCR primer sequences used are as described in table 1 of Estep *et al.* (2012), with the exception of those for *RA1* and *RA2*. Both *RA1* and *RA2* were unreliably amplified, probably due to sequence divergence in the species more distantly related to *S. bicolor*. Therefore, new primer sequences for *RA1* (F, AGCTCAGCTTTGGTGTATAT; R, TAAGCTGAAGATC CAGACG) and *RA2* (F, CACCAGCAACAACTCGGCC; R, GAGGCGCTGATGGCATTCAC) were designed by aligning conserved coding sequences for the same gene regions from other grasses.

TABLE 1. Sorghum *species included in this analysis with their respective accession ID, chromosome number and section*

Species	Accession	Chromosome number	Section
Sorghum angustum S. T. Blake	ausTRC 302605	10	Stiposorghum
S. brachypodum Lazarides	ausTRC 302481	10	Stiposorghum
S. ecarinatum Lazarides	ausTRC 302661	10	Stiposorghum
S. exstans Lazarides	ausTRC 302557	10	Stiposorghum
S. halepense (L.) Pers.	PI 663976	40	Eusorghum
S. halepense (L.) Pers.	PI 271241	40	Eusorghum
S. halepense (L.) Pers.	PI 302268	40	Eusorghum
S. halepense (L.) Pers.	PI 663975	40	Eusorghum
S. interjectum Lazarides	ausTRC 302445	30	Stiposorghum
S. intrans F. Muell. Ex Benth.	ausTRC 302389	10	Stiposorghum
S. laxiflorum Bailey	ausTRC 302510	40	Heterosorghum
S. leiocladum (Hack.) C E. Hubb	ausTRC 300170	10	Parasorghum
S. macrospermum Garber	ausTRC 302367	40	Chaetosorghum
S. matarankense Garber & Snyder	ausTRC 302637	10	Parasorghum
S. plumosum (R. Br.) P. Beauv.	ausTRC 302635	40	Stiposorghum
S. propinquum (Kunth) Hitch.	unnamed	20	Eusorghum
S. propinquum (Kunth) Hitch.	PI 653737	20	Eusorghum
S. purpureosericeum (A. Rich)	ausTRC 318068	10	Parasorghum
S. stipoideum (Ewart & Jean White)	ausTRC 302614	10	Stiposorghum

Polymerase chain reactions contained 50 ng of template DNA, dimethylsulphoxide (DMSO), 0·5 M betaine, 1× Mg-included Taq buffer, 0·8 mM dNTPs, 0·4 pM each forward and reverse primer, and 0·05 U μL^{-1} of *Taq* polymerase (Denville Scientific, South Plainfield, NJ, USA). Reaction conditions were as follows: initial denaturation at 94 °C for 5 min, 32 cycles of denaturation at 94 °C for 1 min, annealing at 5 °C less than the melting temperature for 1 min, and elongation of 72 °C for 1 min, followed by a final extension of 72 °C for 5 min. A touchdown protocol was used for reactions that were difficult to amplify. The touchdown protocol included three rounds of five cycles per round beginning with an annealing temperature of the primer-specific melting temperature in the first round and reducing the annealing temperature by 2 °C in each subsequent round. These initial 15 cycles were followed by 25 cycles with an annealing temperature 10 °C less than the primer melting temperature.

Amplification products were resolved on 1·5 % agarose gels. Bands were excised and purified using the Invitrogen DNA Pure Link Quick Gel Extraction kit (Carlsbad, CA, USA), following the manufacturer's protocol. Purified PCR products were cloned using the Invitrogen TOPO TA Cloning Kit and transformed into One Shot Top 10 electrocompetent *Escherichia coli* cells. Cells were plated on LB agar containing kanamycin, and positive transformants were selected via blue/white screening. Plasmid DNA from eight clones for diploids and 16 clones for polyploids was extracted for sequencing. Each sequencing reaction contained approx. 300–500 ng of template, 1/16× BigDye [BigDye Terminator v.3.1. Cycle Sequencer Kit (Austin, TX, USA), 1× buffer, 0·4 pM M13 forward or 0·4 pM M13 reverse primer and 0·5 M betaine. Sequencing reaction conditions were as follows: 45 cycles of 1 min at 96 °C, 30 s at 50 °C and 4 min at 60 °C. Sequencing reactions were purified by ethanol precipitation and sequenced at the West Virginia University Genomics Core Facility using the ABI 3130*XL*.

TABLE 2. *Phylogenetic summary statistics and parsimony or likelihood results for the eight low-copy nuclear loci used in the analysis*

Locus	Aligned length (bp)	Variable characters	Parsimony informative characters	No. of best trees	Score of best tree
APO1	766	90	70	1498	134
D8	1018	150	96	190	207
EP2_ex7	991	184	82	14	225
EP2_ex8	809	130	59	30	166
LG1	833	163	99	N/A	N/A
RA1	402	108	60	1152	133
RA2	755	86	46	12	105
VT2	999	329	206	N/A	N/A

The number of best trees and score of best tree were determined using a heuristic search in PAUP*. Due to computational intensity, single gene trees for *LG1* and *VT2* were generated in RAxML.

Data analysis

Sequence data were vector screened and trimmed of both vector and primer sequences, and aligned using MUSCLE (Edgar, 2004). Alignments were manually inspected and adjusted in Bioedit v7.3.5 (Hall, 1999) to ensure that the amino acid alignment was maintained and that no aberrant alignment errors were included. Redundant (identical) alleles were removed for each gene from each taxon. All trimmed and inspected reads used in this study are available via GenBank (accession nos KR493932–KR494220). Data statistics, as reported in Table 2, were generated in PAUP* v.4.0b10 (Swofford, 2003). Parsimony analyses on single gene alignments were performed in PAUP using a heuristic search. All nucleotide characters were included, unweighted gaps were treated as missing data, and trees were rooted with outgroup sequences from *Zea mays*. Likelihood analyses on single gene alignments were performed in RAxML v.8 (Stamatakis, 2006),

as described below. The resulting parsimony and likelihood trees were used to determine genome-specific paralogues in polyploid and heterozygous diploid taxa for informing concatenation of alleles into a single multigene matrix for further analysis, as described in Estep *et al*. (2014). Accessions that were homozygous for all genes (e.g. *S. propinquum*, *S. bicolor* and *S. leiocladum*) were represented by a single allele. Further, the *Sorghum* sequences for *EP2_ex7*, *EP2_ex8*, *D8* and *APO* were combined with that of Andropogoneae from Estep *et al*. (2014) (courtesy of Dr Elizabeth Kellogg, Danforth Plant Science Center) for analyses aimed at delineating the placement of *Sorghum* within the tribe.

A maximum likelihood analysis on both the *Sorghum* and Andropogoneae concatenated alignments was performed in RAxML v.8 using the GTR + gamma substitution model over two threads, and employing the autoMRE function for bootstrap replication. For the *Sorghum* alignment, *Z. mays* was designated as the outgroup, and for the Andropogoneae alignment, *Paspalum* and *Plagiantha* were designated as outgroups. Bayesian analysis of both alignments was performed in MrBayes v3.2.3 using rates = invgamma and nst = 6 (Ronquist and Huelsenbeck, 2003). Two separate runs of 50 million generations were performed, sampling each run every 1000 generations. Trees were visualized in FigTree v1.4.2.

RESULTS

Amplification of the eight loci from the accessions listed in Table 1 resulted in a total of 6573 characters, of which 724 (11 %) are parsimony informative. These genes, physically located on nine of the ten maize chromosomes in the B73 reference, are known to play a role in shaping morphological and inflorescence diversity and have been previously identified as useful loci for the determination of evolutionary relationships among grasses (Estep *et al*., 2012). The percentage of parsimony informative characters ranged from 6·1 to 20·6 %; a particularly high number of informative characters was obtained for *VT2*, which contains two diverse introns. Details for each of the genes are listed individually in Table 2.

Individual gene trees were constructed in PAUP for the determination of genome-specific paralogues. Seven of the eight individual gene trees supported two distinct clades, one containing *Eu*/*Chaeto*/*Heterosorghum* (henceforth called Clade I), and a second containing *Stipo*/*Parasorghum* (Clade II). The gene tree for *RA1* placed half of the *S. macrospermum* and *S. laxiflorum* alleles in Clade I with strong bootstrap support (BS = 93) and the other half in Clade II with weak bootstrap support (BS = 42). Interestingly, two of the *S. macrospermum* alleles (a polyploid) always resolved with two of the *S. laxiflorum* alleles (also polyploid), suggesting orthologous relationships. General relationships within *Eusorghum* were consistent across all trees, resolving one of the *S. propinquum* accessions (PI 653737) sister to *S. bicolor*, and placing the second *S. propinquum* (unnamed) basal to all other *Eusorghum* taxa. Placement of various *S. halepense* alleles differed slightly among trees, but they were always sister to the *S. bicolor* + *S. propinquum* PI 653737 clade. Relationships within Clade II were more difficult to distinguish and less consistent across trees, probably due to limited phylogenetic signal leading to very short branch lengths; however, a few relationships were well supported. In all trees, one allele from *S. matarankense* clustered with one allele of *S. interjectum*; *S. intrans* and *S. exstans* were sister to one another. and the *S. plumosum* alleles were dispersed throughout the entirety of Clade II.

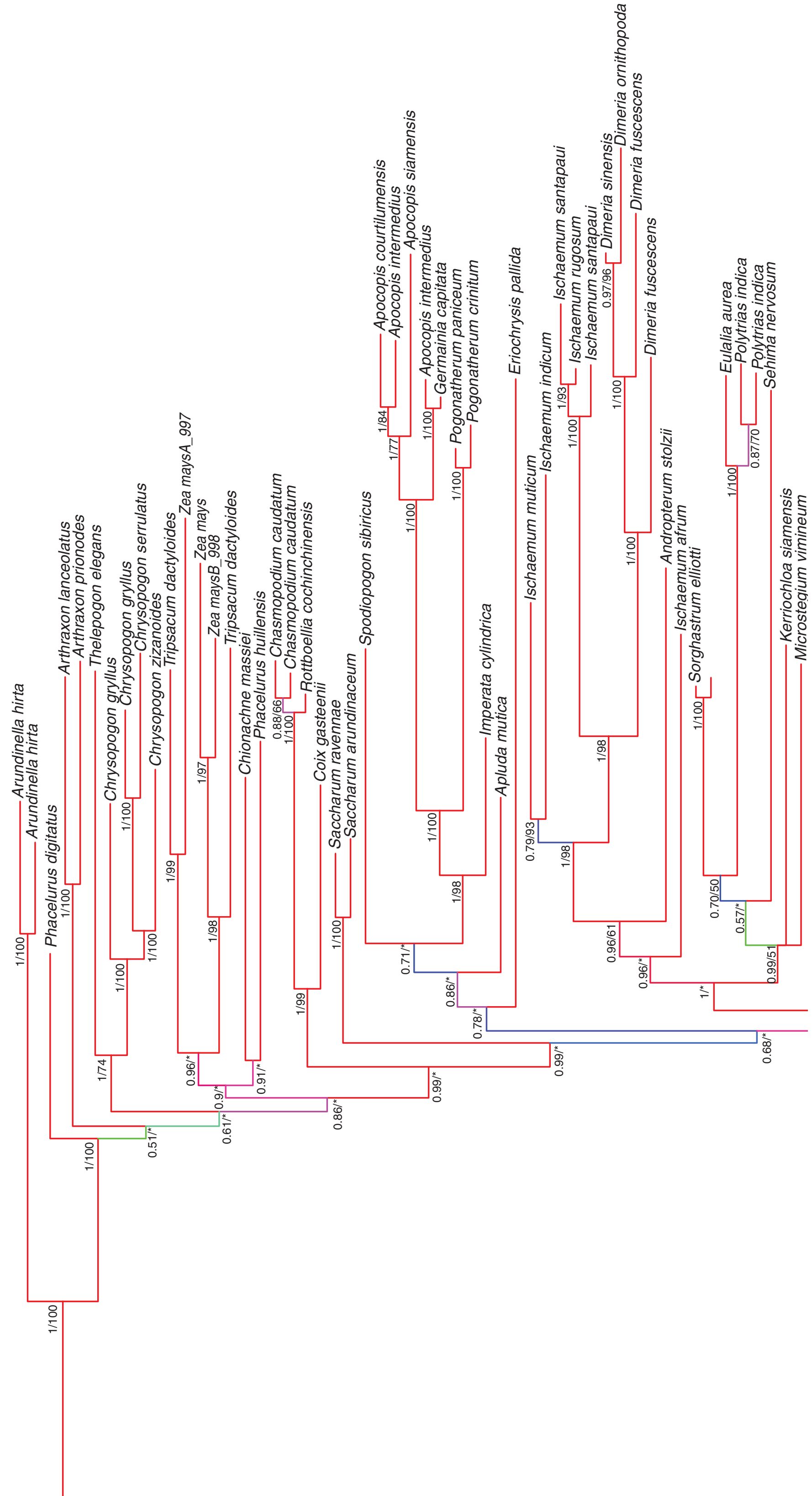
Upon determination of genome-specific paralogues from the individual gene trees, all loci were concatenated into a single data matrix for both maximum likelihood and Bayesian analyses (Fig. 1). Both analyses revealed very strong support for Clade 1 [posterior probability (PP) = 0·99, BS = 80] and Clade II (PP = 1, BS = 100). *Sorghum macrospermum* and *S. laxiflorum* are divided into two basal groups in Clade I, each containing half of the alleles from each species. One of the alleles from an *S. halepense* accession (PI 663975) resolved with *S. propinquum* (PP = 0·99, BS = 59), while the other is sister to *S. bicolor* (PP = 1, BS = 86), as would be expected given the widespread assumption that these are the genome donors to the polyploid *S. halepense* (Paterson *et al*., 1995). In Clade II, there is strong support for the basal placement of *S. purpureosericeum* (PP = 1, BS = 100), a member of the subgenus *Parasorghum*. The only other *Parasorghum* species used in this study, *S. matarankense*, resolved firmly within the *Stiposorghum* and sister to one of the *S. interjectum* alleles (PP = 1, BS = 100). Although support on internal branches throughout Clade II is weak, some relationships were still apparent, such as the sister relationship between *S. intrans* and *S. exstans*, and the resolution of *S. ecarinatum* as sister to the *S. matarankense* + *S. interjectum* clade.

The *Sorghum* data for *APO1*, the two exons from *EP2*, and *D8* were then combined with the same sequence data for numerous members of the Andropogoneae (Estep *et al*., 2014). The topology for the basal Andropogoneae was highly congruent with that of Estep *et al*. (see fig. S2 in Estep *et al*., 2014) with a few minor exceptions (Fig. 2), probably due to the exclusion of sequence data for *Retarded palea1* (*REP1*), which was part of the Estep *et al*. (2014) analysis; however, the inclusion of the additional *Stipo*- and *Parasorghum* species improved internal branch support in this part of the Andropogoneae tree. A sister relationship between *Sorghum* Clade II and the *Miscanthus* + *Saccharum* clade resolves with strong support (PP = 1, BS = 93). *Sorghum* Clades I and II are polyphyletic, with posterior probability support for their separation at 0·67, although bootstrap support at this internal node was <50 %. Nevertheless, there is strong evidence that the *Saccharum* + *Miscanthus* clade is firmly nested within *Sorghum*, and evidence for a sister relationship between Clade I and several members of the core Andropogoneae.

DISCUSSION

Maximum likelihood and Bayesian analyses of the combined eight-locus *Sorghum*-specific data set support interspecific relationships as previously described using various morphological and molecular markers (Garber, 1950; Duvall and Doebley, 1990; Spangler, 2003; Dillon *et al*., 2004, 2007; Price *et al*., 2005; Ng'uni *et al*., 2010; Liu *et al*., 2014). Specifically, *Sorghum* is divided into two major clades, one containing *Eu*/*Chaeto*/*Heterosorghum* (Clade I) and the other composed of the *Stipo*/*Parasorghum* (Clade II). Sub-section *Eusorghum*,

FIG. 1. *Sorghum* phylogenetic tree derived from Bayesian analysis of eight low-copy nuclear loci. For each branch, Bayesian posterior probabilities (PP) and maximum likelihood bootstrap (BS) scores are given in the form of PP/BS. Branch colours indicate higher (red) to lower (green) posterior probability. An asterisk, *, indicates bootstrap scores <50. An × indicates that the maximum likelihood analysis did not support the same topology.



Arundinella hirta
Arundinella hirta
Phacelurus digitatus
Arthraxon lanceolatus
Arthraxon prionodes
Thelepogon elegans
Chrysopogon gryllus
Chrysopogon gryllus
Chrysopogon serrulatus
Chrysopogon zizanoides
Tripsacum dactyloides
Zea maysA_997
Zea mays
Zea maysB_998
Tripsacum dactyloides
Chionachne massiei
Phacelurus huillensis
Chasmopodium caudatum
Chasmopodium caudatum
Rottboellia cochinchinensis
Coix gasteenii
Saccharum ravennae
Saccharum arundinaceum
Spodiopogon sibiricus
Apocopis courtallumensis
Apocopis intermedius
Apocopis siamensis
Apocopis intermedius
Germainia capitata
Pogonatherum paniceum
Pogonatherum crinitum
Imperata cylindrica
Apluda mutica
Eriochrysis pallida
Ischaemum muticum
Ischaemum indicum
Ischaemum santapaui
Ischaemum rugosum
Ischaemum santapaui
Dimeria sinensis
Dimeria ornithopoda
Dimeria fuscescens
Dimeria fuscescens
Andropterum stolzii
Ischaemum afrum
Sorghastrum elliotti
Eulalia aurea
Polytrias indica
Polytrias indica
Sehima nervosum
Kerriochloa siamensis
Microstegium vimineum
1/100
1/100
1/100
1/100
0.51/*
1/74
1/100
1/100
1/100
0.61/*
1/99
0.96/*
1/97
1/98
0.9/*
0.91/*
0.88/66
1/100
0.86/*
1/99
1/100
0.99/*
0.71/*
1/84
1/77
1/100
1/100
1/100
1/100
1/98
0.86/*
0.78/*
0.99/*
0.79/93
1/93
1/100
1/98
1/98
0.97/96
1/100
1/100
0.96/61
0.96/*
1/*
1/100
1/100
0.70/50
0.87/70
0.57/*
0.99/51
0.68/*

FIG. 2. Combined Bayesian tree including four low-copy nuclear loci showing the placement of *Sorghum* within Andropogoneae. Branch support is represented by the Bayesian posterior probability (PP)/maximum likelihood bootstrap (BS) score. Branch colours indicate higher (red) to lower (green) posterior probability. An asterisk, *, indicates bootstrap scores <50. An × indicates that the maximum likelihood analysis did not support the same topology. *Sorghum* Clades I and II are delineated on the right.

which includes the diploids *S. bicolor* and *S. propinquum* and the polyploid *S. halepense*, is strongly supported. *Sorghum halepense*, better known as 'Johnsongrass', is thought to have originated via hybridization between *S. bicolor* and *S. propinquum*, subsequently followed by polyploidization (Paterson *et al*., 1995). Cytogenetic evidence also supports *S. propinquum* as one of the *S. halepense* progenitors (Magoon and Shambulingappa, 1961). We included four *S. halepense* accessions in this study, only one of which clusters with *S. propinquum* with strong support. It should be noted, however, that we originally obtained ten geographically distinct *S. halepense* accessions from the USDA-GRIN seed repository, but during our grow-out we noticed a wide range in both seed and plant morphologies. Therefore, we measured the genome size of each of these accessions and found that only the four accessions used in this study contained the nuclear DNA content expected for *S. halepense* (1C approx. 1600 Mb). This suggests that the phenotypic characteristics associated with *S. halepense* may also easily arise in diploid genotypes, and therefore particular care should be taken in species identification before use in phylogenetic analyses. Further, given that only one of the four *S. halepense* accessions showed a strongly supported relationship with *S. propinquum*, it is also possible that polyploid *S. halepense* has arisen more than once via disparate pathways, and that this convergence may confound inferences regarding its origin.

Sorghum macrospermum and *S. laxiflorum*, the single species belonging to *Chaeto-* and *Heterosorghum*, respectively, are closely related polyploids of $n = 20$ belonging to Clade I. In the work presented here, we show clear genome-specific association of orthologous alleles. Specifically, two of the alleles from *S. macrospermum* cluster with two of the alleles from *S. laxiflorum* with higher support than with the remaining *S. macrospermum* alleles. This implies that these species may have originated from a single polyploidization event or from separate polyploidization events involving the same or similar parental species, and that it is appropriate to merge them into a single subgenus. Although several studies have suggested the merger of these two subgenera (Sun *et al*., 1994; Ng'uni *et al*., 2010), our results provide compelling evidence to support such a reclassification. Further, given the clear interspecific pairing of orthologues, our data do not support the proposal to retain these species as distinct sections within a single sub-section, as suggested by Liu *et al*. (2014). In addition, our results provide strong evidence for the sister relationship of these species with *Eusorghum* (PP = 1, BS = 99), and therefore do not support the proposal by Spangler to classify these species as a distinct genus (*Vacoparis*).

Clade II, composed of *Para-* and *Stiposorghum*, is also strongly supported. Due to low seed viability, only two *Parasorghum* species were included in this study. *Sorghum purpureosericeum* resolved as the basal lineage of Clade II in all of our analyses. This result was expected because (1) *Parasorghum* is considered ancestral to *Stiposorghum*, and (2) *S. purpureosericeum* is the only Clade II taxon included in this study that is endemic to an area outside of Australia. The second *Parasorghum* species, *S. matarankense*, resolved within the *Stiposorghum* with strong support in all of our analyses. Indeed, the two heterozygous *S. matarankense* alleles consistently clustered with *S. interjectum* in all individual locus trees and with *S. interjectum* and the *S. intrans* + *S. exstans* clade in both of our combined loci analyses, suggesting either that *S. matarankense* belongs to *Stiposorghum* or that *Parasorghum* is paraphyletic. We note that *S. matarankense* was originally circumscribed within *Stiposorghum* (Garber, 1950), was included within *Stiposorghum* by Spangler (2003) and was only more recently placed within *Parasorghum*.

The topology within *Stiposorghum* resolved with high posterior probability scores but low bootstrap values, making relationships more difficult to delineate. Nevertheless, some relationships were apparent even in the single gene analyses, and were not only strikingly congruent with the classification of Australian endemics by Lazarides *et al*., and Spangler based on morphology and geographic distribution, but also with the taxonomic treatment of the genus by Garber (Garber, 1950; Lazarides *et al*., 1991, Spangler, 2003). For example, Lazarides discusses polyploid *S. interjectum* and considers it similar to *S. plumosum*, while Spangler considered *S. interjectum* synonymous with *S. plumosum*. Our results show alleles from both species clustering with high support. Lazarides also lists an *S. plumosum* × *S. intrans* hybrid from the Northern Territory (see Table 2), and we also see clustering of one of the *S. plumosum* alleles with the *S. exstans* + *S. intrans* clade. *Sorghum plumosum*, *S. extans* and *S. intrans* are narrowly distributed in the northern part of Australia's Northern Territory, providing further support for these species relationships (see figs 7 and 8 of Spangler, 2003). Finally, Lazarides suggested that *S. intrans*, *S. exstans* and *S. angustum* are the most geographically restricted and morphologically specialized, and that *S. intrans* was probably derived from *S. stipoideum*. Our Bayesian analysis places *S. intrans* + *S. exstans* + *S. angustum* in a clade sister to *S. stipoideum*.

The monophyly of *Sorghum* and its placement within the Andropogoneae has been a contentious topic for several decades, in part due to difficulties in phylogenetic reconstruction in the face of rapid radiation of the tribe leading to continuity in morphological variation (Mathews *et al*., 2002; Estep *et al*., 2014). Early molecular and cytogenetic evidence provided weak support for a para- or polyphyletic origin of *Sorghum*, and suggested that the genus should possibly be reclassified to accommodate distinctions among the subgenera (Duvall and Doebley, 1990; Sun *et al*., 1994; Spangler *et al*., 1999; Dillon *et al*., 2001). Duvall and Doebley noted that the Australian species are highly diverged from the other *Sorghum* species in comparison with divergence rates among other angiosperms, implying that the Australian species may warrant distinct generic status. This idea was echoed by Spangler *et al*. almost 10 years later when analyses of *NDHF* sequences led them to conclude that the Australian group is 'distinct enough to be proposed as a separate taxon'. Shortly thereafter, however, several studies employing a larger number of molecular markers disputed these early findings, and indicated that *Sorghum* is indeed monophyletic, although these studies included a very limited number of non-*Sorghum* species for comparison (Dillon *et al*., 2004, 2007; Price *et al*., 2005; Ng'uni *et al*., 2010; Liu *et al*., 2014).

Our results are congruent with those of Duvall and Doebley, as well as with Spangler *et al*., indicating that *Sorghum* is polyphyletic and supporting reclassification of Clade II (*Para/Stiposorghum*) as a distinct genus, *Sarga* (Duvall and Doebley, 1990; Spangler *et al*., 1999; Spangler, 2003). In both our Bayesian and maximum likelihood analyses, there exists strong

support for a distinction between these clades. The *Sarga* clade is sister to *Saccharum* + *Miscanthus* with a posterior probability score of 1 and bootstrap score of 87 % (Fig. 2). Branch support for the split between this group and the clade containing *Eu*/*Chaeto*/*Heterosorghum* is less convincing, with a posterior probability score of 0·67 but bootstrap score of <50 %. Nevertheless, the sister relationship of *Sarga* with *Miscanthus* + *Saccharum* is evident, and demonstrates a clear polyphyletic relationship within *Sorghum*.

Concluding remarks

The work presented here represents the most comprehensive study of *Sorghum* placement within Andropogoneae to date. Our results are congruent with those of early morphological, cytogenetic and molecular studies arguing that *Sorghum* is polyphyletic. Given the clear polyphyletic placement of *Sorghum*, the distinct base chromosome number for Clade II ($n = 5$) and the strong node support for the sister relationship between Clade II and *Miscanthus* + *Saccharum*, our results support the proposal by Spangler to adopt '*Sarga*' as a distinct genus composed of *Para*- and *Stiposorghum* (Spangler, 2003). Within *Sorghum*, our data suggest paraphyly of *Parasorghum*, but, given the historical variation in placement of *S. matarankense* within the *Stiposorghum*, we can neither support nor refute the monophyly of this sub-section. Our results do, however, suggest that reclassification of *Hetero*- and *Chaetosorghum* as a single sub-section is warranted, but do not support its circumscription as a distinct genus (e.g. *Vacoparis*).

ACKNOWLEDGEMENTS

The authors wish to thank Elizabeth Kellogg, Matt Estep and Mike McKain for providing the Andropogoneae sequence alignment, Bill Rooney for providing *Sorghum propinquum* seed, and Jonah Joffee for his involvement in generating some sequence data for his REU research project. We acknowledge the use of the Super Computing System Spruce Knob at West Virginia University, which is funded in part by the National Science Foundation EPSCoR Research Infrastructure Improvement Cooperative Agreement 1003907, the state of West Virginia (WVEPSCoR via the Higher Education Policy Commission) and West Virginia University. This work was partially funded by NSF-REU DBI 0849917, *Plant Responses to the Environment: From Genes to Ecosystems*, to the Department of Biology at West Virginia University.

LITERATURE CITED

Alvarez I, Wendel JF. 2003. Ribosomal ITS sequences and plant phylogenetic inference. *Molecular Phylogenetics and Evolution* **29**: 417–434.

Celarier RP. 1959. Cytotaxonomy of the Andropogoneae. III. Subtribe Sorgeae, genus *Sorghum*. *Cytologia* **23**: 395–418.

Dahlberg J. 2000. Classification and characterization of *Sorghum*. In: CW Smith, RA Frederiksen, eds. *Crop Science. Sorghum: origin, history, technology, and production*. Chichester, UK: John Wiley & Sons, 99–130.

De Wet JMJ, Harlan JR. 1971. The origin and domestication of *Sorghum bicolor*. *Economic Botany* **25**: 128–135.

De Wet JMJ, Huckabay JP. 1967. The origin of *Sorghum bicolor*. II. Distribution and domestication. *Evolution* **21**: 787–802.

Dillon SL, Lawrence PK, Henry RJ. 2001. The use of ribosomal ITS to determine phylogenetic relationships within *Sorghum*. *Plant Systematics and Evolution* **230**: 97–110.

Dillon SL, Lawrence PK, Henry RJ, Ross L, Price HJ, Johnston JS. 2004. *Sorghum laxiflorum* and *S. macrospermum*, the Australian native species most closely related to the cultivated *S. bicolor* based on ITS1 and ndhF sequence analysis of 25 *Sorghum* species. *Plant Systematics and Evolution* **249**: 233–246.

Dillon SL, Shapter FM, Henry RJ, Cordeiro G, Izquierdo L, Lee LS. 2007. Domestication to crop improvement: genetic resources for *Sorghum* and *Saccharum* (Andropogoneae). *Annals of Botany* **100**: 975–989.

Doggett H. 1988. *Sorghum*, 2nd edn. New York: John Wiley & Sons.

Duvall MR, Doebley JF. 1990. Restriction site variation in the chloroplast genome of *Sorghum* (Poaceae). *Systematic Botany* **15**: 472–480.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**: 1792–1797.

Estep MC, Diaz DV, Zhong J, Kellogg EA. 2012. Eleven diverse nuclear-encoded phylogenetic markers for the subfamily Panicoideae (Poaceae). *American Journal of Botany* **99**: e443–e446.

Estep MC, McKain MR, Diaz DV, *et al.* 2014. Allopolyploidy, diversification, and the Miocene grassland expansion. *Proceedings of the National Academy of Sciences, USA* **111**: 15149–15154.

Garber ED. 1950. Cytotaxonomic studies in the genus *Sorghum*. *University of California Publications in Botany* **23**: 283–361.

Hall TA. 1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Lazarides M, Hacker JB, Andrew MH. 1991. Taxonomy, cytology, and ecology of indigenous Australian *Sorghums* (Sorghum Moench: Andropogoneae: Poaceae). *Australian Systematic Botany* **4**: 591–635.

Liu Q, Liu H, Wen J, Peterson PM. 2014. Infrageneric phylogeny and temporal divergences in *Sorghum* (Andropogonodae, Poaceae) based on low-copy nuclear and plastid sequences. *PLoS One* **9**: e104933.

Magoon ML, Shambulingappa KG. 1961. Karyomorphology of *Sorghum propinquum* and its bearing on the origin of 40-chromosome Sorghum. *Chromosoma* **12**: 460–465.

Mathews S, Spangler RE, Mason-Garner RJ, Kellogg EA. 2002. Phylogeny of Andropogoneae inferred from Phytochrome B, GBSSI, and ndhF. *International Journal of Plant Sciences* **163**: 441–450.

Ng'uni D, Geleta M, Fatih M, Bryngelsson T. 2010. Phylogenetic analysis of the genus *Sorghum* based on combined sequence data from cpDNA regions and ITS generate well-supported trees with two major lineages. *Annals of Botany* **105**: 471–480.

Paterson AH, Schertz KF, Lin Y-R, Liu S-C, Chang Y-L. 1995. The weediness of wild plants: molecular analysis of genes influencing dispersal and persistence of johnsongrass, *Sorghum halepense* (L.) Pers. *Proceedings of the National Academy of Sciences, USA* **92**: 6127–6131.

Price HJ, Dillon SL, Hodnett G, Rooney WL, Ross L, Johnston S. 2005. Genome evolution in the genus *Sorghum* (Poaceae). *Annals of Botany* **95**: 219–227.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Snowden JD. 1935. *A classification of the cultivated Sorghum*. Kew: Royal Botanic Gardens.

Snowden JD. 1936. *The cultivated races of Sorghum*. London: Allard and Son.

Spangler RE. 2003. Taxonomy of *Sarga, Sorghum*, and *Vacoparis* (Poaceae: Andropogoneae). *Australian Systematic Botany* **16**: 279–299.

Spangler RE, Zaitchik B, Russo E, Kellogg EA. 1999. Andropogoneae evolution and generic limits in *Sorghum* (Poaceae) using ndhF sequences. *Systematic Botany* **24**: 267–281.

Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.

Sun Y, Skinner DZ, Liang GH, Hulbert SH. 1994. Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA. *Theoretical and Applied Genetics* **89**: 26–32.

Swofford DL. 2003. *Phylogenetic analysis using parsimony (*and other methods). Version 4*. Sunderland, MA: Sinauer Associates.