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Low-copy nuclear gene and McGISH resolves polyploid history of *Eleusine coracana* and morphological character evolution in *Eleusine*

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Abstract: *Eleusine coracana* (L.) Gaertn. (finger millet) is the third most important cereal crop in semiarid regions of the world, but the degree of relatedness of finger millet with other species in the genus is unverified. The study of morphological character evolution in *Eleusine* Gaertn. has lagged behind due to lack of relevant research. Polyploidy history of finger millet was analyzed using *waxy* sequences together with multicolor genomic in situ hybridization (McGISH). In the *waxy* phylogenetic tree, sequences of 2 homoeologous loci were isolated from 3 tetraploids: *Eleusine coracana*, *E. africana* Kenn.-O'Byrne, and *E. kigeziensis* S.M.Phillips. The 3 species grouped with *E. indica* (L.) Gaertn. and *E. tristachya* (Lam.) Lam. in the W-A1 clade (A-type sequences). The W-B1 and W-B2 clades (B-type sequences) were composed of the *E. africana*-*E. coracana* and the *E. jaegeri* Pilg.-*E. kigeziensis* subclades, respectively. *Eleusine indica* probes produced stronger signals of relative intensities across A-genome chromosomes of finger millet than did the *E. tristachya* probes. The *waxy* phylogenetic tree and McGISH evidence support *E. indica* and *E. tristachya* (or its extinct sister or ancestor) as the primary and secondary A-genome parents for finger millet, respectively. The most likely scenario is that the B-genome donor is extinct. Five morphological characters were found to be homoplasious by optimization on *waxy* gene tree.

Key words: Genome parents, multicolor genomic in situ hybridization, phylogenetic analysis

1. Introduction

Hybridization resulting in the formation of allopolyploid species is a frequent mode of plant speciation and evolution (Soltis and Soltis, 1999; Paun et al., 2009), and 24 % of crops are estimated to have arisen as a result of hybridization events (Meyer et al., 2012). In comparison to the enormous number of molecular phylogenetic studies, relatively few studies have attempted to disentangle the history of allopolyploid crops (Brassac et al., 2012; Triplett et al., 2012), perhaps because of the challenges of working with low-copy nuclear (LCN) markers. Thus, there is a need to estimate the degree of relatedness of crops and their relatives to enable limited conservation resources to be focused on priority species.

Eleusine Gaertn. (Eleusininae, Chloridoideae, Poaceae) includes 6 diploid and 3 tetraploid species and exhibits considerable morphological diversity. The genus is distributed in East Africa and the Americas (Phillips, 1995; Liu and Peterson, 2010), with 7 species confined to East Africa; a single species, *Eleusine tristachya* (Lam.) Lam., endemic to the Americas; and a widespread species, *E. indica* (L.) Gaertn., found in the tropics and subtropics (Phillips, 1972; Lovisolo and Galati, 2007). *Eleusine semisterilis* S.M.Phillips is known in Kenya only from the holotype (Phillips, 1972). The genus is characterized by the annual and perennial habit, panicles with digitately to racemosely arranged branches and subsessile spikelets, and caryopses with simple verrucate or compound reticulate sculpturing (Phillips, 1974; Liu et al., 2007; Jiang et al., 2011). Variation in the palaeoclimate of East Africa has been proposed as an explanation for the rapid diversification of allotetraploids in *Eleusine* (Hilu and Johnson, 1997; Liu et al., 2011).

Eleusine coracana (L.) Gaertn. is an allotetraploid species of enormous agricultural significance; it has an annual production of 4.5×10^6 t and represents the third most important cereal crop in semiarid regions of the world (Barbeau and Hilu, 1993; National Research Council, 1996). However, the putative A-genome parents involved in the evolution of finger millet remain controversial

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(Bisht and Mukai, 2001; Neves et al., 2005; Liu et al., 2011). An ITS phylogenetic tree suggested *E. indica* as the A-genome (maternal) parent of *E. coracana* (Neves et al., 2005). This was also supported by single-probe genomic in situ hybridization (GISH), cpDNA restriction sites, and isozyme analyses (Hilu, 1988; Werth et al., 1994; Bisht and Mukai, 2001, 2002). Recent research based on a biparentally inherited nuclear *Pepc4* gene tree and a maternally inherited plastid 6-gene tree has identified the *E. indica-E. tristachya* clade as possible A-genome progenitors in the formation of *E. coracana* (Liu et al., 2011), yet the degree of relatedness of finger millet and these 2 species was not assessed. Therefore, phylogenetic reconstruction in conjunction with multicolor genomic in situ hybridization (McGISH) can be applied to estimate the degree of relatedness of crops and relatives where the gene pool concept is not understood (Hodkinson et al., 2002; Mahelka and Kopecký, 2010).

Eleusine is morphologically diverse in characters associated with the growth habit, spikelets, and caryopses (Phillips, 1972, 1974, 1995; Liu and Peterson, 2010). Morphological evolution in the genus has not been explored within a phylogenetic framework at the infrageneric level. Two traditional taxonomic groups have been recognized on the basis of growth habit and spikelet characters: an annual group with lower glumes that are 1–3-nerved with winged keels, and a perennial group with glumes that are usually 1-nerved without winged keels (Phillips, 1972). Neither group can be defined in terms of consistent caryopsis characters, although these are very useful for separating the species morphologically. For instance, the annual *E. coracana* can be distinguished from the other 2 annual tetraploids, *E. africana* Kenn.-O'Byrne and *E. kigeziensis* S.M.Phillips, by having globose caryopses with a depressed black hilum and a surface that is finely striate-punctate (Phillips, 1972). In another example, *E. tristachya* can be easily distinguished from *E. indica* by its prominently pointed lemmas, lemmas with a more rounded back, and neatly arranged spikelets being perpendicular to the axis (Hilu, 1980, 2003), although caryopses of *E. indica* and *E. multiflora* Hochst. ex A.Rich. shared the flat ventral face, the grooved hilum, and the compound reticulate sculpturing (Jiang et al., 2011). By contrast, the perennial species display a greater degree of caryopsis diversity than the annual species in hilum shape, ventral face, and compression type (Liu and Peterson, 2010). Therefore, the interspecific phylogenetic relationships are quite confusing due to the morphological diversity. Nevertheless, the biological processes that led to the morphological diversity in *Eleusine* remain unverified.

The granule-bound starch synthase I gene (*waxy* or *GBSSI*) encodes the GBSSI enzyme for amylose synthesis in higher plants (Mason-Gamer et al., 1998, 2010; McIntyre et al., 2008). It has been previously used for accurate phylogenetic assessments in Poaceae (Kellogg and Bennetzen, 2004; Mason-Gamer, 2008; Mahelka and Kopecký, 2010). In this study, sequences of 2 homoeologous loci were isolated from the 3 allotetraploids. The present study aims to: 1) unravel the polyploid history of finger millet, especially to assess the phylogenetic relationships between *E. coracana* and its diploid genome donors using a *waxy* phylogeny together with McGISH evidence; and 2) evaluate the homology of morphological characters in *Eleusine* using the *waxy* gene tree.

2. Materials and methods

2.1. Plant sampling and sequencing

Nine species encompassing the morphological diversity and geographical distribution of *Eleusine* plus 4 species of allied genera as outgroups based on a previous study (Peterson et al., 2010) were included in this study. Materials were collected under the auspices of the ILCA and USDA or originated from specimen collections. Flowering plants were verified by identification, and voucher materials were deposited at IBSC and US (Table 1). The primer combination of *waxy*-8F (5'- AGCAAGGACAAGTACATCGC-3') and *waxy*-11R (5'- ACGACAGTGTCGACGAGTCCA -3') was designed based on sequences from *Eragrostis tef* (Zucc.) Trotter, *Dactyloctenium aegyptium* (L.) Willd., and *Spartina spartinae* (Trin.) Merr. ex Hitchc.

Total genomic DNA was extracted from silica-dried leaves using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. Polymerase chain reactions (PCRs) were performed using 2 μL of genomic DNA (10 ng μL^{-1}), 0.6 μL of each primer (10 pmol), 2.5 μL of dNTPs (10 mM), 1 μL of dimethyl sulfoxide, 1 μL of $MgCl_2$ (2.5 mM), 1 U of Taq polymerase (Bioline, Randolph, MA, USA), 2.5 μL of 10X Mg^{2+}-free reaction buffer, and 0.5 μL of bovine serum albumin (10 mg mL^{-1}) made up to 25 μL under the following protocol: 95 °C/3 min; 15 cycles of 94 °C/20 s, 65 °C /40 s with descending 1 °C/cycle, and 72 °C/90 s; 20 cycles of 94 °C/20 s, 50 °C/40 s, and 72 °C/90 s; and 72 °C/5 min, ending with 4 °C holding.

Amplified PCR products were purified using the PEG method (Hiraishi et al., 1995). Cycle sequencing reactions were conducted in a volume of 10 μL containing 0.25 μL of BigDye 3.1, 0.5 μL of primer, 2.0 μL of purified PCR product, and 1.75 μL of sequencing buffer. The sequencing reactions were run on an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Direct sequencing revealed 2 homoeologues for 3 tetraploids. Therefore, the purified PCR products of 2 LCN loci of as many *Eleusine* accessions as possible (Table 1) were cloned into pCR®4-TOPO vectors and transformed into *Escherichia coli* TOP10 competent cells following the

Table 1. Samples, vouchers, and GenBank accession numbers of nuclear gene (*waxy*) in *Eleusine* and allied genera in this study.

Eleusine africana Kenn.-O'Byrne (2*n* = 36, AABB)
Qing Liu 090 (Columbus 2842); RSA-POM; Mexico; (A1 JN378851, A2 JN378854, A3 JN378852, A4 JN378855, A5 JN378853, B1 JN378867, B2 JN378870, B3 JN378868, B4 JN378869, B5 JN378871)
Eleusine coracana (L.) Gaertn. (2*n* = 36, AABB)
Qing Liu 093 (ILCA 13733); US and IBSC; Ethiopia; (A1 JN378857, A2 JN378856, A3 JN378858, B1 JN378862, B2 JN378863, B3 JN378864, B4 JN378865, B5 JN378866)
Eleusine floccifolia (Forssk.) Spreng. (2*n* = 18, BB)
Qing Liu 099 (ILCA 15383); US and IBSC; Ethiopia; (1 JN378874, 2 JN378875, 3 JN378876)
Eleusine indica (L.) Gaertn. (2*n* = 18, 4*n* = 36, AA)
Qing Liu 092 (ILCA 1042); US and IBSC; Burundi; (1 JN378861, 2 JN378859, 3 JN378860)
Eleusine intermedia (Chiov.) S.M.Phillips (2*n* = 18, AB)
Bogdan 5522; K; Tanzania; (1 JN378836)
Bogdan AB4783; K; Kenya; (2 JN378837)
Eleusine jaegeri Pilg. (2*n* = 20, DD)
Peterson et al. 24299; US; Tanzania; (1 KC756417)
Peterson et al. 24325; US; Tanzania; (2 KC756418)
Eleusine kigeziensis S.M.Phillips (2*n* = 36, AADD)
Qing Liu 109 (ILCA 1079); US and IBSC; Burundi; (A1 JN378850, A2 JN378847, A3 JN378846, A4 JN378841, A5 JN378848, A6 JN378849, B1 JN378879, B2 JN378880, B3 JN378877, B4 JN378878, B5 JN378881)
Eleusine multiflora Hochst. ex A.Rich. (2*n* = 16, CC)
Qing Liu 100 (PI 226067); US and IBSC; Kenya; (1 JN378839, 2 JN378838, 3 JN378840)
Eleusine tristachya (Lam.) Lam. (2n = 18, AA)
Qing Liu 107 (PI 230637); US and IBSC; Mexico; (1 JN378842, 2 JN378844, 3 JN378845, 4 JN378843)
Astrebla lappacea (Lindl.) Domin (chromosome number unavailable)
Qing Liu 089 (PI 284733); IBSC; Australia; (1 JN378834)
Chloris truncata R. Br. (chromosome number unavailable)
Qing Liu 108 (PI 212389); IBSC; Australia; (1 JN378833)
Coelachyrum piercei (Benth.) Bor (chromosome number unavailable)
Qing Liu 104 (PI 197534); IBSC; Ethiopia; (2 JN378832)
Coelachyrum yemenicum (Schweinf.) S.M.Phillips (chromosome number unavailable)
Qing Liu 088 (PI 364502); IBSC; South Africa; (1 JN378835)

Data listed in order: Accession numbers (ILCA, International Livestock Centre for Africa at Addis Ababa, Ethiopia; PI, Germplasm Resources Information Network of United States Department of Agriculture [USDA] at Beltsville); Herbarium (IBSC, South China Botanical Garden Herbarium; K, Herbarium at Royal Botanic Gardens, Kew; RSA-POM, Rancho Santa Ana Botanic Garden Herbarium; US, United States National Herbarium); Origin country; GenBank accession numbers of nuclear gene (*waxy*) followed by sequence number (prefix "A" or "B" indicates A- or B-type sequence for allotetraploid species; interrupted line indicates unavailable sequence); Chromosome numbers downloaded from http://mobot.mobot.org/W3T/Search/ipcn2.html, genome composition referred to Bisht and Mukai (2002).

protocol of TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Transformed cells were plated and grown for 16 h on LB agar (Sigma, St Louis, MO, USA) with X-Gal (Promega, Madison, WI, USA) and ampicillin (Sigma). To assess PCR errors and allelic sequences, 8–24 colonies were selected from each individual via a blue-white screen. Plasmids with inserts were sequenced with vector primers T7 and T3 following the ABI-Prism Big Dye Terminator version 3.1 sequencing method (Applied Biosystems). Sequencing reactions were run on an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems). GenBank accession numbers are presented in Table 1.

2.2. Phylogenetic analyses

Sequencher version 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA) was used to evaluate chromatograms for base confirmation and to edit contiguous sequences. Sequences were initially aligned with Muscle (Edgar, 2004), followed by manual adjustments using Se-Al version 2.0a11 (http://tree.bio.ed.ac.uk/software/seal/). A few recombination and several chimeric sequences, identified by repeated sequencing of the same individual and careful inspection of alignments prior to analysis (Bradley and Hillis 1997; Brassac et al., 2012), were excluded from downstream phylogenetic analyses.

Sequence data was analyzed by 1) maximum parsimony (MP) in PAUP* 4.0b10 (Swofford, 2003) and 2) maximum likelihood (ML) in GARLI 0.951 (Zwickl, 2006). For MP analyses, the most parsimonious trees were estimated using heuristic searches with 1000 random addition-sequence replicates, tree bisection and reconnection branch swapping, and no limiting MaxTrees. MP bootstrap supports (PB) of internal nodes were performed for MP with 1000 bootstrap replicates. ML analysis was implemented starting from random trees and using 10 million generations per search. The substitution model for the *waxy* matrix was determined with the Akaike information criterion as implemented in Modeltest 3.7 (Posada and Crandall, 1998). The log likelihood scores of 56 substitution models ranged from 4716.8540 to 4832.4692, and the best-fitting model was TrN + G. ML bootstrap supports (LB) of internal nodes were estimated using 1000 bootstrap replicates, with runs set for an unlimited number of generations and automatic termination following 10000 generations without a significant topology change (lnL increase of 0.01).

2.3. Chromosome preparations

Healthy root tips (5–10 mm long) of *Eleusine coracana* seedlings were pretreated with 4 mmol L^{-1} 8-hydroxyquinoline for 2 h at room temperature and then fixed in absolute ethanol/glacial acetic acid (3:1, v/v) for 12 h at 4 °C. Somatic chromosome spreads were prepared according to Li et al. (2001) with minor modifications (Schwarzacher et al., 1989). Root tips were macerated at 37 °C for 90 min in a mixture of cellulase and pectinase (1% and 5% w/v, respectively) in sodium citrate buffer (10 mmol L^{-1}, pH 4.8) and then squashed in 45% acetic acid. Slides were air-dried following removal of the coverslip using liquid nitrogen, allowed to stand for 24 h at room temperature, and then kept at –20 °C in a freezer.

2.4. Probe labeling and McGISH

Total genomic DNA of *Eleusine indica* and *E. tristachya* was extracted from fresh blades using the DNeasy Plant Mini Kit (QIAGEN). Accessions of *E. coracana* were analyzed by McGISH according to Leitch et al. (1994) with minor modifications (Li et al., 2001). Probes were labeled with digoxigenin and biotin using the DIG-Nick Translation Kit or Biotin-Nick Translation Kit following the manufacturer's protocol (Roche, Mannheim, Germany) after preshearing by ultrasonic processor (Jiang et al., 2012). Detection of the digoxigenin-labeled probe was done using rhodamine-conjugate anti-DIG antibody (Roche, Penzberg, Germany) and biotin-labeled probe using the avidin-FITC antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The stringency was calculated using the equation described by Meinkoth and Wahl (1984). Chromosomes were counterstained with 2 μg mL^{-1} 4',6-diamidino-2-phenylindole (DAPI) in Vectashield antifade solution (Vector Laboratories, Burlingame, CA, USA). Slides were examined by an Axioplan 2 imaging fluorescence microscopy (Carl Zeiss, Oberkochen, Germany) equipped with filters 3 (DAPI), 4 (FITC), and 5 (rhodamine). All images were observed with the Zeiss Apochromat × 100/1.4 oil immersion objective lens. Digital images were captured with an Axiocam under image acquisition modules in Zeiss Axiovision version 4.6, and were then imported into Adobe Photoshop CS2 (Adobe, San Jose, CA, USA) for final processing.

In order to assess the degree of relatedness of *Eleusine coracana* and putative A-genome donors quantitatively, the relative intensity of the signal from a single-channel image was assessed using Zeiss Axiovision version 4.6 by 5-time analyses of the same metaphase spread (Iourov et al., 2005). Descriptive statistics are presented as mean ± standard deviation values.

2.5. Optimization of morphological characters

Morphological characters and the character state coding matrix are listed in Table 2, and the character state coding matrix was edited from herbarium specimens and the literature (Avdulov, 1931; Chennaveeraiah and Hiremath, 1973; Phillips, 1974; Hilu, 1980; Hiremath and Chennaverraiah, 1982; Watson and Dallwitz, 1992; Liu et al., 2005, 2006; Liu and Peterson, 2010; Jiang et al., 2011). Five characters including 1) plant longevity, 2) rhizome, 3) lower glume nerve, 4) polyploid level, and 5) caryopsis compression were mapped onto the *waxy* phylogeny (Figure 1). The character states of allotetraploid species

Table 2. Coding matrix of morphological characters of *Eleusine* used in this study.

Species \ Character no.	1 Plant longevity	2 Rhizomes formation	3 Lower glume nerve number	4 Ploidy level	5 Caryopsis compression
Eleusine africana	[1] annual or biennial	[0] absent	[1] 3 or more nerves	[1] tetraploid	[0] lateral to nearly terete
Eleusine coracana	[1] annual or biennial	[0] absent	[1] 3 or more nerves	[1] tetraploid	[0] lateral to nearly terete
Eleusine floccifolia	[0] perennial	[1] present	[0] 1-nerve	[0] diploid	[1] dorsiventral
Eleusine indica	[1] annual or biennial	[0] absent	[0] 1-nerve	[a] di/tetraploid	[0] lateral to nearly terete
Eleusine intermedia	[0] perennial	[1] present	[0] 1-nerve	[0] diploid	[1] dorsiventral
Eleusine jaegeri	[0] perennial	[1] present	[0] 1-nerve	[0] diploid	[1] dorsiventral
Eleusine kigeziensis	[0] perennial	[1] present	[0] 1-nerve	[1] tetraploid	[1] dorsiventral
Eleusine multiflora	[1] annual or biennial	[0] absent	[0] 1-nerve	[0] diploid	[0] lateral to nearly terete
Eleusine tristachya	[0] perennial	[0] absent	[0] 1-nerve	[0] diploid	[1] dorsiventral
Coelachyrum piercei	[0] perennial	[0] absent	[0] 1-nerve	[0] diploid	[1] dorsiventral

[a] = 0 /1.

were marked only once at the lowest point on the tree where *E. coracana* and *E. africana* separated in order to avoid distortion caused by different *waxy* homoeologous form, i.e. these characters evolved only once in the *waxy* gene tree of *Eleusine*.

3. Results

3.1. Phylogenetic analyses

The *waxy* matrix comprised 860 characters, including exons 8, 9, 10; partial exon 11; and introns 8, 9, and 10, measuring 42 bp, 181 bp, 192 bp, 44 bp, 115 bp, 138 bp, and 148 bp in length, respectively. Among 860 characters, 190 were parsimony-informative (22.09%) with 54.01% of GC content. The ML tree with bootstrap supports (PB and LB) is presented in Figure 1, and the monophyly of *Eleusine* was supported by MP and ML analyses (PB = 87%, LB = 80%).

A single sequence type for the *waxy* locus was identified for 6 diploid species of *Eleusine* [*E. floccifolia* (Forssk.) Spreng., *E. indica* (both diploids and tetraploids have been reported), *E. intermedia* (Chiov.) S.M.Phillips, *E. jaegeri* Pilg., *E. multiflora*, and *E. tristachya*; Figure 1], and this formed 4 groups including the *E. multiflora* subclade (PB = 100%, LB = 100%), the *E. jaegeri* subclade (PB = 100%, LB = 100%), the *E. floccifolia-E. intermedia* subclade (PB = 92%, LB = 75%), and the *E. tristachya* subclade (PB = 100%, LB = 100%), together with *E. indica* sequences nested within clade W-A. Two (A- and B-) types of *waxy* sequences were identified in 3 accessions of *E. africana*, *E. coracana*, and *E. kigeziensis*, consistent with these species being tetraploid. The A- and B-type *waxy* sequences are separated by 7 mutations (3–19 bp indels). These sequences fell into 3 distinct groups. Clade W-A (PB = 92%, LB = 84%) contained the *E. tristachya* subclade, the A-type sequences of *E. kigeziensis* (PB = 84%, LB = 67%), and a subclade comprising *E. indica* and the A-type sequences of *E. africana* and *E. coracana* (PB = 89%, LB = 89%). Clade W-B1 contained B-type sequences of *E. africana* and *E. coracana* (PB = 99%, LB = 95%), and clade W-B2 contained B-type sequences of *E. kigeziensis* (PB = 100%, LB = 100%) and the *E. jaegeri* subclade (PB = 100%, LB = 100%). Clade W-A was sister to clade W-B1 with low support (PB = 63%), and then clades W-A and W-B1 were sister to the *E. floccifolia-E. intermedia* subclade; this group, in turn, was sister to clade W-B2, and the *E. multiflora* subclade was sister to all remaining species of *Eleusine* (Figure 1).

3.2. McGISH

The representative somatic metaphase of *Eleusine coracana* following McGISH is shown in Figures 2A–2F. At 83.95% stringency (posthybridization washing at 41 °C), the *E. indica* probes yielded the most intense and uniform signals embedded in the 18 chromosomes of *E. coracana* (Figures 2B and 2D). At 83.95% stringency, the *E. tristachya* probes yielded the most intense and dispersed signals embedded in the 18 chromosomes of *E. coracana* (Figures 2C and 2E). In all cases, the combined image of double GISH showed the most intense and nonuniform yellow signals

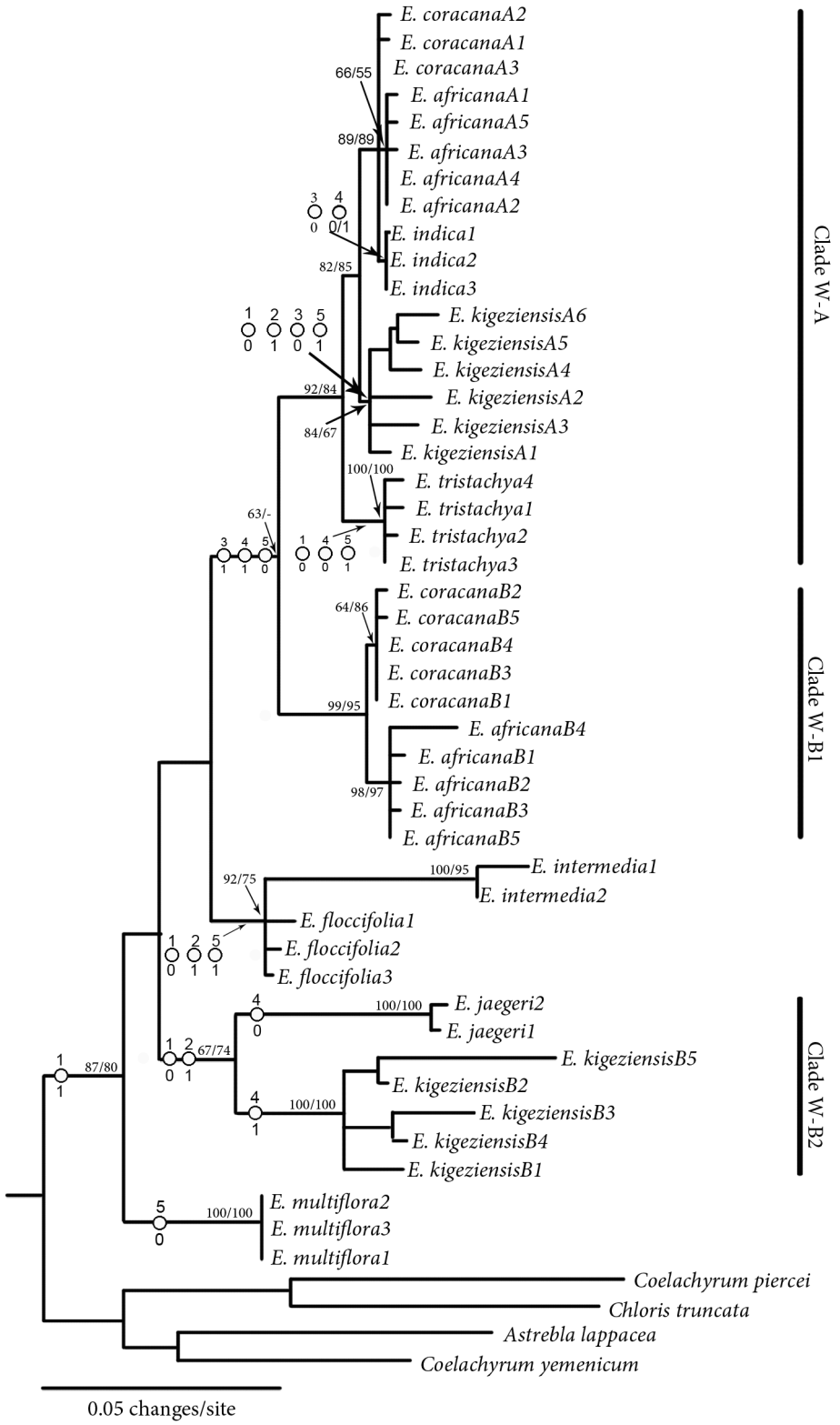

Figure 1. Maximum likelihood tree of *Eleusine* inferred from nuclear *waxy* data. Numbers close to nodes indicate MP and ML bootstrap values (PB/LB). Circles indicate 5 homoplasious characters. Numbers above circles indicate character, and numbers below circles indicate character state.

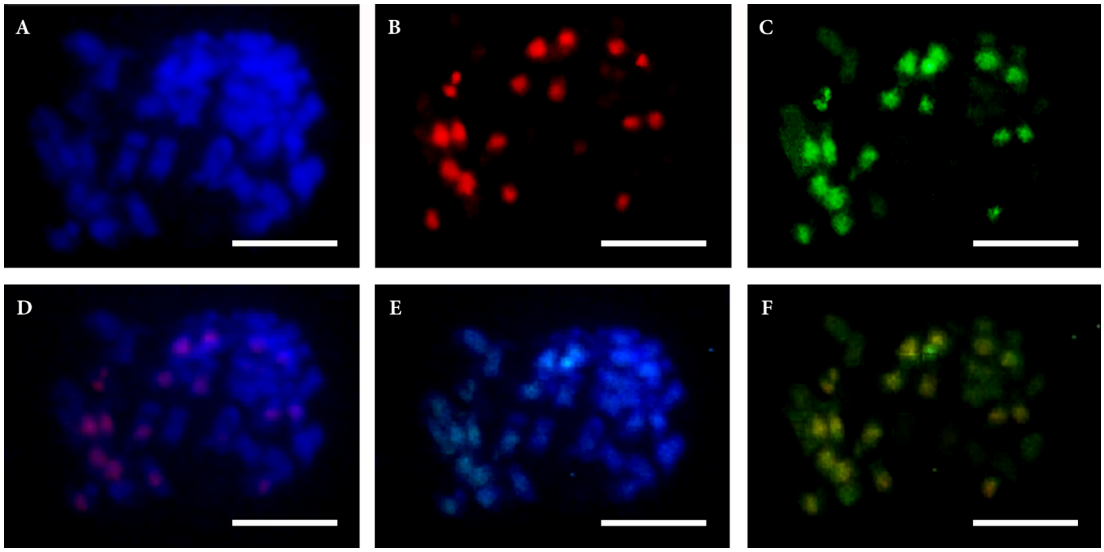

Figure 2. Location of total genomic DNA of *E. indica* and *E. tristachya* probes on metaphase chromosomes of *Eleusine coracana* (2*n* = 36) using GISH at 83.95% stringency condition. A- chromosomes stained with DAPI (blue), B- fluorescent signals of total genomic DNA of *E. indica* probe labeled with digoxigenin (red), C- fluorescent signals of total genomic DNA of *E. tristachya* probe labeled with biotin (green), D- merged images of Figure 2A and 2B, E- merged images of Figure 2A and 2C, F- merged images of Figure 2B and 2C showing the overlapping *E. indica* and *E. tristachya* signals (orange) on *E. coracana* A-genome chromosomes. Scale bars = 5 μm.

(red/green overlapping image) for the same 18 *E. coracana* chromosomes (Figure 2F). The same relative intensities of hybridization signals were detected separately following McGISH with *E. indica* and *E. tristachya* probes when a stronger 84.95% stringency (posthybridization washing at 42 °C) was applied.

McGISH demonstrated 2 features for the *Eleusine coracana* A-genome chromosomes. The relative intensities of the signals for centromeric and pericentromeric regions were 183.1 ± 16.5 pixels for the *E. indica*-specific subgenome (Figure 2B) and 133.3 ± 12.8 pixels for the *E. tristachya*-specific subgenome (Figure 2C); no hybridization signals were detected in the distal regions of *E. coracana* A-genome chromosomes (Figures 2D and 2E).

3.3. Morphological character evolution

Five morphological characters were homoplasious when optimized on the *waxy* gene tree (Figure 1). The annual habit (character: state = 1:1) arose once in the *Eleusine* clade but was reversed at least 3 times in the *E. jaegeri-E. kigeziensis*, *E. tristachya*, and *E. floccifolia-E. intermedia* clades. Rhizomes (2:1) were developed in at least 2 clades, *E. floccifolia-intermedia* and *E. jaegeri-E. kigeziensis*. Three or more nerves on the lower glume (3:1) arose once in the *Eleusine* clade and reversed in *E. indica* and *E. kigeziensis*. Polyploidy (4:1) could have arisen once in *E. africana*, *E. coracana*, *E. kigeziensis*, and *E. indica* clade and reversed in *E. tristachya*.

4. Discussion

4.1. Phylogenetic analyses

The nuclear *waxy* gene has been successfully employed to disentangle the genome origins for some allopolyploids in Poaceae (Mason-Gamer, 2004; Fortune et al., 2007; Jakob and Blattner, 2010). To our knowledge, there is no information available about the boundaries of 3 gene pools for finger millet. We show evidence for the allopolyploidization in the ancestry of finger millet and identify additional species that may be part of its broader gene pool. In the *waxy* phylogeny of *Eleusine* (Figure 1), sequences of 2 homoeologous loci were isolated from 3 tetraploids. The *waxy* data support a single independent allotetraploid origin for the *E. africana-E. coracana* subclade. This event may have involved *E. indica* and *E. tristachya* (or its extinct sister or ancestor) as the A-genome progenitors with differential degrees of relatedness; the differential degree of relatedness between progenitor-descendant relationships was the significant difference with previous nuclear gene phylogenies (Neves et al., 2003; Liu et al., 2011). Even though the cladogram indicates the polyploidization event before the split of *E. tristachya* (Figure 1), it is obvious that the tree illustrates the evolutionary history of *waxy* and these characters are hypothetically placed on this tree.

In clade W-A, the A homoeologues of the *E. africana-E. coracana* subclade shared a common ancestor with *E. indica*, and this lineage, in turn, grouped with

A homoeologues of *E. kigeziensis*; all these taxa share a common ancestor with *E. tristachya*. Regarding the close phylogenetic relationships of diploids *E. indica* (also known to have tetraploid individuals) and *E. tristachya* with *E. africana*, *E. coracana*, and *E. kigeziensis* in both cpDNA (Neves et al., 2005) and nrDNA *Pepc4* (Liu et al., 2011) and *waxy* (Figure 1) phylogenies, we propose that diploids *E. indica* and *E. tristachya* might be the A-genome parents of the *E. africana-E. coracana* subclade. This conclusion is substantiated by 2 previously reported results. First, the *E. africana-E. coracana-E. indica-E. kigeziensis* clade is composed of *E. indica* and 3 tetraploids, with *E. tristachya* as its sister in the *trnT-F* phylogeny (Neves et al., 2005). The relationship is also observed in our *waxy* phylogeny, which indicates that *E. indica* shares a recent common ancestor with the *E. africana-E. coracana* subclade rather than with *E. tristachya*. This result is compatible with our previous combined plastid tree that placed the *E. indica-E. tristachya* subclade as sister to the 3 tetraploid species (Liu et al., 2011). Therefore, *E. indica* and *E. tristachya* contributed to the A (i.e. maternal) homoeologues. Second, the close relationship between *E. indica* and *E. tristachya* in the chronogram suggests that *E. tristachya* has arisen recently in the Americas (late Pliocene) (Liu et al., 2011); this finding could account for the derivation of the American *E. tristachya* (18 bp, 45 bp, and 24 bp insertions in *trnT-F*, *ndhA* intron, and *ndhF*, respectively), possibly from a long-distance dispersal event. Various studies on plants and animals have shown that species with higher dispersal capacities tend to be found with greater frequency towards the margins of their range (Thomas et al., 2001; Darling et al., 2008) and that these species tend to have more genetic variation (Hanski et al., 2004). *Eleusine tristachya* is sister to the remaining members within the A-genome lineage in the *waxy* phylogeny (Figure 1, clade W-A), suggesting that *E. tristachya* is 1 of the maternal parents in the hybridization event leading to the speciation of the 3 tetraploids. Multiple maternal origins have been reported for several allopolyploids, e.g., *Aegilops triuncialis* L., *Stebbinsoseris heterocarpa* (Nutt.) K.L.Chambers, *Thinopyrum intermedium* (Host) Barkworth & D.R.Dewey, and *Tragopogon miscellus* G.B.Ownbey (Wallace and Jansen, 1995; Vanichanon et al., 2003; Chester et al., 2012; Zeng et al., 2012).

The B homoeologues of *E. africana* and *E. coracana* formed the strongly supported clade W-B1 in our *waxy* phylogeny. Bisht and Mukai (2002) also found a similar genome composition based on chromosome banding pattern. Artificial hybrids between these 2 species had intermediate morphologies (Mehra, 1962), and a close relationship between these 2 species was confirmed by previous molecular data (Hilu and De Wet, 1976; Neves et al., 2005; Liu et al., 2011). However, the B homoeologues in clade W-B1 do not have any diploid taxa (possible paternal parents), as confirmed by ITS, *Pepc4*, and *EF-1α* phylogenies (Neves et al., 2005; Liu et al., 2011). Three hypotheses (the extra-*Eleusine* origin, divergence, and extinction) have been proposed to explain why the paternal parents remain unidentified for the *E. africana-E. coracana* subclade (Liu et al., 2011). In *waxy* data, B-type *waxy* sequences of *E. africana* and *E. coracana* can be easily distinguished from those of outgroups by 15 mutations (11 insertions and 4 deletions, 1–19 bp), and thus the divergence relatedness degree of sequences between A-type *waxy* sequences of *E. africana* and *E. coracana* and outgroup sequences was higher than those between A- and B-type *waxy* sequences of *E. africana* and *E. coracana* [7 mutations (3–19 bp indels)]. The natural distribution of *E. coracana* also excludes the possibility of a genetic contribution by extra-African diploid taxa (Liu and Peterson, 2010). Since the monophyly of *Eleusine* received robust support from 3 former nuclear gene trees and moderate support from the *waxy* gene tree, and the internal branching suggested a relationship between clade W-B1 and clade W-A (PB = 63%), it is likely that the paternal parents were closely related species that should be members of the genus. It seems unlikely that the B-genome of tetraploids has diverged so much that *Eleusine* markers were not detected in it, because *Eleusine* primers worked well in non-*Eleusine* species herein. The most likely scenario is that the B-genome donor is extinct and hence not yet identified.

The *waxy* phylogeny provided an explanation for the origin of *E. kigeziensis*, which may be a recent allotetraploid. A short genetic distance separates the *E. indica-E. africana-E. coracana* subclade, *E. tristachya*, and A homoeologues of *E. kigeziensis* in clade W-A, and the *E. jaegeri* subclade is sister to the B homoeologues of *E. kigeziensis* with moderate support (PB = 67%, LB = 74%) in clade W-B2, suggesting that *E. indica*, *E. tristachya*, and *E. jaegeri* shared a recent common ancestor with *E. kigeziensis*. Moreover, these 3 latter species might be the diploid progenitors to *E. kigeziensis*. So far, sequence data suggest a recent polyploidy origin for *E. kigeziensis* owing to the varied phylogenetic position in *trnT-F* and ITS phylogenies (Neves et al., 2005). Furthermore, the sum of 2C DNA contents of *E. indica* (2.85 pg) and *E. jaegeri* (3.33 pg) is higher than those of *E. kigeziensis* (5.93 pg). Therefore, the origin of *E. kigeziensis* is far more complex than previously reported (Bisht and Mukai, 2002) and needs to be further explored.

4.2. McGISH evidence

McGISH results demonstrated 2 features for *Eleusine coracana* A-genome chromosomes. The stronger signal relative intensities of *E. indica* probes indicate that *E. coracana* has a large number of *E. indica*-specific repeats, while the weaker signal relative intensities of *E. tristachya* probes indicate that *E. coracana* has fewer *E. tristachya*-specific repeats (Kubis et al., 1998; Dechyeva et al., 2003). Such a differential relatedness degree of finger millet and *E. indica* and *E. tristachya* was also supported by single-probe GISH results, such that the *E. indica* probe produced higher intensity signals (226.3 ± 21.2 pixels) than did the *E. tristachya* probe (125.1 ± 16.7) across 18 finger millet chromosomes (Jiang et al., 2011; Jiang, 2012). The presence of highly similar repeats in *E. indica* and *E. coracana* could indicate that the tetraploid is relatively young. The lower relatedness between *E. tristachya* and *E. coracana* repeats may indicate that *E. tristachya* diverged early, possibly before the hybridization event took place, or that an extinct sister or common ancestor of *E. tristachya* may have served as the A-genome parent, and hence a weaker GISH. However, the distal regions of *E. coracana* A-genome chromosomes produced no hybridization signals, and this may be due to extremely weak GISH intensities or probe hybridization difficulty. It should be noted that in the metaphase spreads selected for GISH experiments, the chromosomes are usually not sufficiently condensed to facilitate loci mapping. No fluorescence signals were detected in distal regions, probably due to probe hybridization's difficulty to target DNA; in other words, the distal regions may possess a lower abundance of repeats than the centromeric and pericentromeric regions (Flavell, 1986; Seijo et al., 2007). Because the polyploidy history of *E. africana* and *E. kigeziensis* was beyond the scope of this study, we have not attempted to validate genome donors for them using McGISH.

4.3. Morphological character evaluation

A single origin of polyploidy is indicated by the clade with *Eleusine coracana-E. africana-E. indica-E. kigeziensis* in Figure 1. *Eleusine kigeziensis* and *E. coracana-E. africana* occur in 2 places, but these are not necessarily where the species should align, and so we know that it has occurred only once. The *waxy* phylogeny provides first evidence that all 5 characters (plant longevity, rhizomes formation, lower glume nerve number, ploidy level, and caryopsis compression) were homoplasious, perhaps a consequence of interspecific hybridization. The dorsiventral caryopsis (5:1) appears to have originated at least 3 times in the *waxy* tree. Caryopsis compression has been used to define groups at various taxonomic levels in Poaceae (Wang et al., 1986; Jiang et al., 2011), and it is not surprising that dorsiventral caryopses evolved 4 times in *Eleusine*. The probable interpretation is that the caryopsis phenotype may evolve rapidly under simple genetic control (Drea et al., 2005), since caryopsis size (i.e. starch accumulation) is critical for species survival (Satoh et al., 2008). Caryopsis compression may reflect functional rather than evolutionary relationships for the Poaceae (Jiang et al., 2011). We found no nonhomoplasious characteristics (synapomorphies) shared by 2 or more species in the *waxy* gene of *Eleusine*, and we suspect that the interspecific hybridization events may have contributed to the high level of homoplasy in morphological characters (see also Lowrey et al., 2001; Terzioğlu et al., 2012; Yücedağ and Gailing, 2013).

In summary, the *waxy* data support a single allotetraploid origin for the *Eleusine africana-E. coracana* subclade. Furthermore, this event may have involved *E. indica* and *E. tristachya* as the A-genome donors, with a differential degree of relatedness to *E. coracana*. McGISH supports *E. indica* as the primary A-genome parent and *E. tristachya* (or its extinct sister or ancestor) as the secondary A-genome parent for the derivation of finger millet (*E. coracana*). The most likely scenario is that the B-genome donor is extinct. Five morphological characters (plant longevity, rhizome formation, lower glume number, ploidy level, and caryopsis compression) were homoplasious, perhaps a consequence of interspecific hybridization. This study presented findings that are vital in guiding our efforts to expand the genetic base of finger millet by the elucidation of its gene-pool range in the future.

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