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Original Article

Phylogenetic analysis of the genus *sorghum* based on the combined sequence data from *cpDNA* regions and the *ITS* generated strongly bootstrap supported trees with two major lineages.

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1 **ABSTRACT**

2 • *Background and Aims* Wild *Sorghum* species provide novel traits for both biotic and
3 abiotic stress resistance and yield for the improvement of cultivated sorghum. A better
4 understanding of the phylogeny in genus *Sorghum* will enhance use of the valuable
5 agronomic traits found in the wild sorghum.

6 • *Methods* Four regions of chloroplast DNA (cpDNA; *psbZ-trnG*, *trnY-trnD*, *trnY-*
7 *psbM*, and *trnT-trnL*) and the internal transcribed spacer (ITS) of nuclear ribosomal
8 DNA were used to analyze the phylogeny of sorghum based on maximum parsimony.

9 • *Key Results* Parsimony analyses of the ITS and cpDNA regions as separate or
10 combined sequence datasets formed strongly bootstrap supported trees with two
11 lineages, the *Eu-sorghum* species, *S. laxiflorum* and *S. macrospermum* in one and
12 *Stiposorghum* and *Para-sorghum* in the other. Within *Eu-sorghum*, *S. bicolor-3*, 11
13 and 14 originating from southern Africa form a distinct clade. *S. bicolor-2*, originally
14 from Yemen, is distantly related from other *S. bicolor* accessions.

15 • *Conclusion* *Eu-sorghum* species are more closely related to *S. macrospermum* and *S.*
16 *laxiflorum* than to any other Australian wild *Sorghum* species. *S. macrospermum* and
17 *S. laxiflorum* are so closely related that it is inappropriate to classify them in separate
18 section. *S. alnum* is closely associated with *S. bicolor* suggesting that the latter is its
19 maternal parent considering that cpDNA is maternally inherited in angiosperms. *S.*
20 *bicolor-3*, 11 and 14, from southern Africa are closely related but distantly related
21 from *S. bicolor-2*.

22 **Key words:** Molecular phylogeny, *Sorghum* Moench, *Eu-sorghum*, *Zea mays*, non-
23 coding regions, cpDNA, ITS.

24

1 INTRODUCTION

2 *Sorghum* Moench is highly heterogeneous which with *Cleistachne* Bentham form Sorghastrae
3 (Garber 1950), one of the sixteen subtribes belonging to tribe Andropogoneae. Species of the
4 genus *Sorghum* have chromosome numbers of $2n=10, 20, 30$ or 40 (Garber 1950; Lazarides *et*
5 *al.* 1991). There are five recognized sections and twenty five species within *Sorghum*. The
6 sections are *Eu-sorghum*, *Chaetosorghum*, *Heterosorghum*, *Para-sorghum* and *Stiposorghum*
7 (Garber 1950; Lazarides *et al.* 1991). *Eu-sorghum* includes cultivated sorghums and their
8 closest wild relatives (De Wet and Huckay 1967). According to De Wet (1978) three species
9 were recognized in section *Eu-sorghum*; including two perennial species *S. halepense* and *S.*
10 *propinquum* and an annual, *S. bicolor*. However, in the earlier classification by Snowden
11 (1935), *Eu-sorghum* consists of two subsections: *Arundinacea* and *Halepensia*. The
12 subsection *Arundinacea*, commonly found in tropical Africa and India, consists of *S. bicolor*
13 (L.) Moench, *S. arundinaceum* (Desv.) Stapf and *S. drummondii* (Steud.) Millsp. *S.*
14 *propinquum* (Kunth) Hitchcock, *S. halepense* (L.) Pers and *S. alnum* Parodi form subsection
15 *Halepensia*, and found in the Mediterranean region and Southeast Asia.

16
17 The wild Australian *Sorghum* species constitute over two thirds of the recognized *Sorghum*
18 species, of which one species each belong to *Chaetosorghum* and *Heterosorghum*. The
19 section *Para-sorghum* comprises seven species. Of these, five are native to the northern
20 monsoonal Australia, Africa and Asia (Garber 1950; Lazarides *et al.* 1991). *Stiposorghum*
21 consists of ten species that are endemic to northern Australia (Garber 1950; Lazarides *et al.*
22 1991). The wild and weedy *Sorghum* species present a valuable source of agronomic traits
23 such as pest and disease resistance (Kamala *et al.* 2002; Komolong *et al.* 2002; Sharma and
24 Franzmann 2001) for introgression into *S. bicolor*. Exploitation of these valuable traits

1 requires a thorough understanding of the phylogenetic relationships between cultivated
2 sorghum and the wild sorghum genepool.

3

4 The Chloroplast genome is useful in providing information on the inference of the
5 evolutionary patterns and processes in plants (Raubeson and Jansen 2005). The genome has,
6 either solely or combined with other genomes, been widely used for inferring phylogenetic
7 relationships of different taxa including *Hordeum*, *Triticum*, and *Aegilops* -(Gielly and
8 Taberlet 1994), *Guizotia* (Geleta 2007), Solanaceae (Melotto-Passarin *et al.* 2008) and
9 *Sorghum* (Dillon *et al.* 2007). The noncoding chloroplast regions are phylogenetically more
10 informative than the coding regions at lower taxonomic levels because they are under less
11 functional constraints and evolves rapidly (Gielly and Taberlet 1994). One of the cpDNA
12 regions, *trnT-trnL* used in this study was reported to possess enough phylogenetic signals for
13 studies at lower taxonomic levels (Shaw *et al.* 2005).

14

15 The ITS region of the 18S-5.8S-26S nuclear ribosomal DNA (nrDNA) has been commonly
16 used for phylogenetic inference at the generic and infrageneric level in plants. The ITS loci
17 properties that include biparental inheritance, universality of primers, intragenomic uniformity
18 and intergenomic variability merit their utility for purposes of phylogenetic reconstruction
19 (Baldwin *et al.* 1995). The two regions (ITS1 and ITS2) generally evolve more rapidly than
20 coding regions and have shown to be equally informative being able to differentiate between
21 closely related species (Baldwin 1992) and more specifically to resolve phylogenetic
22 relationships of sorghum and related species (Dillon *et al.* 2001; Guo *et al.* 2006; Sun *et al.*
23 1994).

1 This study sought to resolve the phylogenetic relationships between the species within the
2 genus *Sorghum* based on four regions of the cpDNA: *trnY-trnD*, *psbZ-trnG*, *trnY-psbM* and
3 *trnT-trnL* and the ITS of nrDNA and also to evaluate the usefulness of the five non-coding
4 regions of cpDNA in resolving relationships among the closely related species within section
5 *Eu-sorghum*.

6 MATERIALS AND METHODS

7

8 *Plant material*

9 Details of twenty two *Sorghum* species along with genebank germplasm and Genbank
10 sequence accession numbers used in this study are shown in Table 1. The germplasm
11 accessions included the wild sorghum and some cultivated sorghum obtained from the
12 Australian Tropical Crops Genetic resource Centre, Biloela, Australia. In addition, five
13 accessions of *S. bicolor* and one accession of *S. arundinaceum* were obtained from the
14 Zambian National Plant Genetic Resources Centre (ZNPGR).

15

16 *DNA extraction, PCR and sequencing*

17 Each *Sorghum* species was represented by 1-2 accessions, except for *S. bicolor* where eleven
18 accessions were used. Genomic DNA was extracted from fresh leaf tissues of seedlings raised
19 in the green house approximately at two weeks of age using a modified CTAB extraction
20 method (Doyle and Doyle 1987). The quality of the DNA was analysed by agarose gel
21 electrophoresis and DNA concentration was determined using a Nanodrop ® ND-1000
22 spectrophotometer (Saveen Werner, Sweden).

23

1 The primers for amplification and sequencing of the *trnS-trnfM*, *trnY-psbM* and *trnT-trnD*
2 regions were designed for this study while *trnT-trnL* region was amplified and sequenced
3 using the universal primers designed by Taberlet et al., (1991). A primer pair was used for
4 each of the cpDNA regions. However, two primer pairs were designed for the amplification of
5 the *trnY-psbM* region. Universal primers, ITS4 and ITS5 (White *et al.* 1990), were used for
6 the amplification and sequencing of the ITS.

7

8 The sequences of the primers and information on specific primers supplied by Eurofins MWG
9 GmbH used in this study are given in Table 2. A GeneAMP PCR system 9700 thermocycler
10 was used for amplification at the following temperature regime: Denaturation at 94°C for 3
11 min and final 7 min extension at 72°C with intervening 30 cycles of 1 min denaturing at 94°C,
12 1 min primer annealing temperature at 51°C and 2 min primer extension at 72°C. Successfully
13 amplified samples were purified using the QIAquick PCR purification kit (Qiagen GmbH,
14 Germany) and the microcentrifuge according to the manufacturer's instructions. Nine
15 microlitres of purified PCR products was mixed with 1 µl of sequencing primers and sent to
16 the sequencing facility in the University of Oslo, Norway (<http://www.bio.uio.no/ABI-lab/>),
17 where DNA sequencing was done. The quality of the sequences was evaluated using Sequence
18 Scanner version 1.0 (Applied Biosystems) and only high quality sequences were used for the analysis.
19 All regions were sequenced using both forward and reverse primers. The sequences from the
20 forward and reverse primers were aligned for each sample in order to generate consensus
21 sequence. Since the sequences were of high quality, the forward and reverse sequences are in
22 complete agreement, except in few cases. Such few discrepancies were resolved by repeating
23 PCR and sequencing.

24

1 *Sequence alignment and data analyses*

2 The quality of the sequences was visually inspected using Sequence Scanner version 1.0
3 (Applied Biosystems). Multiple sequence alignment was performed using ClustalX version
4 2.1.10 (Larkin *et al.* 2007). The sequences were edited using BioEdit version 7.0.9 (Hall
5 1999) and PAUP* 4.0 Beta 10 was used for phylogenetic analyses. The phylogenetic analyses
6 were approached in three ways. In the first approach, the four non-coding regions of the
7 cpDNA were analyzed separately. In the second approach, a combined analysis included the
8 cpDNA regions and the ITS. In these two approaches gap positions were treated as missing
9 data. In the final approach, a combined analysis of the cpDNA regions and the ITS was
10 undertaken, but to exploit the utility of indel positions, parsimony informative indels were
11 coded as binary characters according to Simmons and Ochoterena (2000). *Zea mays* L.
12 (Genbank U04796) was used as an outgroup species.

13

14 **RESULTS**

15 *Sequence characteristics of the Sorghum species*

16 The sequence characteristics and parsimony analyses based tree statistics of four non-coding
17 regions of cpDNA and ITS have been summarized in Table 3. The aligned sequences derived
18 from all the cpDNA regions and the ITS revealed some differences in sequence length
19 between the *Sorghum* species. The longest sequences were obtained from the *trnY-psbM*
20 spacer ranging from 1028 (*S. drummondii*) to 1053 (*S. exstans*) to nucleotides. The eight *S.*
21 *bicolor* sequences from this spacer exhibited 2-3 nucleotides differences between them.
22 Comparatively, the *psbZ-trnG* spacer provided the shortest sequences that ranged between
23 286 (*Eu-sorghum* species) and 291 (*S. intrans*) nucleotides. The similarity in sequence length
24 between the *Eu-sorghum* species could be attributed to the occurrence of five nucleotide

1 indels within the *psbZ-trnG* intergenic spacer. Indels of similar magnitude at corresponding
2 positions were also observed in *S. laxiflorum* and *S. macrospermum*. Sequence length
3 variations were also observed between *Sorghum* species in the *trnT-trnL* spacer, ranging in
4 number of nucleotides from 684 (*S. arundinaceum*) to 693 (*S. leiocladum* and *S. laxiflorum*).
5 Low sequence length differences of 2 nucleotides in the *trnT-trnL* spacer were observed
6 among the *S. bicolor* accessions. Significant sequence variations arising from transitions and
7 transversions were observed at eight positions which resulted in the discrimination of *S.*
8 *bicolor*-12, *S. bicolor*-13 and *S. bicolor*-14 from the rest of the *S. bicolor* accessions. The
9 sequences derived from *trnY-trnD* spacer were between 318 (*S. amplum*, *S. angustum*) and
10 329 (*S. exstans*) nucleotides. The sequences obtained from the ITS showed narrow length
11 differences between the *Sorghum* species in the range of 528-534 nucleotides. Sequence
12 differences between *Sorghum* species were observed with base substitutions in the ITS1
13 accounting for most of the variation. The *S. bicolor* accessions exhibited sequence length
14 differences arising from a single nucleotide indel in ITS1 region.

15

16 **Parsimony analysis of the ITS sequences**

17 The aligned sequences of the ITS of the nrDNA provided comparatively the highest number
18 of parsimony informative characters (69; 12.8%) of the regions used in this study, which
19 could be attributed to an overall faster rate of base substitutions in the ITS than in the non-
20 coding regions of the chloroplast DNA. The ITS revealed the consistency and retention
21 indices of 0.87 and 0.97 respectively (Table 3). The 50% majority rule consensus of 91 trees
22 is shown in Figure 1. Two lineages A and E were resolved. Lineage A was resolved with
23 strong bootstrap support (100%) that contained the *Eu-sorghum* species (clade B, 100%
24 bootstrap) and clade C with similar bootstrap support containing *S. laxiflorum* and *S.*

1 *macrospermum*. The moderately supported internal clade D (61%) contains unresolved
2 relationships of *S. bicolor* accessions with other *Eu-sorghum* species but excludes *S. bicolor* -
3 2 originally from Yemen. The other lineage, E, with 92% bootstrap support contained the
4 remaining native Australian *Sorghum* species which except for *S. nitidum* are contained in
5 clade F that is moderately bootstrap supported (88%; Figure 1).

6

7 **Analysis of the non-coding regions of cpDNA sequence data**

8 The cpDNA regions, *psbZ-trnG*, *trnY-psbM*, *trnY-trnD* and *trnT-trnL*, revealed some
9 differences in the number of parsimony informative characters, consistency and retention
10 indices (Table 3). The cpDNA data show less homoplasy than the ITS data (Table 3), resulting in
11 more fully resolved 50% majority rule consensus trees and generally greater bootstrap values for
12 various nodes. Comparatively, the *trnY-psbM* spacer provided the highest number of
13 parsimony informative characters, (32; 3.9%). The *psbZ-trnG* region provided the lowest
14 number and percent parsimony informative characters (8; 2.7%). The *trnT-trnL* and *trnY-*
15 *trnD* intergenic spacers generated sequences that had 19 (2.7%) and 12 (3.6%) parsimony
16 informative characters, respectively. As measures of accuracy for the topologies obtained,
17 consistency and retention indices were highest (0.94 and 0.98 respectively) for *psbZ-trnG* on
18 comparison of the cpDNA regions used. The *trnY-psbM* spacer had the lowest consistency
19 index (0.69) and retention index (0.93). The other non coding regions of the cpDNA had the
20 consistency and retention indices in between these ranges. The 50% majority rule consensus
21 of 100 trees most parsimonious trees is shown in Figure 2. Lineage A is resolved includes all
22 the *Eu-sorghum* species, clade B with strong support (100%), *S. laxiflorum* and *S.*
23 *macrospermum* (clade C) with an equal bootstrap support. The strongly bootstrap supported
24 (94%) Clade D includes all the *Eu-sorghum* species but excludes *S. arundinaceum*. The

1 strongly bootstrap supported (96%) internal clade H containing *S. alnum* and *S. bicolor* -2
2 from Yemen excludes *S. drummondii* -2. All wild Sorghum species from Australian except *S.*
3 *laxiflorum* and *S. macrospermum* form the second lineage (lineage J) that has very strong
4 bootstrap support (100%; Figure 2). Clade K with moderate bootstrap support (71%) includes
5 all *Stiposorghum* species and some *Parasorghum* species except *S. leiocladum* and *S. nitidum*.
6 The internal relationships within Clade K are either moderately to strongly supported by
7 bootstrap data (76-95%) or remain unresolved (Figure 2).

8

9 **Combined analysis of cpDNA and ITS sequence data**

10 The combined cpDNA and ITS sequences generated a total of 3096 characters of which 140
11 characters (4.5%) were parsimony informative (Table 3). The maximum parsimony (MP)
12 analysis involving the combined data from the cpDNA regions and the ITS sequence data
13 with the gaps either considered as missing values (Figure 3) or when the gaps are scored as
14 presence or absence characters (not shown), produced two main lineages. Lineage A contains
15 all the *Eu-sorghum* species (clade B) that includes all *S. bicolor* and their immediate wild
16 relatives, *S. x alnum*, *S. halepense*, *S. drummondii* and *S. arundinaceum* with 100% bootstrap
17 support. The other lineage, lineage J, consists of all Australian wild *Sorghum* species except
18 *S. laxiflorum* and *S. macrospermum* with high bootstrap support (Figures 3). *S. laxiflorum* and
19 *S. macrospermum* not only form the single clade (C), with strong bootstrap support but are
20 also more closely related to the *Eu-sorghum* species with 100% bootstrap support than with
21 other Australian wild *Sorghum* species. Within the *Eu-sorghum* section, clade D excludes *S.*
22 *arundinaceum* from the rest of the species but a subgroup comprising *S. halepense*-1, *S.*
23 *drummondii*, *S. alnum* and four accessions of *S. bicolor*-1, 2, 5 and 13 is formed as clade F
24 with 99% bootstrap support (Figure 3). The strongly bootstrap supported (94%) Clade E

1 consists of three accessions of *S. bicolor*-3, 11 and 14. The *S. bicolor* accessions in this clade
2 originated from southern Africa, one accession from Zimbabwe (*S. bicolor*-3) and other two
3 accessions from Zambia. *S. bicolor*-2, an accession from Yemen seems to be distantly related
4 with *S. bicolor* accessions from southern Africa but forms stronger association (clade H) with
5 *S. alnum* with strong bootstrap support (Figure 3).

6

7 *Stiposorghum* and *Para-sorghum* form one clade J with 100% bootstrap support (Clade J;
8 Figure 3). The internal nodes of this particular clade, however, lack strong bootstrap support.
9 Most of the *Para-sorghum* and all the *Stiposorghum* species form clade K with moderate
10 bootstrap support and the two accessions of *S. nitidum* form a single clade (L) with equally
11 moderate bootstrap support (Figure 3). Clade M consists of *S. brachypodium* and *S. exstans*
12 with 95% bootstrap support. *S. intrans* and *S. stipoides* -1 form clade N whereas *S. amplum*
13 and *S. ecarinatum* form clade O but with a moderate bootstrap support of 78% (Figures 3).

14

15 **DISCUSSION**

16 Comparative DNA sequencing has become a widespread tool for inferring phylogenetic
17 relationships and systematic studies as it is relatively fast and convenient. Phylogenetic
18 inference and elucidation of the evolutionary processes that generate biological diversity have
19 been accomplished even at lower taxonomic levels using non-coding regions of the
20 chloroplast genome and the internal transcribed spacers of the nuclear ribosomal DNA
21 (Kårehed *et al.* 2008; Mort *et al.* 2007). In this study, all the five cpDNA primers used in this
22 study successfully amplified the target regions in the *Sorghum* species. Mort *et al.*,(2007)
23 assessed the phylogenetic utility of the ITS and nine rapidly evolving cpDNA loci including
24 *trnS-trnfM*, *-trnD-trnT*, *psbM-trnD* and *trnT-trnL* involving six taxa sets of 13-23 taxa using

1 published primer sequences (Shaw *et al.* 2005). Failure of PCR amplification was reported in
2 *Tolpis* (Asteraceae) and *Chrysosplenium* (Saxifragaceae) with the primer pair, *trnD-trnT*.
3 Attempts to amplify *trnT-trnL* region was not successful in all the taxa used. This implies that
4 successful amplification using published primers for some cpDNA regions of one taxon may
5 not have universal application across taxa. In this study, *trnY-psbM* provided the highest
6 number of parsimony informative characters while *trnT-trnL* and *trnY-trnD* were second and
7 third respectively. Based on the potentially informative characters generated, *trnT-trnL* and
8 *psbM-trnD* were identified as suitable for low taxonomic level phylogenetic studies (Shaw *et*
9 *al.* 2005). Of the cpDNA regions used in this study, *trnY-psbM*, *trnT-trnL* and *trnY-trnD*
10 intergenic spacers were proven to be useful in the inference of phylogenetics at low
11 taxonomic level in general and in the genus *Sorghum* in particular.

12

13 In the ITS analysis, all the *Stiposorghum* and *Para-sorghum* were resolved into a lineage
14 separate from the *Eu-sorghum*, *Heterosorghum* and *Chaetosorghum* species with a strong
15 bootstrap support (92%). Our results are consistent with the findings based on the analysis of
16 the ITS sequences (Dillon *et al.* 2001; Sun *et al.* 1994). However, on the whole the internal
17 relationships between species within section are unresolved (Figure 1). As implied and going by
18 its utility in numerous studies, the ITS is a useful marker for resolving phylogenetic relationships at
19 various taxonomic levels, in particular infrageneric. However, caution need to be taken when
20 analysing ITS sequence data to avoid problems resulting from concerted evolution on the ribosomal
21 DNA arrays. Concerted evolution may homogenize different paralogous gene copies in a genome
22 leading to the loss of all but one of the copies, i.e., different copies may be present in different
23 organisms by chance and consequently this will create disagreement between the gene trees and
24 species trees (A'lvarez and Wendel 2003). A fundamental requirement for historical inference based
25 on nucleic acid or protein sequences is that the genes compared are orthologous as opposed to

1 paralogous. However, there are inherent risks in relying exclusively on rDNA sequences for
2 phylogenetic inferences given the ‘nomadic’ nature of the rDNA loci between inclusion of paralogous
3 genes and exclusion of orthologous comparisons (A'lvarez and Wendel 2003).

4

5 The combined analysis of the cpDNA and ribosomal ITS sequence data as the case when only
6 combined cpDNA dataset was used resolved two major lineages (Figure 2 & 3). In one
7 lineage A, the *Eu-sorghum* species form a clade B with 100% bootstrap support. These results
8 indicate a close association between species within the section *Eu-sorghum*. Our results are in
9 agreement with the findings from an assessment of phylogenetic relationships among
10 *Sorghum* taxa based on 30 allozyme loci (Morden *et al.* 1990), which could not show clear
11 delimitation between the *Eu-sorghum* taxa. Weedy form(s) of sorghum and as an example, *S.*
12 *drummondii* occur wherever cultivated sorghum and *S. arundinaceum* grow sympatrically (De
13 Wet 1978). Sympatric speciation, one of the theoretical models for the phenomenon of
14 speciation, is the genetic divergence of various populations from a single parent species
15 inhabiting the same geographic region, such that these populations become different species.
16 However, our study has shown emergence of two subgroups within *Eu-sorghum* with strong
17 bootstrap support (Figure 2). A strong phylogenetic affinity was obtained between *S. bicolor-*
18 3, an accession from Zimbabwe and three others *S. bicolor* accessions (11, 12 and 14) from
19 Zambia and *S. halepense-1*, as shown in clade E. The other subgroup, clade F, contains all
20 other *S. bicolor* accessions (1, 2, 5 and 13; Figure 2). Within this clade, *S. alnum* is closely
21 associated with *S. bicolor-2*, an accession from Yemen. *S. alnum* is believed to be a recent
22 fertile hybrid between *S. halepense* and *S. bicolor* (Doggett 1970). As the chloroplast
23 genomes are believed to display maternal inheritance in the majority of angiosperms (Keeling

1 2004; Mogensen 1996; Udall and Wendel 2006), our phylogenetic results suggest that *S.*
2 *bicolor* could be the maternal parent of *S. alnum*.

3

4 *S. drummondii*, commonly known as Sudan grass, is believed to be a segregate from a natural
5 hybrid between *S. bicolor* and *S. arundinaceum* and said to have originated in the region from
6 southern Egypt to the Sudan (Hacker 1992). The cultivated species, *S. bicolor* is allied to *S.*
7 *arundinaceum*, which according to Lazarides *et al.*, (1991) is the wild progenitor of *S. bicolor*.
8 This is consistent with our results which place *S. arundinaceum* in close relationship with *S.*
9 *bicolor* with 100% support (Figures 3).

10

11 Various models of the origin of *S. halepense* have been suggested. Generally, the species is
12 believed to have arisen as a segmental allotetraploid derived from the cross of two diploids
13 (n=10) species. Doggett (1970) suggested that *S. halepense* was derived from the rhizomatous
14 perennial, *S. propinquum* and the annual, *S. arundinaceum*. In the allozyme variation study
15 involving *Eu-Sorghum*, *S. halepense* could not be differentiated from *S. bicolor* suggesting
16 that the latter was one of the parental species of *S. halepense* (Morden *et al.* 1990). Our results
17 (Figures 1 and 2) support the suggestion that *S. bicolor* is one of the parents of *S. halepense*.

18 *Eu-sorghum* species are closely related to *S. macrospermum* and *S. laxiflorum* with strong
19 bootstrap support (Figures 3), consistent with the previous reports that were based on
20 combined ITS1/*ndhF/adh1* (Dillon *et al.* 2007) and ITS sequence data (Sun *et al.* 1994). This
21 study has also revealed a very close relationship between *S. macrospermum* and *S. laxiflorum*
22 with 100% support (Figure 1), which suggests the inappropriateness of classifying these
23 species under different sections. The close association between these two species has already

1 prompted a suggestion to combine *Chaetosorghum* and *Heterosorghum* into a single section
2 (Dillon *et al.* 2004; Sun *et al.* 1994), which is strongly supported by this data. The ancestry of
3 cultivated sorghum has not been well understood. Based on the ease of formation of crosses
4 (Doggett 1970) and chromosome morphological similarities (Gu *et al.* 1984) within *Eu-*
5 *sorghum*, it has been assumed that no other sections except *Eu-sorghum* provided the
6 ancestral material for cultivated sorghum (Oosterhout van 1992). However, the close
7 association of *S. macrospermum* and *S. laxiflorum* with section *Eu-sorghum* indicates that
8 there is strong sequence homology among them suggesting that these species are
9 phylogenetically closely related.

10 The phylogenetic relationships among the Australian wild *Sorghum* species have been
11 elaborately described (Dillon *et al.* 2001; Dillon *et al.* 2007; Dillon *et al.* 2004; Price *et al.*
12 2005; Spangler 1997; Spangler 2003; Spangler *et al.* 1999; Sun *et al.* 1994). The internal
13 relationships among the Australian wild Sorghums are moderately bootstrap supported. *S.*
14 *intrans* and *S. stipoides* (2) belonging to section *Stiposorghum* form a clade N with
15 moderate support (Figure 1). These species have also been reported to be comparable in
16 morphology and distribution (Lazarides *et al.* 1991).

17 The analysis of the combined data set involving ITS and cpDNA resulted in a tree that is
18 identical to that inferred from cpDNA alone. Similar results were obtained using the two loci
19 on *Crassula* (Mort *et al.* 2007). In contrast to a cpDNA-based approach, phylogenetic studies
20 using nuclear DNA sequences have traditionally been hampered difficulties distinguishing
21 between orthologous and paralogous sequences (Small *et al.* 2004). The practice of obtaining
22 sequence data from two or more loci that can reasonably provide independent tests of
23 phylogeny is proven means of avoiding obtaining well supported but incorrect phylogenies

1 that is not tracking organismal phylogeny (Mort *et al.* 2007). Chloroplast DNA loci, which are
2 often assumed to be uniparentally inherited and non-recombining, have been extensively used
3 for systematics and phylogenetics. However, the rate of evolution of the cpDNA genome is
4 slower than that of the nuclear genome. Correspondingly, the cpDNA regions that have been
5 used for phylogenetic studies are less variable than the most extensively used nuclear loci,
6 internal transcribed spacers of nuclear ribosomal DNA (ITS) (Mort *et al.* 2007; Small *et al.*
7 2004). It is often difficult to obtain adequate resolution of any phylogeny of closely related
8 taxa using few cpDNA loci due to the low number of phylogenetically informative characters
9 (Rokas *et al.* 2003). Hence, the practice of acquiring sequence data from several loci is a
10 proven means of acquiring a better resolved phylogeny (Mort *et al.* 2007; Rokas and Carroll
11 2005). In this study, the phylogeny of the genus *Sorghum* is well resolved when the combined
12 data from *ITS* and four cpDNA regions were used.

13

14 **Conclusion**

15 The cpDNA regions used in this study have shown ability to infer phylogenetic relationships
16 even at low taxonomic level. The *trnY-psbM*, *trnT-trnL* and *trnY-trnD* intergenic spacers
17 have specifically been identified to be more useful in inferring phylogenetics even at
18 infraspecies level. The close relationship between *S. macrospermum* and *S. laxiflorum* suggest
19 the inappropriateness of classifying them under different sections and thus the result strongly
20 back the proposal for merging of sections *Chaetosorghum* and *Heterosorghum*. The results
21 also indicated that the *Eu-sorghum* species are more closely related with *S. macrospermum*
22 and *S. laxiflorum* than with any other Australian wild *Sorghum* species. *S. alnum* is more
23 closely associated with *S. bicolor* than with *S. halepense*, its known parents. As the
24 chloroplast genome is maternally inherited, the results suggest that *S. bicolor* is the most

1 probable maternal parent of *S. alnum*. The *S. bicolor* accessions (3, 11 and 14) from southern
2 Africa form a distinct and strong bootstrap supported clade. *S. bicolor*-2 originally from
3 Yemen is distantly related to other *S. bicolor* accessions in this study. These results have
4 indications of existence of opportunities for utilization of sorghum gene pools outside the
5 section *Eu-sorghum* for the cultivar development and improvement.

6

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13

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6

7

8 **Figure legends**

9 Figure 1. The 50% majority rule consensus tree (1000 bootstrap replicates with 100 random
10 additions; MaxTrees = 100) the parsimonious tree generated from a phylogenetic analysis
11 DNA sequence data from the internal transcribed spacers of the nrDNA of twenty one
12 *Sorghum* species and *Zea mays* as an outgroup species. The indels are treated as missing data.
13 The letters below the branch denote clade. Bootstrap values greater than 50% are indicated
14 above the branches.

15

16 Figure 2. The 50% majority rule consensus tree (1000 bootstrap replicates with 100 random
17 additions; MaxTrees = 100) the parsimonious tree generated from a phylogenetic analysis
18 DNA sequence data from the four cpDNA of twenty one *Sorghum* species and *Zea mays* as an
19 outgroup species. The indels were coded as binary characters and included in the analysis.
20 The letters below the branch denote clade. Bootstrap values greater than 50% are indicated
21 above the branches.

22 Figure 3. The 50% majority rule consensus tree (1000 bootstrap replicates with 100 random
23 additions; MaxTrees = 100) the parsimonious tree generated from a phylogenetic analysis
24 DNA sequence data from the four cpDNA regions and the internal transcribed spacers of the
25 nrDNA of twenty one *Sorghum* species and *Zea mays* as an outgroup species. The indels were
26 coded as binary characters and included in the analysis. The letters below the branch denote
27 clade. Bootstrap values greater than 50% are indicated above the branches.

28

1 Table 1: Accession identity and geographic origin of each accession of *Sorghum* species used in the
 2 study

Species	Section	Germplasm accession number	DNA sequence accession number				
			trnY-trnD	psbZ-trnG	trnY-psbM	trnT-trnL	ITS
<i>S. alnum</i>	<i>Eu-sorghum</i>	AusTRCF302386 ^A	GQ121828	GQ121769	GQ121810	GQ121791	GQ121750
<i>S. amplum-1</i>	<i>Stiposorghum</i>	AusTRCF302455 ^A	N/A	N/A	N/A	N/A	N/A
<i>S. amplum-2</i>	<i>Stiposorghum</i>	AusTRCF302623 ^A	GQ121822	GQ121755	GQ121799	GQ121783	GQ121727
<i>S. angustum-1</i>	<i>Stiposorghum</i>	AusTRCF302588 ^A	GQ121824	N/A	GQ121793	GQ121775	GQ121737
<i>S. angustum-2</i>	<i>Stiposorghum</i>	AusTRCF302606 ^A	N/A	GQ121761	N/A	N/A	N/A
<i>S. arundinaceum</i>	<i>Eu-Sorghum</i>	ZMB 7203 ^{Zm}	GQ121832	GQ121766	GQ121806	GQ121790	GQ121746
<i>S. bicolor-1</i>	<i>Eu-Sorghum</i>	AusTRCF304111 ^{TA}	N/A	N/A	N/A	N/A	N/A
<i>S. bicolor-2</i>	<i>Eu-Sorghum</i>	AusTRCF304113 ^{YA}	N/A	N/A	N/A	N/A	GQ121748
<i>S. bicolor-3</i>	<i>Eu-Sorghum</i>	AusTRCF304114 ^{ZwA}	N/A	N/A	N/A	N/A	N/A
<i>S. bicolor-4</i>	<i>Eu-Sorghum</i>	AusTRCF304115 ^{BA}	N/A	N/A	N/A	N/A	GQ121745
<i>S. bicolor-5</i>	<i>Eu-Sorghum</i>	AusTRCF312813 ^{ZmA}	N/A	N/A	N/A	N/A	N/A
<i>S. bicolor-14</i>	<i>Eu-Sorghum</i>	ZMB 5395 ^{Zm}	N/A	N/A	N/A	N/A	N/A
<i>S. bicolor-12</i>	<i>Eu-Sorghum</i>	ZMB 5757 ^{Zm}	GQ121829	GQ121770	GQ121813	GQ121792	GQ121743
<i>S. bicolor-15</i>	<i>Eu-Sorghum</i>	ZMB 6665 ^{Zm}	N/A	N/A	N/A	N/A	N/A
<i>S. bicolor-10</i>	<i>Eu-Sorghum</i>	ZMB 7016 ^{Zm}	N/A	N/A	N/A	N/A	GQ121744
<i>S. bicolor-11</i>	<i>Eu-Sorghum</i>	ZMB 7034 ^{Zm}	N/A	N/A	N/A	N/A	N/A
<i>S. bicolor-13</i>	<i>Eu-Sorghum</i>	ZMB 7112 ^{Zm}	N/A	N/A	N/A	N/A	N/A
<i>S. brachypodium-1</i>	<i>Stiposorghum</i>	AusTRCF302480 ^A	GQ121818	GQ121756	GQ121802	GQ121774	GQ121736
<i>S. brachypodium-2</i>	<i>Stiposorghum</i>	AusTRCF302481 ^A	N/A	N/A	N/A	N/A	N/A
<i>S. bulbosum-1</i>	<i>Stiposorghum</i>	AusTRCF302418 ^A	N/A	N/A	N/A	N/A	N/A
<i>S. bulbosum-2</i>	<i>Stiposorghum</i>	AusTRCF302646 ^A	GQ121823	GQ121758	QG121803	GQ121781	GQ121732
<i>S. drummondii-1</i>	<i>Eu-Sorghum</i>	AusTRCF300263 ^{EA}	N/A	N/A	N/A	N/A	N/A
<i>S. drummondii-2</i>	<i>Eu-Sorghum</i>	AusTRCF300264 ^{KA}	GQ121831	GQ121765	GQ121809	GQ121789	GQ121747
<i>S. ecarinatum-1</i>	<i>Stiposorghum</i>	AusTRCF302450 ^A	GQ121821	GQ121754	GQ121800	GQ121784	GQ121730
<i>S. ecarinatum-2</i>	<i>Stiposorghum</i>	AusTRCF302662 ^A	N/A	N/A	N/A	N/A	N/A
<i>S. exstans-1</i>	<i>Stiposorghum</i>	AusTRCF302401 ^A	N/A	N/A	N/A	N/A	N/A
<i>S. exstans-2</i>	<i>Stiposorghum</i>	AusTRCF302473 ^A	GQ121816	GQ121759	GQ121796	GQ121782	GQ121735
<i>S. halepense-1</i>	<i>Eu-Sorghum</i>	AusTRCF300167 ^A	GQ121830	GQ121768	GQ121808	GQ121788	N/A
<i>S. halepense-2</i>	<i>Eu-Sorghum</i>	AusTRCF300188 ^A	N/A	N/A	N/A	N/A	GQ121749
<i>S. interjectum-1</i>	<i>Stiposorghum</i>	AusTRCF302396 ^A	GQ121817	GQ121753	GQ121797	GQ121772	GQ121738
<i>S. interjectum-2</i>	<i>Stiposorghum</i>	AusTRCF302433 ^A	N/A	N/A	N/A	N/A	N/A
<i>S. intrans</i>	<i>Stiposorghum</i>	AusTRCF302390 ^A	GQ121825	GQ121752	GQ121795	GQ121780	GQ121733
<i>S. laxiflorum-1</i>	<i>Heterosorghum</i>	AusTRCF302503 ^A	GQ121833	GQ121771	GQ1218011	GQ121786	GQ121741
<i>S. laxiflorum-2</i>	<i>Heterosorghum</i>	AusTRCF302607 ^A	N/A	N/A	N/A	N/A	N/A
<i>S. leiocladum-1</i>	<i>Para-sorghum</i>	AusTRCF300148 ^A	GQ121814	N/A	GQ121805	N/A	N/A
<i>S. leiocladum-2</i>	<i>Para-sorghum</i>	AusTRCF300170 ^A	N/A	GQ121763	N/A	GQ121778	GQ121739
<i>S. macrospermum</i>	<i>Chaetosorghum</i>	AusTRCF302367 ^A	GQ121834	GQ121767	GQ121812	GQ121787	GQ121742
<i>S. matarankense-1</i>	<i>Para-sorghum</i>	AusTRCF302521 ^A	GQ121826	GQ121757	GQ121804	GQ121776	GQ121731
<i>S. matarankense-2</i>	<i>Para-sorghum</i>	AusTRCF302636 ^A	N/A	N/A	N/A	N/A	N/A
<i>S. nitidum-1</i>	<i>Para-sorghum</i>	AusTRCF302539 ^A	N/A	N/A	N/A	GQ121785	N/A
<i>S. nitidum-2</i>	<i>Para-sorghum</i>	AusTRCF302558 ^A	GQ121815	GQ121764	GQ121807	N/A	GQ121740
<i>S. plumosum-1</i>	<i>Stiposorghum</i>	AusTRCF302399 ^A	GQ121819	GQ121762	GQ121798	N/A	N/A
<i>S. plumosum-2</i>	<i>Stiposorghum</i>	AusTRCF302489 ^A	N/A	N/A	N/A	GQ121773	GQ121729
<i>S. plumosum-3</i>	<i>Stiposorghum</i>	AusTRCF302635 ^A	N/A	N/A	N/A	N/A	N/A
<i>S. stipoides-1</i>	<i>Stiposorghum</i>	AusTRCF302393 ^A	GQ121827	GQ121751	GQ121794	N/A	GQ121734
<i>S. stipoides-2</i>	<i>Stiposorghum</i>	AusTRCF302669 ^A	N/A	N/A	N/A	GQ121779	N/A
<i>S. timorensis-1</i>	<i>Para-sorghum</i>	AusTRCF302381 ^A	GQ121820	GQ121760	GQ121801	GQ121777	GQ121727
<i>S. timorensis-2</i>	<i>Para-sorghum</i>	AusTRCF302459 ^A	N/A	N/A	N/A	N/A	N/A

3 The two capitalized letter superscripts at the end of the accession number denote country of origin and donor of
 4 that particular accession. A single letter means the country is a donor and origin of the accession. A=Australia;
 5 B=Burundi; E=Ethiopia; K=Kenya; T=Tanzania; Y=Yemen, Zm=Zambia and Zw=Zimbabwe.
 6
 7
 8

1 Table 2. Primers used to amplify and sequence the five non-coding regions of cpDNA and the ITS of
 2 nrDNA

Region of cpDNA	Primer name	Primer sequence (5'→3')	Source of primer sequences
<i>psbZ-trnG</i>	tnSM –fw ²	TGC TTC TCC TGA TGG TTG GT	This study
	tnSM –rv ²	GCT CGC TAC ATT GAA CTA CGC	
<i>trnY-psbM</i>	psBD –fw ¹	CTG TCA AGG CGG AAG CTG	This study
	psBD –rv ²	GGG TCA CAT AGA CAT CCC AAT	
	trYB –fw ²	GGT TAA TGG GGA CGG ACT	
	trYB –rv ²	AGG AAG TTA AGA TGA GGG TGG	
<i>trnY-trnD</i>	trTD –fw ²	TGA CGA TAT GTC TAC GCT GGT	This study
	trTD –rv ¹	AAT CCC TGC GGG GTG TAT	
<i>trnT-trnL</i>	trTL –fw ²	CAT TAC AAA TGC GAT GCT CT	(Taberlet <i>et al.</i> 1991))
	trTL –rv ²	TCT ACC GAT TTC GCC ATA TC	
ITS	ITS5 –fw ²	GGA AGT AAA AGT CGT AAC AAG G	(White <i>et al.</i> 1990)
	ITS4 –rv ²	TCC TCC GCT TAT TGA TAT GC	

3 ¹ Primer was used for amplification only

4 ² primer used for both PCR amplification and sequencing

5

6 Table 3: Sequence characteristics and tree statistics of the cpDNA and ITS regions from maximum
 7 parsimony (MP) analysis

	cpDNA regions					Combined cpDNA regions	Combined cpDNA regions and ITS
	<i>psbZ-trnG</i>	<i>trnY-trnD</i>	<i>trnY-psbM</i>	<i>trnT-trnL</i>	ITS		
LAS	286-291	318-329	1028-1053	684-693	528-534	2316-2366	2844-3111
PICs^a	8(2.7%)	12(3.6%)	32(3.9%)	19(2.7%)	69(12.8%)	71(3.0%)	140(4.5%)
TL	16	48	101	57	190	536	743
CI	0.9375	0.8958	0.6931	0.8947	0.8737	0.6250	0.6743
HI	0.0625	0.1048	0.31	0.1053	0.1263	0.3750	0.3257
RI	0.9846	0.9734	0.93	0.9757	0.9764	0.8463	0.8938
RC	0.9231	0.8720	0.6489	0.8730	0.8531	0.5252	0.6027

8 ^a Inclusive of the outgroup.

9 LAS=Length of aligned sequences.

10 PICs=Parsimony informative characters (number & percent).

11 TL=Tree length.

12 CI=Consistency index.

13 HI=Homoplasy index.

14 RI=Retention index.

15 RC=Rescaling consistency index.

16

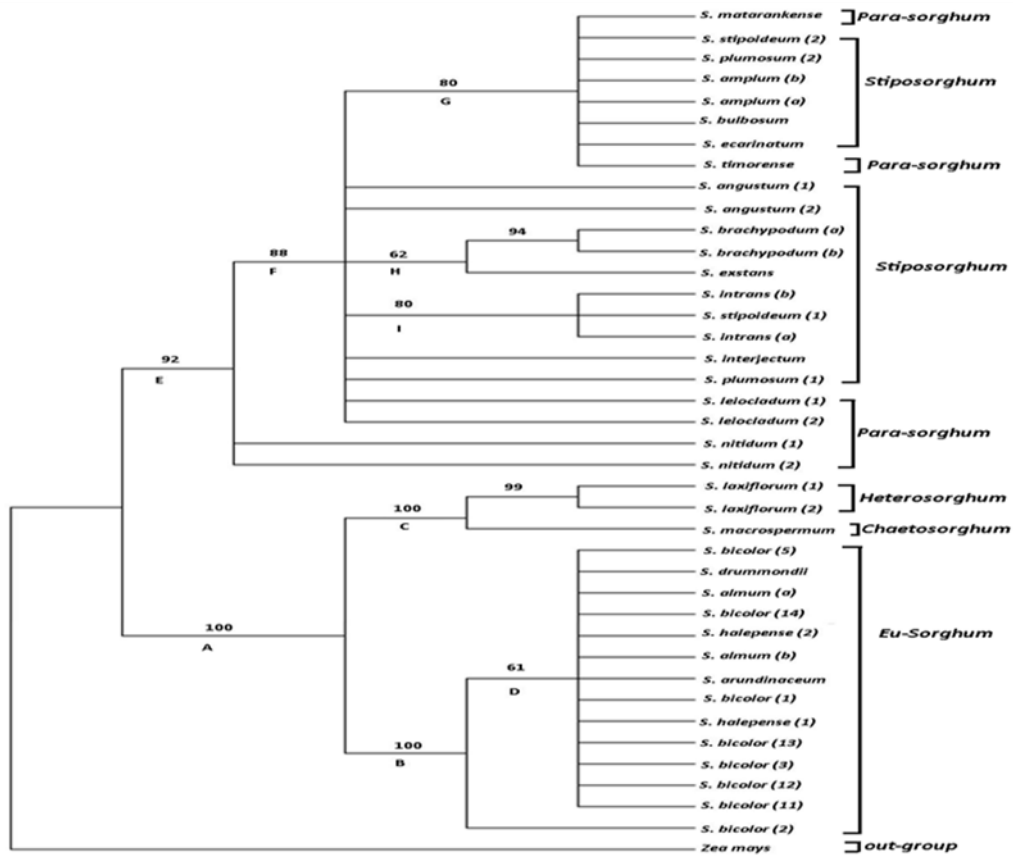


Figure 1: The 50% majority rule consensus tree of 96 most parsimonious trees (1000 bootstrap replicates with 100 random additions; MaxTrees = 100) generated from phylogenetic analysis of the ITS sequence data of twenty one *Sorghum* species and *Zea mays* as an outgroup species. The indels are treated as missing data. The letters below the branch denote clade. Bootstrap values greater than 50% are indicated above the branches.

1

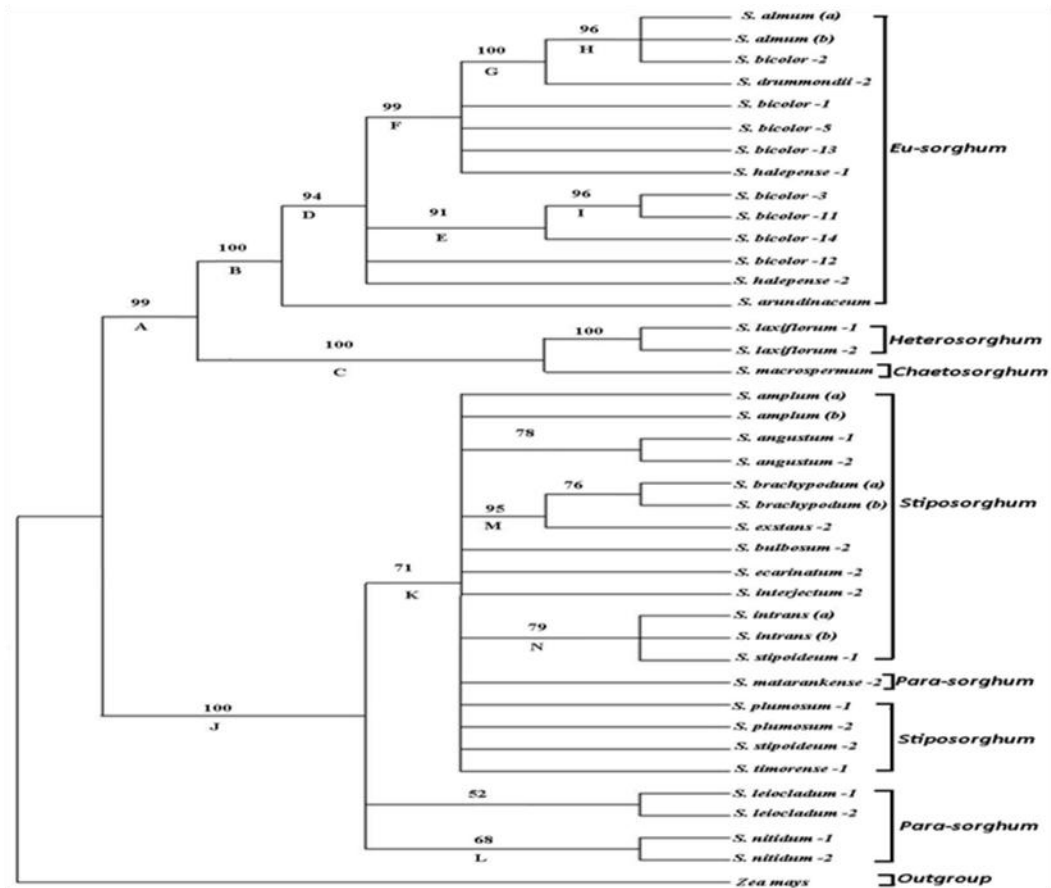


Figure 2 The 50% majority rule consensus tree of the 100 most parsimonious tree (1000 bootstrap replicates with 100 random additions; MaxTrees = 100) generated from phylogenetic analysis of sequence data from the four cpDNA regions of twenty one *Sorghum* species and *Zea mays* as an outgroup species. The indels are treated as missing data. The letters below the branch denote clade. Bootstrap values greater than 50% are indicated above the branches.

1

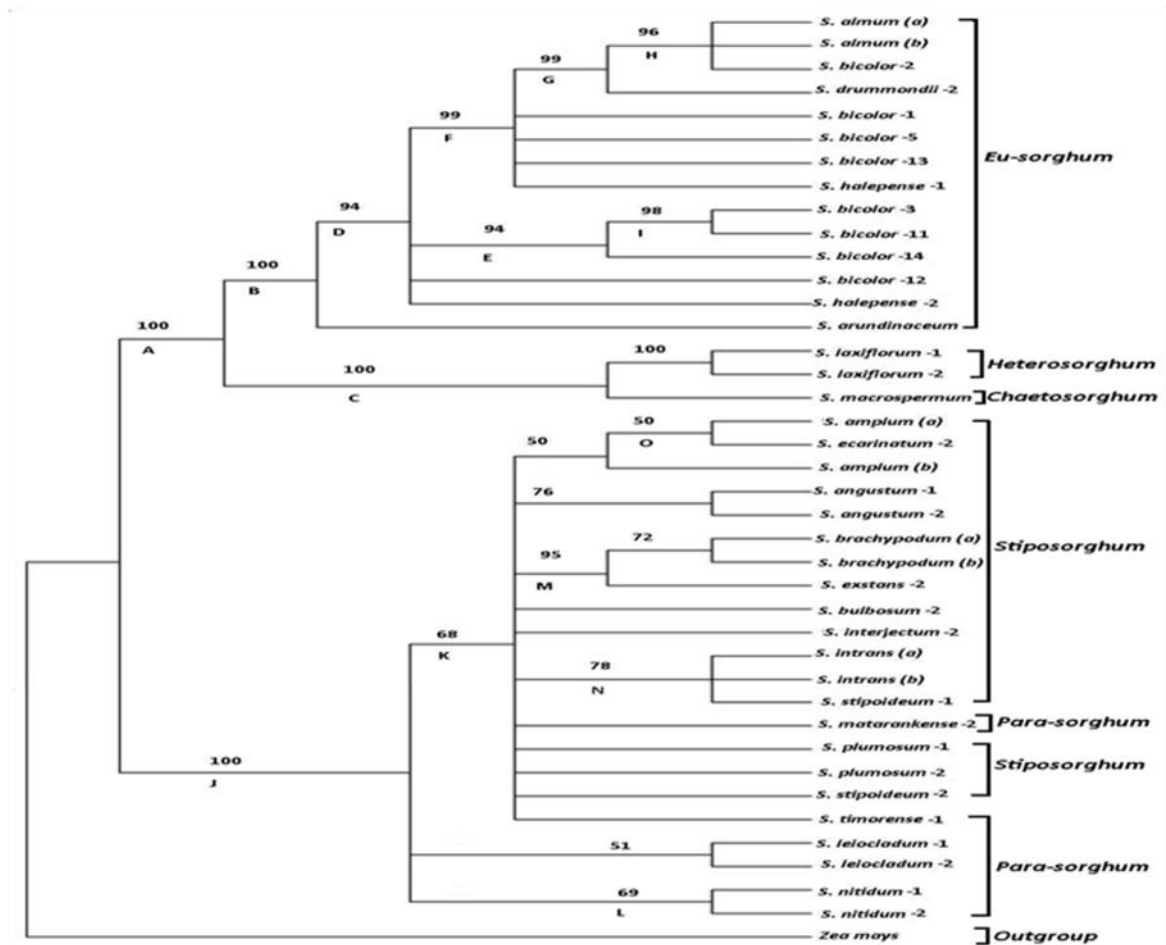


Figure 3 The 50% majority rule consensus tree of parsimonious tree (1000 bootstrap replicates with 100 random additions; MaxTrees = 100) generated from phylogenetic analysis of combined sequence data from the four cpDNA regions and the ITS of the nrDNA of twenty one *Sorghum* species and *Zea mays* as an outgroup species. The indels are treated as missing data. The letters below the branch denote clade. Bootstrap values greater than 50% are indicated above the branches.

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