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RESEARCH ARTICLE

A novel genome of C and the first autotetraploid species in the *Setaria* genus identified by genomic *in situ* hybridization

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Abstract Genomic *in situ* hybridization (GISH) was used to investigate the genomic relationships among some newly collected species of genus *Setaria*. Previous work identified that *S. viridis* and *S. adhaerens* carry genomes A and B, respectively. GISH patterns obtained in this report clearly distinguished the genome of *S. grisebachii* from the known genomes A and B, and indicated its new genomic constitution which we suggest to name genome C of the *Setaria* genus. The two sets of chromosomes of tetraploid *S. queenslandica* hybridized well with the A genome of *S. viridis* indicating its autotetraploid nature. This is the first autotetraploid identified in the *Setaria* genus, which should be classified into the primary A genome gene pool rather than the tertiary gene pool as previously classified. GISH patterns did not distinguish the genome of *S. leucopila* from the A genome of *S. viridis* and *S. italica*, suggesting its close relation with foxtail millet. Strong hybridization signals were observed when *S. adhaerens* genomic DNA was used as probe to hybridize the chromosomes of diploid *S. verticillata*, inferring its B genome nature. Combined with morphological observation and previous work, we deduce that diploid *S. verticillata* and *S. adhaerens* are probably taxonomically the same species with different names.

Keywords Genome analysis · Genomic *in situ* hybridization · *Setaria*

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Introduction

The genus *Setaria* Beauv. belongs to the tribe Paniceae, subfamily Panicoideae and family Poaceae in the grass family. There are about 125 species worldwide occurring in tropical, sub-tropical and temperate regions. Seventy four species originate from Africa, America has 25 native species, and others are distributed in Eurasia (Hubbard 1915; Rominger 1962). Foxtail millet (*S. italica* (L.) Beauv.), which is one of the oldest cereal crops contributed to human civilization not only in the past but still used as a staple food in China and India, belongs to this group of species. Some species in

genus *Setaria*, such as foxtail millet, *S. leiantha* Hackel (Argentinean foxtail millet) and *S. splendida* Stapf var. *splendida* are cultivated for grazing and hay in America, Africa and Southern Asia (Wanous 1990). The genus also has members of the worst weed species that are deleterious to world agriculture management, such as *S. viridis* (L.) Beauv. (green foxtail), *S. faberi* Herrm. (giant foxtail), *S. verticillata* (L.) Beauv. (bristly foxtail) and *S. glauca* (Weigel) Hubb. (yellow foxtail) (Dekker 2003).

The nuclear genome of *Setaria* spp. typically has a complement of nine chromosomes in its monoploid form (Avdulov 1931) and different ploidy levels were identified in the genus including diploid ($2n = 2x = 18$, *S. viridis*, *S. italica*, *S. adhaerens* (Forssk.) Link ex Chiov., *S. grisebachii* Fourn. ex Hemsl., *S. leucopila* K. Schum., and *S. verticillata* (L.) Beauv.), tetraploid ($2n = 4x = 36$, *S. verticillata*, *S. faberi*, *S. pumila* (Poir.) Roem. et Schult., and *S. queenslandica* Domin), hexaploid ($2n = 6x = 54$, *S. verticillata* and *S. pumila*) and octoploid species ($2n = 8x = 72$, *S. pumila* and *S. geniculata*) (Avdulov 1931; Brown 1948; Gupta and Singh 1977; Dekker 2003; Wang et al. 2007). Observations drawn from interspecific hybridization, karyological studies, and genomic *in situ* hybridization (GISH) indicated that foxtail millet and its putative wild ancestor *S. viridis*, the green foxtail, carry the A genome, and *S. adhaerens* carries the B genome. Both *S. verticillata* and *S. faberi*, which are tetraploid species, are composed of A and B genomes (Li et al. 1945; Harlan and de Wet 1971; Darmency and Pernes 1987; Benabdelmouna et al. 2001). The genome composition of many other wild species is still unidentified and needs to be reliably established, which is useful in foxtail millet breeding and related genetic researches.

GISH offers a powerful tool for investigating genome composition and genetic relationship among species. It has been successfully used to identify closely related species in Triticeae (Anamthawat-Jónsson et al. 1990) and to investigate genomic relationship between *Thinopyrum intermedium* and *Th. ponticum* (Chen et al. 1998). It is a direct and visual method of distinguishing different genomes (Mukai et al. 1993). This technique has been applied in *Setaria* species genomic composition study (Benabdelmouna et al. 2001), and successfully used for genomic constitution study of *Oryza* and *Brassica* species (Li et al. 2001; Hasterok and Maluszynska 2005).

In our previous study, we have studied three diploid (*S. grisebachii*, *S. leucopila*, and *S. verticillata*) and one tetraploid (*S. queenslandica*) species (Wang et al. 2007), which are morphologically close to both the A and B genome species of the genus, but their genome compositions are not investigated. In this study, we used GISH technique to identify the genome constitution of these species, which will be useful in establishment of phylogenetic relationship of *Setaria* group of species.

Materials and methods

Plant materials

Wild and cultivated species of *Setaria* used in this study and their characteristics, are listed in Table 1. Seeds were germinated on moistened filter paper at 25°C in Petri dishes. Root tips of about 2 cm in length were cut and treated with 8-hydroxyquinoline for 2 h to accumulate metaphases. All treatments were performed at room temperature in dark. After

Table 1 Origin, chromosome numbers, and putative genome attribution of *Setaria* species used in this study

Species	Accession no or cultivar	Ploidy level	Haploid genome	Origin
S. adhaerens	25001	$2n = 2x = 18$	B	France
S. viridis	N33	$2n = 2x = 18$	A	China
S. italica	Yugu 1	$2n = 2x = 18$	A	China
S. verticillata (tetraploid form)	08004	$2n = 4x = 36$	AB	France
S. verticillata (diploid form)	08001	$2n = 2x = 18$	B	Hawaii, USA
S. grisebachii	03001	$2n = 2x = 18$	C	Mexico
S. leucopila	16001	$2n = 2x = 18$	A	Germany
S. queenslandica	20001	$2n = 4x = 36$	AA	Australia

rinsing with distilled water, the root tips were then fixed in freshly prepared 1:3 glacial acetic acid and ethanol (100%) for at least 24 h and stored at 4°C until use. Seeds of each species were grown, germinated in autoclaved soil and transferred into plastic pots in an open air field. Young leaves of plants in an age at 5- to 6-leaves stage were used to isolate DNA for probes in GISH analysis.

Chromosome preparation

The fixed root tips were rinsed with distilled water and a single root tip was transferred in a drop of 45% acetic acid onto a clean slide before gentle squashing. The slides were then scanned for good chromosome spreads at prophase or metaphase stages with phase-contrast microscope and the selected slides were stored at −80°C until use.

Isolation and labeling of genomic DNA

Total genomic DNAs of five diploid species, *S. viridis*, *S. italica*, *S. adhaerens*, *S. grisebachii*, and *S. leucopila*, were extracted from young leaves using the method of Li et al. (1998) with some modification. About 1 μg of the total genomic DNA from each sample was labeled with digoxigenin-11-dUTP using a nick translation kit (Roche Company) following the instructions provided by the manufacturer.

GISH analysis

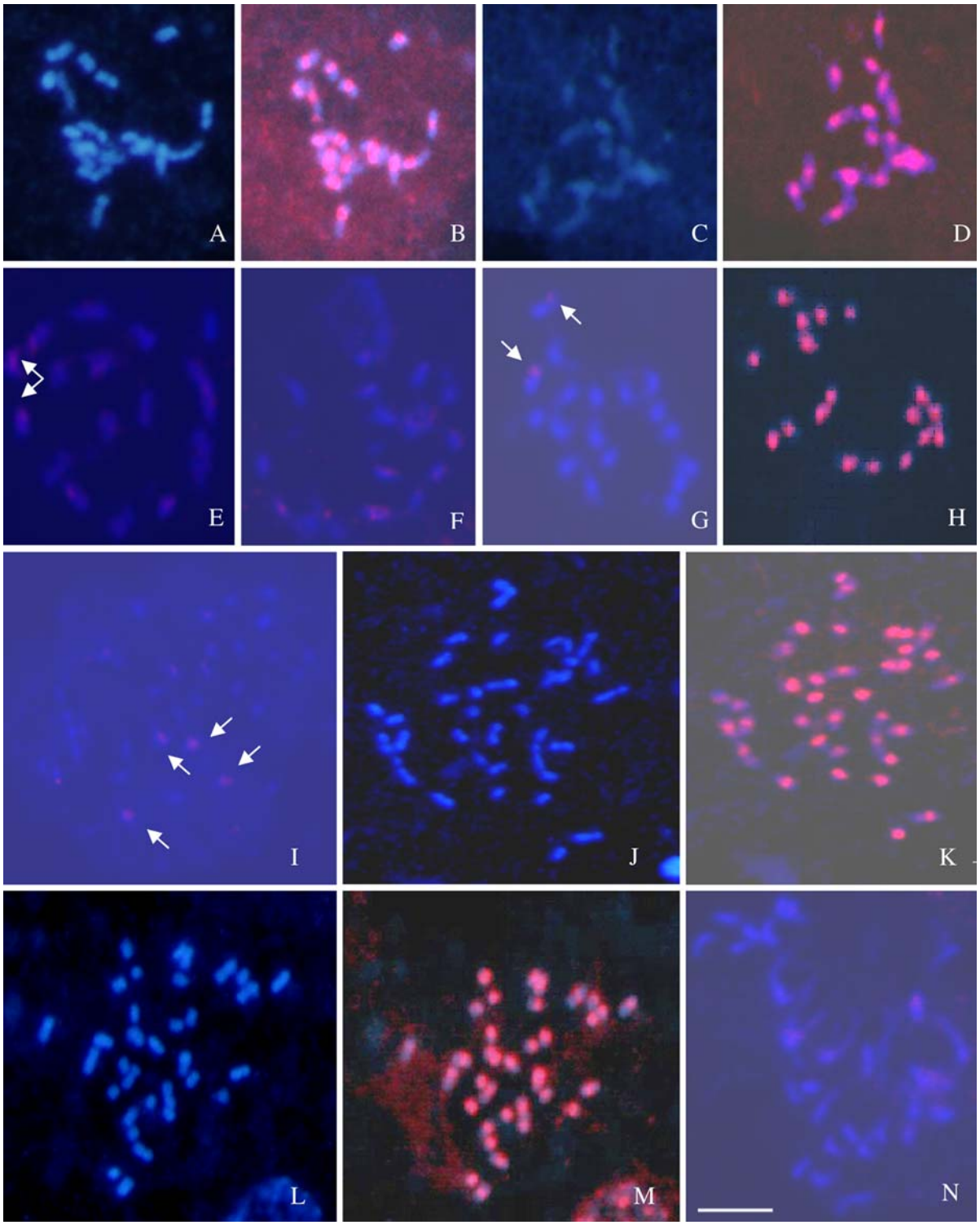
GISH was carried out following Bisht and Mukai (2001) with some modifications. The hybridization mixture contained 50% formamide, 10% dextran sulfate, 2× SSC, 3 ng/μl of DNA probe, and 1 ng/μl salmon sperm DNA. Chromosomal DNA on the slides was denatured by immersion in 70% formamide in 2× SSC for 3 min at 80°C. After removing the cover with a razor blade, the slides were rapidly dehydrated in an ethanol series (70%, 95%, and 100%) for 3 min each at −20°C. The hybridization mix was denatured for 10 min at 95°C and then immediately chilled on ice for 10 min, and 20 μl of probe mix was applied to each slide. DNA–DNA *in situ* hybridization was carried out overnight in a moist chamber at 37°C. Following hybridization the slides were washed with high stringency in 2× SSC at 32°C, 20% formamide in 2× SSC at 32°C, 20% formamide in 0.1× SSC at 32°C for 5 min each, and 2× SSC and 4× SSC for 5 min each at room temperature, the slides were placed in the BSA (Bovine serum albumin) blocking solution (5% BSA-2× SSC) for 15 min at 37°C. To ensure the washing stringency being strong enough for a certified result we also carried out slide washing following Bennett et al. (1992) for some slides, which was carried out in 2× SSC at room temperature, 50% formamide in 2× SSC at 42°C, 2× SSC at 42°C, and 2× SSC at room temperature, for 10 min each. According to Schwarzacher et al. (1989), these conditions allow hybridization between DNA–DNA duplexes sharing approximately 80% sequence homology.

Hybridization signals were detected with anti-Digoxigenin-Rhodamine. The slides were washed twice in 4× SSC for 5 min each at room temperature. The chromosomes were finally counterstained with 4′-6-diamidio-2-phenylindole (DAPI). Chromosome preparations were visualized using an Olympus epifluorescence microscope with appropriate filters, and photos were taken digitally.

Results

Genomic relationship of the diploid *Setaria* species

Genome identification of the three newly identified diploid species, *S. grisebachii*, *S. leucopila*, and *S. verticillata* was carried out using the known A genome (*S. viridis*) and B genome (*S. adhaerens*) DNAs separately as probes without blocking DNA. Total genomic DNA from *S. viridis* or *S. adhaerens* was digoxigenin labeled and used as probe on metaphase chromosomes from the target species. When genomic DNA from *S. viridis* was hybridized to chromosome preparations of *S. leucopila*, all its chromosomes had strong signals in the pericentromeric regions. The terminal regions were faintly labeled for most chromosomes (Fig. 1a, b). The same hybridization pattern was obtained in the reciprocal experiment using *S. leucopila* genomic DNA as probe on chromosome preparations of *S. viridis* (Fig. 1c, d).



When the probe prepared from total genomic DNA of *S. italica* was hybridized on chromosomes of *S. leucopila*, the same results as in *S. viridis* experiments were obtained (data not shown). In GISH using *S. viridis* genomic DNA as probe, weak hybridization signals or no signals were observed on

◀ **Fig. 1** GISH patterns obtained on different *Setaria* species. **a** The metaphase chromosomes of *S. leucopila* were counterstained blue with DAPI. **b** The same metaphase cell hybridized with the probe of *S. viridis* digoxigenin-labeled genomic DNA (*red*). **c** DAPI counterstained metaphase cell from *S. viridis*. **d** The same metaphase cell hybridized with the probe of *S. leucopila* digoxigenin-labeled genomic DNA. A strong and total painting on nearly entire length of all chromosomes of *S. leucopila* was observed. **e** GISH was carried out using *S. viridis* genomic DNA as probe hybridizing on chromosome preparations of *S. grisebachii*, and chromosomes of this species showed weak signals as indicated by arrows. **f** Genomic DNA of *S. adhaerens* was applied to *S. grisebachii* chromosomes, only a few weak signal points were observed by dispersed pattern. **g** Chromosomes of *S. leucopila* hybridized with *S. adhaerens* genomic DNA as probe also showed faint signal, only two major spots signals were detected (*arrowhead* shows the two signals). **h** Metaphase of diploid *S. verticillata* probed with *S. adhaerens* total genomic DNA. A strong and total painting on the entire length of all the chromosomes was observed. **i** B genome of *S. adhaerens* genomic DNA was used as probe, very weak signals of pointed distribution were observed on a few chromosomes of *S. queenslandica*. **j** The metaphase of *S. queenslandica* was counterstained with DAPI. **k** The same metaphase cell hybridized with the genomic DNA of *S. viridis*, showing that 36 chromosomes of *S. queenslandica* were hybridized thoroughly with the *S. viridis* A genome. **l** The chromosomes of tetraploid *S. queenslandica* were counterstained blue with DAPI. **m** The same metaphase cell hybridized with genomic DNA of *S. viridis* (*red*). All the chromosomes were hybridized with strong signals. **n** Chromosomes of tetraploid *S. verticillata* hybridized with genomic DNA of *S. grisebachii*, some dispersed and faint spots signals were detected. Bar 5 μm

chromosome preparations of *S. grisebachii* (Fig. 1e) and diploid *S. verticillata* (data not shown).

Total genomic DNA of *S. adhaerens*, which carries the *Setaria* B genome, was also used as a probe to identify the three target species, *S. grisebachii*, *S. leucopila* and diploid *S. verticillata*. When genomic DNA of *S. adhaerens* was applied to *S. grisebachii* chromosomes, only a few weak signals were observed by dispersed pattern (Fig. 1f), indicating the genome of *S. grisebachii* was distinct from the B genome. Chromosomes of *S. leucopila* hybridized with *S. adhaerens* genomic DNA as probe also showed faint signal or no signal at all (Fig. 1g). A complete different pattern was obtained when diploid *S. verticillata* was used as the target for probes prepared from total genomic DNA of *S. adhaerens*, all the chromosomes of diploid *S. verticillata* were strongly labeled almost entirely (Fig. 1h), indicating diploid *S. verticillata* carries the B genome of *Setaria*.

Genomic constitution of *S. queenslandica*

To investigate the genome constitution of tetraploid *S. queenslandica*, total genomic DNAs of *S. viridis* and *S. adhaerens* were used as probes on chromosome preparations of *S. queenslandica*, respectively. When the B genome of *S. adhaerens* genomic DNA was used as probe, no labeling signal or only very weak signals on few chromosomes of the species with 36 chromosomes were observed, implying that there is no B genome in *S. queenslandica* genomic constitution (Fig. 1i). When total genomic DNA of the *Setaria* A genome, *S. viridis*, was used as probe to test the chromosome constitution of *S. queenslandica*, all the 36 chromosomes were strongly hybridized with labeling signals throughout the nearly entire chromosomes (Fig. 1j, k). Using genomic DNA of *S. italica* as a probe, a nearly identical signal pattern was obtained (data not shown). To eliminate the possible influence of slide washing stringency on the result, using a different strong stringent washing protocol of Bennett et al. (1992), the 36 chromosomes of *S. queenslandica* all gave strong signals by the hybridization of *S. viridis* genomic DNA as probe (Fig. 1l, m). These results clearly indicate that *S. queenslandica* is an autotetraploid with the genome constitution of AAAA.

Identity test of the *S. grisebachii* genome with tetraploid species

To further understand the genome constitution of *S. grisebachii*, GISH analysis was carried out in tetraploid species with genomic DNAs of different diploid species as probes. When total genomic DNA of the A genome of *S. viridis* (AA) was used as a probe, only one set of 18 chromosomes of the tetraploid *S. verticillata* was labeled (data not shown). When genomic DNA of *S. adhaerens* (BB) was used as probe, also only 18 chromosomes showed signals. Those results were consistent with the known chromosome constitution of AABB of tetraploid *S. verticillata* demonstrated by Benabdelmouna et al. (2001). Using genomic DNA of *S. grisebachii* as a probe, no or weak hybridization signals were observed in the A and B genomes of tetraploid *S. verticillata* (Fig. 1n), which further indicates that the genome constitution of *S. grisebachii* is neither A nor B. We also hybridized genomic DNA of

S. grisebachii onto the chromosomes of *S. queenslandica*, only very faint labeling signals was observed (data not shown).

Discussion

S. grisebachii probably carries the *Setaria* genome C

Compared to other genera of the grass family, the genome constitutions of *Setaria* are poorly understood although there are 125 species in the genus worldwide. From interspecific hybridization and karyological studies, Li et al. (1945) first suggested *S. italica* (foxtail millet) and its putative wild ancestor *S. viridis* were composed of genome A, and *S. faberi* Herrm. was an allotetraploid composed of A and another unknown genome. GISH by Benabdelmouna et al. (2001) indicated that chromosomes of *S. adhaerens* could not hybridize with the A genome of *S. viridis* and *S. italica* genomic DNA, and hybrid weakness between *S. adhaerens* and *S. viridis* also confirmed the different genomes of these two species. The genome of *S. adhaerens* was designated B and further analysis indicated that *S. faberi* and tetraploid *S. verticillata* were all allotetraploids, which were composed of A and B genomes (Benabdelmouna et al. 2001). In this study, genomic DNA of *S. grisebachii* hybridized neither to the A genome of *S. viridis* nor to the B genome of *S. adhaerens*, and reciprocal performance gave the same results. It is reasonable to assume that *S. grisebachii* carries a new genome, designated genome C, which is different from the known genomes A and B. This point of view is well supported by the known fact that *S. grisebachii* originated from Mexico, while *S. viridis* and *S. adhaerens* originated from Eurasia. Our three years field observations indicate that botanical morphology of *S. grisebachii* also was clearly different from *S. viridis* and *S. adhaerens*, which supports the new genome hypothesis. Our data from phylogenetic analysis of *Setaria* species with DNA sequences from genes of *Adh*1, *Adh*2 and *Gpa*1 also clearly distinguishes *S. grisebachii* from *S. viridis* and *S. adhaerens* (not published data), which also support the C genome constitution of *S. grisebachii*.

The A genome species and the first autotetraploid in *Setaria*

Clear understanding of genomic constitution and relationship will be important for germplasm management and breeding by use of wild resources. Probes from both *S. viridis* and *S. italica* genomic DNA hybridized well with chromosomes of *S. leucopila*, indicating that *S. leucopila* is a diploid species of the A genome. So far three A genome diploid *Setaria* species were identified including *S. viridis*, *S. italica*, and *S. leucopila*. *S. viridis* was thought to be the putative wild ancestor of domesticated *S. italica*, and the close genetic relationship of these two species was verified by interspecific hybridization and karyological studies (Li et al. 1945; Darmency and Pernes 1987), RAPD classification (Li et al. 1998), RFLP based genetic map construction (Wang et al. 1998) and GISH analysis (Benabdelmouna et al. 2001). Accumulated data made probably that these species are taxonomically the same species, which was first suggested by Harlan and de Wet (1971). Our GISH results showed that the genome of *S. leucopila* could not be differentiated from *S. viridis* and *S. italica*, indicating their close karyological relationship. This makes *S. leucopila* a valuable genetic resource for improvement of foxtail millet because of its possession of original genetic information. Morphological observation found that *S. leucopila* is someway intermediate between *S. viridis* and *S. italica* with fewer tillers and larger seeds than *S. viridis*, but the seed shattering habit indicates its wild species character. Crossability of *S. leucopila* with *S. viridis* and *S. italica* must be further investigated in order to determine their genetic evolution relationship.

Although many *Setaria* species are tetraploid, most of their genome constitutions are not clear except for two allotetraploid species composed of AABB, *S. faberi* and tetraploid *S. verticillata* (Benabdelmouna et al. 2001). In this research, the two sets of chromosomes of *S. queenslandica* all hybridized well with *S. viridis* and *S. italica* genomic DNA, indicating that *S. queenslandica* probably is an autotetraploid composed of AAAA. Using different washing protocols with high stringency, multiple independent hybridizations of chromosome preparations of *S. queenslandica* with genomic DNA of *S. viridis* as probe all gave the same result (Fig. 1j–m),

which eliminated the possibility of an inaccurate result generated by not enough washing stringency and ensured the autotetraploid AAAA genome constitution of *S. queenslandica*. But we do not know whether the two sets of A genome in *S. queenslandica* are identical or in some degree identical, or in other words whether its genome constitution is AAAA or AAA_1A_1. Further experiments with DNA sequence data need to be done to clarify this question. Because artificially made autotetraploid lines of *S. italica* from foxtail millet cultivars all suffer from certain degree of seed setting sterility (Ahanchede et al. 2004), which made polyploid breeding in foxtail millet not successful, the finding of a naturally formed fertile autotetraploid might be helpful for polyploid breeding in foxtail millet.

The *Setaria* genus was previously classified into three gene pools. The primary gene pool is composed of *S. viridis*, *S. italica* and its close relatives (Harlan and de Wet 1971; Jusuf and Pernès 1985). The secondary gene pool is composed of more related wild species, including *S. adhaerens*, *S. verticillata*, and *S. faberi*. The remaining wild *Setaria* species were classified into the tertiary gene pool, which includes *S. queenslandica* (Zangré et al. 1992; Panaud 2006). Our experiment in this report clearly identified the close genetic relationship of *S. queenslandica* with *S. viridis* and *S. italica*. Hence, it is reasonable to classify *S. queenslandica* into the primary gene pool, which is the only natural autotetraploid species in *Setaria* so far identified.

Diploid *S. verticillata* and *S. adhaerens* are taxonomically the same species

Our GISH results showed that the genome of diploid *S. verticillata* and *S. adhaerens* were closely related and could not be separated. In morphology, there was no distinct difference between the two species; they are all with thin and fragile stems, and the same structure of panicle and spikelet. Based on GISH experiments and morphology observation, we deduce that diploid *S. verticillata* and *S. adhaerens* are probably taxonomically the same species with different names. Using GISH experiment Benabdelmouna et al. (2001) identified that the A genome of tetraploid *S. verticillata* comes from *S. viridis* and its B genome from *S. adhaerens*. From isoenzyme analysis and cytogenetical studies with F_1 hybrid between *S. italica* ($4n$) and tetraploid *S. verticillata*, Wu and Bai (2000) deduce that *S. viridis* and diploid *S. verticillata* were the ancestors of tetraploid *S. verticillata*. The results of these two studies strongly support our conclusion that diploid *S. verticillata* and *S. adhaerens* are taxonomically the same species.

Because of its worldwide distribution and broad genetic variation, the A genome species of *S. viridis* was thought to be the primary gene pool of the *Setaria* genus (Harlan and de Wet 1971; Zangré et al. 1992; Panaud 2006). The diploid species *S. verticillata* or *S. adhaerens* had probably evolved from the A genome *S. viridis* or from an unknown diploid *Setaria* ancestor. Two allotetraploid species, *S. verticillata*, and *S. faberi*, were probably descended from the hybrid genome duplication between two diploid species of the A and B genome after the evolution of the B genome species. Successful hybridization between *S. viridis* and *S. adhaerens* (Benabdelmouna et al. 2001) strongly supports this point of view.

The nomenclature of *S. verticillata*

A wide range of chromosome complements have been reported by many literature papers with the same taxonomical name of *S. verticillata*, including diploid $2n = 18$, tetraploid $2n = 36$ and hexaploid $2n = 54$, and the situation was well summarized by Dekker (2003). Whether this is due to misidentification, or a product of the complex taxonomy of the genus, is not apparent. If these reports are true, they raise important questions about intra-specific fertility, and may indicate reproductive barriers within species, and we know that polyploidization (either alloploidy or autoploidy) has played an active role in speciation and delimiting taxa in *Setaria* (Khosla and Sharma 1973). Auguier (1979) named the diploid form with *S. verticillata* and the tetraploid form with *S. verticiformis* Auguier and Wu and Bai (2000) supported this nomenclature. Our result, combined with the GISH result of Benabdelmouna, et al. (2001) and the isoenzyme result of Wu and Bai (2000), clearly indicate that *S. verticillata* and *S. adhaerens* are taxonomically the same species. So misidentification and different names with the synonymous species made the complex situation for some *Setaria* species. We suggest that diploid *S. verticillata* should be named *S. adhaerens* and the tetraploid form be kept the commonly accepted name of *S. verticillata*.

Because we do not have the hexaploid *S. verticillata* sample, its genome constitution and nomenclature need to be studied in the future.

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Author contributions Xianmin Diao conceived and designed the experiments. Yongqiang Wang and Hui Zhi performed the experiments. Hui Zhi, Wei Li, Haiquan Li, Yongfang Wang and Zhanjing Huang contributed to reagents, materials planting and data analysis. Xianmin Diao wrote the paper.

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