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Reproductive System and Molecular Phylogenetic Relationships of Fonio Millets (*Digitaria spp.*, Poaceae) with Some Polyploid Wild Relatives

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Abstract Fonio millets (*Digitaria exilis, D. iburua*) are minor but important indigenous cereals in the semiarid areas of West-Africa. Recent interest in breeding strategies for these crops requires a better understanding of their biology and genetics. Amplified Fragment Length Polymorphism (AFLP) markers were employed to assess the phylogenetic relationships among cultivated fonio species and some polyploid wild relatives and examine proposed hypotheses on fonio ancestry. The AFLP analysis was found quite suitable for identifying each species. A very strong genetic affinity (over 92% similarity) was detected between the wild *D. longiflora* and *D. ternata* and the cultivated *D. exilis* and *D. iburua*, respectively. These data provided additional molecular evidence supporting the previous view of direct domestication of fonio millets from these two wild species. High genetic divergences were expectedly found between fonio species and the other taxonomically distant *Digitaria* taxa investigated. The results also revealed *D. ciliaris* and *D. sanguinalis* as separate species sharing close ancestry. Selfing experiments and subsequent progeny analyses using three isozymes supplemented by AFLPs were further conducted to determine the reproductive system in fonio millets. The results revealed apomixis as absolute mode of reproduction of these crops, except *D. exilis* in which 2% residual sexuality was detected. Additional data documented on seed set and pollen viability suggested that apomixis in fonio would be of pseudogamous type. The data also revealed fonio crops as highly self-compatible and of allopolyploid origin. This study adds new information about the reproductive system and the evolution of fonio, contributing to the knowledge on their biology, and thus providing useful subsides for future genetic improvement of these valuable crops.

Keywords AFLPs · *Digitaria spp.* · Fonio · Isozymes · Phylogeny · Pseudogamous apomixis

Abbreviations

APS	Ammonium persulfate
AFLP	Amplified fragment length polymorphism
BS	Bootstrap support
CTAB	Cetyl trimethyl-ammonium bromide
EDTA	Ethylene diamine tetra-acetic acid
PCR	Polymerase chain reaction
PGI	Phosphoglucoisomerase
PGM	Phosphoglucomutase
NJ	Neighbor-Joining
RAPD	Random amplified polymorphic DNA

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SKDH	Shikimate dehydrogenase
UPGMA	Unweighted pair-group method with arithmetic average
TEMED	Tetramethylethylenediamine

Introduction

The grass genus *Digitaria* Haller is one of the largest genera of the family Poaceae including up to 325 annual and perennial species native to the tropics and subtropics (Clayton and Renvoize 1986; Henrard 1950). This large species diversity within the genus may be attributed to its great antiquity, but also to its important rate of speciation. A considerable range of variation in quantitative morphological characters occurs both within and between species (Hayward and Hacker 1980). Henrard (Henrard 1950) divided the genus into four subgenera and 32 sections, the latter merely based on the hair type of the spikelet. The taxonomy of many of these groups remains nonetheless confused requiring further detailed studies to delineate taxonomic/species boundaries (Killeen and Deagrasar 1992). Polyploidy is common in the genus and constitutes an important mechanism in its evolution. *Digitaria* species are generally C_4 plants with digitate inflorescences but rarely paniculate type is also observed. Like most of the economically important genera in the Paniceae tribe, many *Digitaria* species are valuable forage grasses and some others are cultivated as cereal crops. *Digitaria exilis* (Kipp.) Stapf and *D. iburua* Stapf, known as white and black fonio, respectively, are indigenous millets cultivated since prehistoric times in semiarid areas of West-Africa. Their grains are used to make a variety of food products including traditional couscous, stiff or thin porridge, boiled either alone or with beans and vegetables, etc. that serve as staple food for millions of low income people. The two species are morphologically variable and overlap in inflorescence and spikelet characteristics, two main features by which they are distinguished. Recent cytological data indicate that both fonio species are tetraploids with a chromosome number of $2n=4x=36$ and having relatively small genome sizes (1C ranging from 904 to 956 Mbp) (Adoukonou-Sagbadja et al. 2007a).

Fonio millets are among the most suitable native cereals for production in infertile and drought-prone areas of West-Africa. Precise figures on the amount of fonio grown in West-Africa are not available, but it is estimated that approx. 300,000 ha are devoted yearly to its cultivation in the region (Bezpaly 1984). While the crops are appreciated for their traditional dishes, their productivity has not been improved through conventional breeding. Yields are low (0.6–0.9 t/ha, often under 0.2 t/ha in the Sahelian zone) and highly influenced by climate hazards (Adoukonou-Sagbadja et al. 2006; Haq and Ogbe 1995). Plant breeding is, potentially, an even more efficient way to achieve higher yield and good quality products. There is, however, very limited genetic variation within fonio species. Isozyme (unpublished data) and AFLP (Adoukonou-Sagbadja et al. 2007b) studies carried out to date have shown extremely (*D. iburua*) to relatively (*D. exilis*) low overall genetic diversity in traditional landraces grown by farmers in the major growing areas. It is therefore necessary, for breeding purposes, to broaden the genetic basis of these millets. Such broadening may be achieved by inter-specific hybridizations with closely related species. Understanding the life history and determining the ancestries of fonio millets are closely related to these objectives.

Reproductive system is one of the most important life history traits of a plant species. It has a large impact on plant population genetic structure and diversity and is particularly important for designing appropriate breeding procedures and genetic conservation strategies (Hamrick and Godt 1989). The genus *Digitaria* presents different reproductive systems: outbreeding, inbreeding, intermediate and vegetative reproduction (Killeen and Deagrasar 1992; Watson and Dallwitz 1992; Wipff and Hatch 1994). In fonio, the mating system remains less understood and the available information is mostly speculative. For some authors, fonio species are likely self fertilized crops (Sarker et al. 1993); however the possibility of outbreeding system has also been advocated (Fogg 1976; Hilu et al. 1997). In a recent study focusing on genetic diversity and population differentiation in West-African fonio millets, Adoukonou-Sagbadja et al. (2007b) confirmed the self-oriented mating behavior but noted that apomixis can be a conceivable alternative mating system to inbreeding in fonio. Apomixis or agamospermy is an asexual mode of reproduction in which the ovule develops into a seed without involving meiosis and fertilization (Nogler 1984). Since apomixis results in a transmission of an exact copy of the maternal genotype, it is then possible to discriminate between the two systems as genotypic deviation from maternal profile within the offspring is expected in sexual mating conditions (Marshall and Brown 1974; Siena et al. 2008).

A number of investigators have speculated on the origins and evolution of the domesticated fonio millets. Based on botanical affinities, Stapf (1915) and Dalziel (1937) earlier proposed the wild *D. longiflora* (Retz.) Pers., an annual weed widely distributed in tropical Africa, as the possible progenitor of *D. exilis*. In contrast, Henrard (1950) noted that *D. longiflora* is botanically more close to *D. fuscescens* Henr. and rather claimed affinities of *D. exilis* with the wild *D. barbinodis* Henr., also commonly found in West-Africa and generally exploited as wild cereal in Nigeria or Togo (Haq and Ogbe 1995). Regarding *D. iburua*, Stapf (1915) proposed the wild *D. ternata* Stapf while Portères (1976) considered *D. barbinodis* as its possible progenitor. Other wild species like *D. tricostulata* (Hackel) Henr. and *D. atrofusca* (Hackel) Camus are cited as morphologically close to *D. iburua* but they are geographically remote from the areas of diversity of the crop (Haq and Ogbe 1995). Using random amplified polymorphic DNA (RAPD) markers, Hilu et al. (1997) revealed that, in addition to morpho-botanical affinities, only *D. longiflora* and *D. ternata* displayed high genetic relatedness to *D. exilis* and *D. iburua*, respectively. Recent cytological information data pointed to the same conclusion (Adoukonou-Sagbadja et al. 2007a). Like the cultivated species, these two aggressive weeds are also tetraploids and display approximately similar genome size. The only consistent morphological distinctions observable between them and the cultivated fonio are the presence of fine pubescence on the spikelet of the wild species and heavy shattering as natural means of seed dispersion. Based on this information, it is likely that cultivated fonio crops are direct domesticates of these two wild species in which several key traits (e.g. spikelet hairiness, seed shattering) have been altered through generations of human selection. Identification and genetic studies of ancestral species of crop plants are a central issue in plant breeding. Diverse agronomically useful characters including resistance and tolerance to pests and diseases are generally known to be present in wild relatives of crop plants (Ochatt et al. 2004). Other important traits like resistance to lodging and especially big seed size critically useful for fonio breeding are also expected to be found in wild *Digitaria* species (Kuta et al. 2003). Successful gene introgression from wild to cultivated crop species essentially relies on the degree of speciation and phylogenetic relationships among the two gene pools. A better understanding of these relationships is crucial for the desired traits from the wild relatives to be used in fonio improvement programs.

During the past decades, classical morphological and isozyme methods in plant genetics and breeding have been complemented by modern molecular techniques targeting directly DNA sequences in the plant genome. These novel DNA techniques have been used for various purposes including population genetics and diversity analysis in a large number of crop plants (e.g. Ayele et al. 1999; Dida et al. 2008), molecular taxonomy and phylogeny investigations (e.g. Bänfer et al. 2004; Dasmahapatra et al. 2009; Milla et al. 2005), as well as mating system determination (e.g. Hovmaln et al. 2004; Karasawa et al. 2007; Kollmann et al. 2000), etc., and have in that way considerably facilitated the breeding work, mainly in major food crops. In fonio millets, the use of RAPDs in studying genetic diversity and evolution has been reported (Hilu et al. 1997). While this marker technique provides better information data than morpho-botanical traits, its efficiency, particularly for phylogenetic analysis, is controversially discussed for theoretical and technical reasons (Van de Zande and Bijlsma 1995). Amplified Fragment Length Polymorphism (AFLP) (Vos et al. 1995) analysis is currently a method of choice in molecular studies and has been, as in many other crops (e.g. Ayele et al. 1999; Bänfer et al. 2004; Seehalak et al. 2006), applied with success in genetic diversity and population differentiation analysis in West-African fonio germplasm (Adoukonou-Sagbadja et al. 2007b). The AFLP technique has at least three important advantages: its applicability to all organisms without previous sequence information, its high multiplex ratio and wide genome coverage, and its high reproducibility and robustness comparably to most of other multi-locus marker systems (Bänfer et al. 2004).

As part of ongoing comprehensive characterization of West-African fonio genetic resources (Adoukonou-Sagbadja et al. 2007a, b; Clottey et al. 2006; Hilu et al. 1997; Kuta et al. 2005; Sanou 1993), the present study first aims at evaluating, using AFLP marker technique, hypotheses on the ancestry of fonio species by analyzing representative accessions of *D. exilis* and *D. iburua* gene pools and the two wild species that have been considered as their possible progenitors, i.e. *D. longiflora* and *D. ternata*. We choose AFLPs because they have already been shown to generate sufficient markers to easily identify genotypic diversity and differentiation in fonio (Adoukonou-Sagbadja et al. 2007b). To confirm the close phylogenetic relationships of these species, accessions of three other taxonomically distant wild species are included. Besides, as the second objective of the study, AFLP fingerprinting technique is additionally used as complement to isozyme markers in progeny analysis to infer the reproductive system of fonio species.

Results

AFLPs and Phylogenetic Relationships

The AFLP analysis, conducted on the 17 accessions of fonio and wild *Digitaria* species (cf. Supplementary Table 1), produced distinct profiles for the 6 primer-pairs used in the study. The number of polymorphic bands generated by each primer set varied from 37 to 96 (average of 65), in a size range from 50 to 515 bp (Table 1). In total, 391 distinct bands were scored; fourteen (3.6%) are present across individuals within the seven species, 316 (80.8%) are shared between groups of two to six species and 61 (15.6%) are strictly specific to a single species. These latter markers are more important in wild species than cultivated ones with *D. iburua* showing the lowest (2) and the highest (24) specific markers obtained with *D. sanguinalis*. Mean

Table 1 Number and size range of polymorphic bands scored per primer combination during AFLP analysis of *Digitaria* species

Primer combination [a]	SPB [b]	Size range of SPB	R [b]
E-AAG/M-CAA	87	50–400 bp	99
E-AGG/M-CGT	37	125–515 bp	100
E-ACA/M-CAT	75	69–400 bp	97
E-ATG/M-CAC	96	50–430 bp	99
E-AGG/M-CCT	47	55–390 bp	100
E-CTC/M-GTA	49	70–350 bp	99

[a] *E= 5′-GACTGCGTACCAATTC-3′, M= 5′-GATGAGTAGTCCTGAG-3′*

[b] *SPB* number of scored polymorphic bands; *R* repeatability

reproducibility values, calculated as the percentage of bands that were identical in the two duplicates, are very high and range from 97% to 100% for the six primer-pairs (Table 1).

Based on the computed pairwise genetic distance, consensus phylogenetic trees were constructed using both unweighted pair group method with arithmetic average (UPGMA) and neighbor-joining (NJ) method. In general, these two methods generated trees of identical topologies with only minor differences within *D. exilis*; the one constructed by the NJ method is hereafter presented (Fig. 1a). Globally, three robust groups with very high (100%) bootstrap support (BS) were identified. The first group (I) included members of cultivated fonio species and their two presumed progenitors. In this group, two sub-clusters were formed with 99.9% BS: the first sub-cluster (Ia) is composed of all *D. exilis* accessions and its presumed wild progenitor *D. longiflora* while the second sub-cluster (Ib) groups *D. iburua* with its proposed wild progenitor *D. ternata*. In each sub-cluster, species clustered separately. The second group (II) formed also two well defined sub-groups (100% BS) including on the one hand *D. ciliaris* accessions (IIb) and on the other hand *D. sanguinalis* accessions (IIa). The last group (III) of the phylogenetic tree includes the single accession of *D. lecardii*.

AFLP-based mean genetic distances calculated between and within the *Digitaria* species are given in Table 2. White and black fonio showed pronounced genetic differences with Nei and Li distance estimated at 0.78. *D. iburua* and its presumed wild progenitor, *D. ternata*, showed the lowest

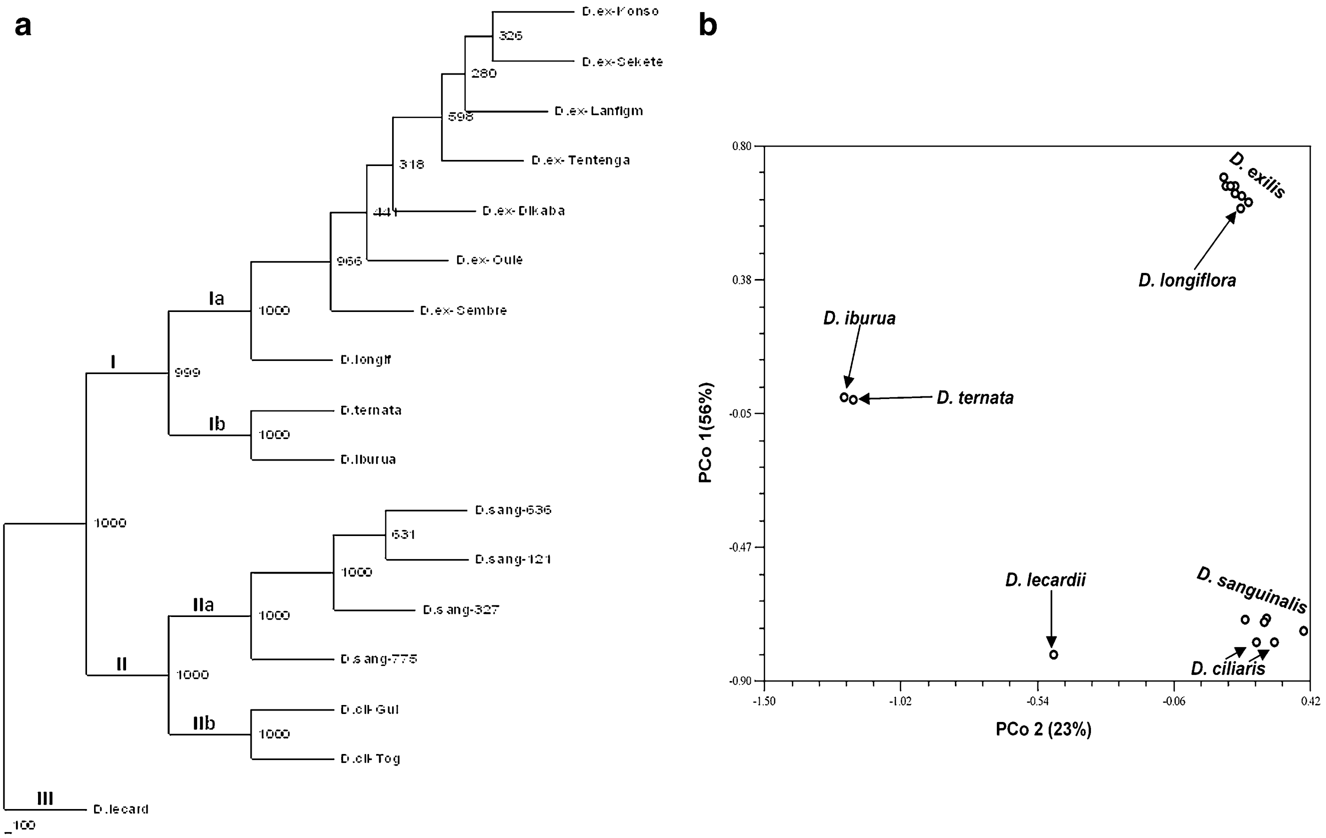

Fig. 1 Phenetic analysis of 17 accessions of 7 *Digitaria* species based on 371 AFLP markers: **a** Consensus neighbor-joining (NJ) tree showing the phylogenetic relationships of investigated accessions, **b** Principal Coordinate Analysis (PCoA) illustrating the general grouping of the different species analyzed. *Numbers at the dendrogram nodes represent bootstrap values; the genotype abbreviations include the abbreviation of the species (*e.g. *D. ex for D. exilis) followed by that of accession name or origin if relevant (*e.g. *−121 for accession n° 121 of D. sanguinalis, -Tog for Togo)*

genetic distance (0.018) and clustered very closely. A similar pattern is apparent for *D. exilis* with its proposed wild progenitor, *D. longiflora*, with a genetic distance of 0.072 (Table 2). The taxonomically distant species (i.e. the hexaploids *D. lecardii* and *D. ciliaris*, and the polyploid *D. sanguinalis*) showed the largest genetic distances to both cultivated fonio species ranging from 0.694 to 0.809. Between these three wild species, *D. sanguinalis* and *D. ciliaris* appears to be closely related with a genetic distance of 0.283.

Genetic relationships among the species investigated are further illustrated by the results of principal coordinate analysis (Fig. 1b). Variations along the first two axes accounted for 79% of the total variation, cumulatively. The species clustered together in a similar manner to the NJ analysis with *D. exilis/D. longiflora*, *D. iburua/D. ternata*, and *D. ciliaris/D. sanguinalis* associations and the differentiation of *D. lecardii* in an isolated group confirmed. The first axis (56% of the total variation) clearly differentiates the cultivated/proposed wild progenitors gene pools (NJ group I) from the taxonomically divergent species (*D. lecardii, D. ciliaris*, *D. sanguinalis*). The second axis (23% of the total variation), confirming species affinities, clearly resolved the high genetic differentiation between *D. exilis/D. longiflora* and *D. iburua/D. ternata* complexes in contrast to the NJ clustering which grouped them in the mega-group I (Fig. 1a).

Intra-specific variability in AFLPs was evident in both cultivated and wild species in which at least two accessions were studied i.e. *D. exilis, D. ciliaris* and *D. sanguinalis* (Table 2). The degree of variability within each species depends on its status: the highest variability was observed in wild species while the cultivated *D. exilis* was the least variable when considering the 391 AFLP markers surveyed.

Reproductive Characteristics of Fonio Millets

To examine the reproductive system in fonio, a series of progeny tests was performed using both isozymes and AFLP markers. In total, 270 progenies of six families (five for *D. exilis* and one for *D. iburua*) were screened. At the three isozyme loci surveyed, all progenies within each family showed fixed heterozygous profiles identical to each other and to that of their respective maternal genotype. The profiles for phosphoglucose isomerase (PGI) and shikimate dehydrogenase (SKDH) are presented as examples in supplementary Fig. 1. For AFLP analysis, similar results were obtained, except in *D. exilis* where two of the 20 progenies of the maternal genotype Oulè-Oulè showed a deviating profile (Table 3) revealed by the primer-pair E-ACA/M-CCA. The polymorphism shown by this primer-pair is due to the absence in the two deviating individuals of a single AFLP band (see arrow supplementary Fig. 2), differentiating them from the remaining 18 progenies which were identical to the mother plant. They represent 10% in the target population but only 2% at the species level when considering the whole 100 progenies fingerprinted by AFLPs. In the other progeny groups of *D. exilis* and in *D. iburua*, no deviating progeny from the maternal genotype was observed (Table 3) whatever the primer-combination employed. In conclusion to progeny tests, the results indicate that the mode of reproduction in fonio species is predominantly a vegetative multiplication, i.e. by apomixis.

Percent seed set under self- and open pollination conditions documented for all the six families is also presented in Table 3. In *D. exilis*, seed set varied from 86% to 99% after selfing and from 90% to 98.1% after open-pollination with overall mean percentage in almost the same order (91% and 93.8%, respectively). Similarly, high seed set was also documented in *D. iburua* under self- and open-pollination conditions (95% and 94%, respectively). Pollen viability in the analyzed *D. exilis* landrace populations ranged from 81% to 90% with an overall mean of 85.8% (Table 3). In *D. iburua*, the pollen viability documented for the unique landrace was 84% which falls within the range observed for *D. exilis*. In general, the two

Table 2 Estimated genetic distance values between (lower triangle) and within (diagonal) different species of *Digitaria* based on mean character differences between individuals using 391 AFLP markers

Code	Species	1	2	3	4	5	6	7
1	*D. exilis*	0.050[a]						
2	*D. iburua*	0.780	–[a]					
3	*D. longiflora*	0.072	0.774	–				
4	*D. ternata*	0.775	0.018	0.776	–			
5	*D. ciliaris*	0.754	0.816	0.734	0.807	0.125		
6	*D. lecardii*	0.791	0.694	0.788	0.585	0.402	–	
7	*D. sanguinalis*	0.756	0.809	0.741	0.806	0.283	0.425	0.105

[a] A mean genetic distance of 0.23 was previously detected within *D. exilis* using 118 accessions and 1,050 AFLP markers while a mean distance of 0.03 was detected between four genotypes of *D. iburua* using the same number of markers (Adoukonou-Sagbadja et al. 2007b).

Table 3 Percent seed set under self- and open-pollinations, pollen viability and polymorphism in offspring of *D. exilis* and *D. iburua* populations

Species	Landraces' population	Percent seed set under		% pollen viability	No. of analyzed progenies	No. of deviating progenies	
		SP [a] (bagged)	OP [a] (control)			Isozymes	AFLPs
D. exilis	I3-Sèkètè	86.0	98.1	90.0	25 (20) [b]	0	0
	IV-Oulè-Oulè	90.2	95.6	81.0	25 (20)	0	2
	BUF69-Foni Femba	99.0	90.0	84.0	25 (20)	0	0
	TKB74-Sémbre	87.8	96.5	82.0	25 (20)	0	0
	BEN08-Tentenga	93.0	89.0	89.0	25 (20)	0	0
	Overall	91.2	93.8	85.8	125 (100)	0	2
D. iburua	BEN39b-Péi	95.0	94.0	84	25 (20)	0	0

[a] *SP* self pollination; *OP* open pollination

[b] in brackets, No. of progenies analyzed with AFLPs, others are by isozymes

fonio species displayed high and approximately similar pollen fertility, indicating a normal meiosis (i.e. microsporogenesis) in the anthers.

Discussion

The primary objectives of the present study are to assess the reproductive system in fonio millets and clarify their phylogenetic relationships to some polyploid wild relatives. Except the RAPD study by Hilu et al. (1997) that partly dealt with the origin and evolution of fonio crops, no specific attempt has so far been made to investigate these important aspects of fonio biology and genetics. The useful results and inferences drawn from this study contribute to the general understanding of these millets.

AFLP Efficiency and Phylogenetic Relationships

In the present study, AFLP markers were successfully used to survey the genetic variations and relationships among the two cultivated fonio species and five wild *Digitaria* taxa. Recent characterization of fonio genetic diversity by Adoukonou-Sagbadja et al. (2007b) revealed that AFLP is an efficient molecular tool to strengthen the resolution of morpho-botanical approaches in identification of fonio species and could be useful for taxonomic and evolutionary investigations in the genus *Digitaria*. The use of this technique in the present study supports this observation. The six informative primer-pairs assayed revealed variability in AFLP markers among as well as within species, resulting in a clear differentiation of the two fonio species and the five wild relatives investigated (Fig. 1). Furthermore, the relationships obtained among species were in general concordant with the ones expected based on their taxonomy and/or ancestral relationships. The very high repeatability of the technique (97–100%) whatever the primer-combinations used in the study (Table 1) and the concordance obtained between the clustering approaches (i.e. UPGMA and NJ) indicate the reliability of AFLP technique for fingerprinting in *Digitaria*. Besides, the presence of important species-specific markers (about 15.6% of the total polymorphic markers detected) suggests the possibility of developing probes to effectively discriminate and adequately exploit wild *Digitaria* genetic resources in fonio breeding.

Previous hypotheses on the close relationships between *D. exilis* and *D. longiflora* on the one hand, *D. iburua* and *D. ternata* on the other hand, based upon morphology (Dalziel 1937; Henrard 1950; Stapf 1915), RAPD (Hilu et al. 1997), and recent cytological data (Adoukonou-Sagbadja et al. 2007a) were here confirmed by the present study. Although *D. barbinodis* has until now not been included in any molecular study because of its unavailability in gene banks, important morpho-botanical traits such as the shape of the rachis, the relative length of the upper glume to the lemma or the number of nerves on the glume distinguish this wild species from the crops (Clayton 1972). In contrast, the only divergent traits of *D. longiflora* and *D. ternata* from the cultivated species are the fine pubescence of their spikelets and the heavy shattering of their mature grains as a way of natural seed dispersion. The high degree of genetic relatedness obtained in the present study (more than 92% similarity, 100% BS) clearly supports the view of direct domestication of fonio crops from these two wild tetraploid species. However, in contrast to Hilu et al. (Hilu et al. 1997), our AFLP data do not suggest multiple domestications of fonio crops since the unique genotype investigated in *D. longiflora* was well separated from *D. exilis* accessions carefully selected to cover the range of diversity in the crop (Fig. 1a). The idea of unique domestication already arose from our previous genetic diversity study in fonio millets (Adoukonou-Sagbadja et al. 2007b). However, more thorough sampling of both *D. longiflora* and *D. ternata* will be required to confirm this finding.

As expected, pronounced genetic differentiation was observed between fonio species and the other wild *Digitaria* taxa investigated (i.e. *D. ciliaris, D. lecardii* and *D. sanguinalis*). This finding supports the differences in morphology (Henrard 1950) and cytology (Adoukonou-Sagbadja et al. 2007a) among these two groups of species and indicates that they belong to distinct evolutionary lines. A similar conclusion can also be drawn when considering the two fonio species/wild progenitors complexes since a comparably high genetic divergence was also documented between them (Table 2). This observation is globally concordant with previous RAPD (Hilu et al. 1997) and AFLP (Adoukonou-Sagbadja et al. 2007b) studies and supports the view of differences in genomic composition of these crops (Adoukonou-Sagbadja et al. 2007a). However, in contrast to these wild species which morphologically differ greatly from the cultivated gene pools, fonio species display a considerable resemblance in their morphologies (Haq and Ogbe 1995; Portères 1976). The major differences among the two species are related to the inflorescence morphology, and spikelet size, structure and pigmentation. Plasticity in quantitative floral traits and human artificial selection for specific agricultural traits could lead to such morphological overlap as has already been reported in *Echinochloa* millets (Hilu 1994). It is important to note here that the genetic relationships observed between the *D. exilis* accessions in the present phylogenetic tree (Fig. 1a) do not follow those previously reported between their genetic groups of origin (see Adoukonou-Sagbadja et al. 2007b). This finding may not be taken as a contradictory result since the clustering observed here within this crop species was mostly supported by very low BS, ndicating that the number of AFLP loci surveyed, if sufficient for resolving inter-specific differentiation, remains limited for the inference of stable and strong genetic relationships within the cultivated fonio crops.

Among the wild species investigated, *D. ciliaris* (syn. *Digitaria adscendens*) and *D. sanguinalis* are the most genetically distant from both cultivated fonio species. The taxonomic distinction of these studied wild species has been discussed (Ebinger 1962; Henrard 1950). They are morphologically similar and lie at the center of a complex of somewhat intergrading and weedy species sometimes difficult to be distinguished (Shouliang and Philips 2006) despite their different origins: *D. ciliaris* is a pantropical species while *D. sanguinalis* is from temperate regions (Henrard 1950). The present AFLP data confirm the genetic distinction of the two species (Fig. 1a) but suggest that they may share a close ancestry (Fig. 1b). The genetic differentiation of these two related wild species from *D. lecardii* is also supported by their morpho-botanical divergence (Henrard 1950; Vega et al. 2009).

Although the seven accessions investigated in *D. exilis* were carefully selected to cover the range of genetic diversity of the crop, the genetic variation observed here within this cultivated species was lower than that detected in *D. ciliaris* and *D. sanguinalis*, despite the comparably smaller sample size of these wild species (Table 2). The decreased genetic diversity of the cultivated species compared to the wild taxa is indicative of a domestication bottleneck, a common feature in crops (e.g. Dida et al. 2008). Crop domestication is a relatively recent (about 10,000 years) evolutionary process from a few wild ancestral populations. As a consequence, crop populations represent only a subset of the variability of the wild ancestral species. The success in broadening crop genetic variability by gene introgression from wild species relies essentially on the degree of speciation and phylogenetic relationships among the two gene pools. Having recognized the species most closely related to fonio such as the two presumed wild progenitors, it may now be possible to use them in the improvement of the crops. The other wild species investigated in this study, because of their cytological and high genetic divergences are of less immediate significance in the improvement of fonio crops.

Reproductive System in Fonio Millets

Segregation patterns within progeny arrays for isozyme and AFLP markers were further studied with the purpose of determining the reproductive mode in the cultivated fonio crops. The inference of the mode of reproduction by assaying progeny arrays for molecular genetic markers is a direct and robust procedure that is more conclusive than the traditional cyto-embryological approaches (Clegg 1980). To our knowledge, this study is the first that specifically deals with the determination of the reproductive system of fonio species. The results concordantly revealed that fonio crops reproduce essentially by apomixis. This is shown by the fixed heterozygosity (multi-banded patterns) observed at the three isozyme loci and the large number of identical AFLP fingerprints of the progenies from a single maternal plant (98% and 100% for *D. exilis* and *D. iburua*, respectively). Besides, almost all apomictic plants are known to be polyploids (Asker and Jerling 1992). The tetraploid level of both fonio species (Adoukonou-Sagbadja et al. 2007a) is then concordant with their apomictic reproduction. Apomixis has previously been described in other *Digitaria* taxa such as *D. arenicola* Beetle and *D. cognata* Pilger (Wipff and Hatch 1994) and is known to be a widespread evolutionary phenomenon within the Poaceae, particularly in Chlorideae and Paniceae (Nogler 1984). In many well characterized apomictic species, apomixis is often associated with hybridization and allopolyploidy (Asker and Jerling 1992). The fixed heterozygous isozyme profiles observed in this study attests the hybrid genomic nature of fonio species and suggests that the tetraploid level of these crops (and thus their two wild progenitors) is of allopolyploid origin, as it has also been recently demonstrated by Shinohara et al. (2010) in *Lepisorus thunbergianus* Ching (Polypodiaceae). Questions nonetheless remain on the identities of the genome contributors of these tetraploid cultivated/wild progenitor complexes. These may be sought among the closely related diploid *Digitaria* species, particularly those of West-African origin.

Despites the complete genetic uniformity observed in progeny arrays of both cultivated fonio species, 2% of the seedlings in *D. exilis* showed genotypic deviation from the common maternal banding pattern (Table 3). As it was already reported in many other species, these aberrant seedlings were most likely the product of rare sexual recombination events including natural hybridizations and automixis, i.e. the fusion and subsequent parthenogenetic development of two egg nuclei in a reduced embryo sac (e.g. Antonius and Nybom 1995; Bayer et al. 1990; Kollmann et al. 2000; Thomson and Ritland 2006). These potential sexual seedlings are characterized by a missing band detected by one of the six primer-pairs used. Based on the present results and following the above authors, it is well conceivable to classify *D. iburua* as an obligate apomict and *D. exilis* as a highly apomictic species with residual (i.e. 2%) sexuality. Such a conclusion is well in agreement with the extremely (H=0.02) or relatively (H = 0.267) low genetic diversity detected in these crops as well the high genetic differentiation observed among *D. exilis* populations (Adoukonou-Sagbadja et al. 2007b). The percentage of sexual seedlings detected in *D. exilis* is clearly lower than that reported in many facultative apomicts (Kollmann et al. 2000; Nybom 1995; Thomson and Ritland 2006, etc.). Since the experiments were only conducted under greenhouse conditions, further investigations is needed on whether or not sexually derived seedlings appear more often under field conditions.

Seed set and pollen viability are important reproductive characteristics in plants. In our experiments, the percentages of seed set and pollen viability determined for the two fonio species were high, almost always exceeding 80% (Table 3). Similar results regarding pollen viability have been reported in *D. eriantha* (Pozzobon et al. 2006). High seed set and pollen viability are characteristics of most pseudogamous apomictic plants (a form of apomixis in which pollination and fertilization of polar nuclei are required for endosperm development, thus for good seed set) (e.g. Acuña et al. 2008; Vogel and Burson 2004). Since hand emasculation is often unsuccessful due to the miniature size of fonio florets, the effect of outbreeding on seed set could not be assessed. However, the high seed set obtained under self-pollination conditions clearly suggest that fonio crops are highly self-compatible. On the other hand, the high pollen viability indicates that fonio plants are potentially male fertile and can be used as pollen donor in cross-breeding. According to Brown and Emery (1958), the mechanism of apomixis in the Paniceae tribe (which contains both fonio species) is apospory, i.e. the development of unreduced embryo sacs from nucellar cells in the ovule. Apospory has already been described in *D. cognata and D. arenicola* by Wipff and Hatch (1994). Since these aspects seem to be relevant for a complete understanding of the apomixis mechanisms in fonio, further cyto-embryological examinations could be a valuable complement to the present molecular (isozyme and AFLP) approaches.

Conclusion

The present study provides additional molecular (AFLP) genetic support to the origin and evolution of fonio crops as shown by previous RAPD (Hilu et al. 1997) and cytological (Adoukonou-Sagbadja et al. 2007a) data. It confirms the genetic differentiation of the two fonio species as well the tetraploid *D. longiflora* and *D. ternata* as their putative wild progenitors. The study also provides useful information regarding the reproductive system of fonio millets, as that the crops essentially reproduce by apomixis. Overall, the AFLP markers displayed better resolution than isozymes in the determination of the mode of reproduction in fonio species. Besides, the study reveals AFLPs to be well conserved marker traits and more reliable indicators of genetic relationships in *Digitaria* when comparing our results to those obtained with RAPD in a previous study (Hilu et al. 1997). These findings clearly indicate that AFLPs have the potential of complementing conventional approaches in reconstructing the phylogenetic history and assessing the reproductive system in the genus *Digitaria*. The dearth of information on fonio biology and genetics remains the major limitation for the breeding of these millets. The major results presented here along with those of previous studies (Adoukonou-Sagbadja et al. 2007a, b; Clottey et al. 2006; Hilu et al. 1997; Kuta et al. 2005; Sanou 1993) can be used to initiate promising breeding programs in fonio millets.

Methods

Plant Material

The plant material used in the study included 13 accessions of *D. exilis* (7), *D. iburua* (1), *D. longiflora* (1), *D. ternata* (1), *D. ciliaris* Koeler (2) and *D. lecardii* Stapf (1) taken from the germplasm maintained as research collection at the

Laboratory of Genetics and Biotechnology (Univ. Abomey-Calavi, Cotonou, Benin) and previously analyzed at cytological and/or molecular levels by Adoukonou-Sagbadja et al. (2007a, b). Samples of *D. exilis* were carefully selected to cover the three gene pools previously identified in West-African fonio germplasm based on AFLP markers (Adoukonou-Sagbadja et al. 2007b). Because of the extremely low genetic diversity detected in *D. iburua* germplasm (Adoukonou-Sagbadja et al. 2007b), this species is represented by only one accession in the present study. Further seeds of four accessions of *D. sanguinalis* L., obtained from the gene bank of the Leibniz Institute of Plant Genetic and Crop Plant Research (IPK, Gatersleben), were included as reference species for comparison. A list of all germplasm used, their country of origin if available, as well their ploidy status is given in supplementary Table 1. Voucher specimens of all the studied samples exist at the Herbarium Gatersleben (GAT) of IPK gene bank. Plants were grown in the greenhouse at Giessen from grains in small pots.

Two random individual plants from five of the seven of pot-grown *D. exilis* accessions (i.e. the landraces Sèkètè, Oulè-Oulè, Foni Femba and Sémbre, supplementary Table 1) and the one (Péi) of *D. iburua* were considered in the reproductive system analysis. The plants were earlier transferred to new pots and grown to maturity: one set was self-pollinated at anthesis by isolating inflorescences in a glassine bag; the second set of plants was left to grow without bagging as control in open pollination conditions. At physiological maturity, panicles were separately harvested for both set of plants and threshed. Two aliquots of 50 harvested seeds from each selfed genotype were further sown in pots in greenhouses at Abomey-Calavi, Benin (aliquot A) and Giessen, Germany (aliquot B). Seedlings derived from the same representative maternal plant are defined as belonging to the same family and termed the progeny array.

Phylogeny Inference

Six AFLP primer-combinations (Table 1) adequately chosen among the 24 informative primer-pairs previously employed by Adoukonou-Sagbadja et al. (2007b) were used to fingerprint (described below) the 17 *Digitaria* accessions. AFLP banding patterns of the analyzed genotypes were scored as presence (1)/absence (0) of the bands using RFLPScan 2.1 software package (Scananalytics, Fairfax USA) to generate a binary data matrix. During the scoring, only bands showing unambiguous polymorphism were considered; other faint, fuzzy and monomorphic bands were discarded. Genetic distances between *Digitaria* accessions were computed from the generated binary raw data matrix using Dice similarity index (Dice 1945) which is equivalent to equation 21 of Néi and Li (1979). This distance measure is appropriate for AFLPs as it takes into account only the shared presence of bands as indication of similarity and ignores the absence of fragments, known to be more likely homoplasious (Dasmahapatra et al. 2009). Phylogenetic phenograms were generated by the unweighted pair group method with arithmetic average (UPGMA) (Sokal and Michener 1958) and the neighbor-joining (NJ) (Saitou and Nei 1987) clustering procedures using PHYLIP 3.6 software package (Felsenstein 1985). The reliability and robustness of the phylogenetic trees were obtained by comparing trees from different methods and by bootstrap analysis after 1,000 replications. To further confirm the global grouping pattern of the analyzed species, the binary distance matrix was also used to perform Principal Coordinate Analysis (PCoA) using NTSYS pc version 2.20e software program (Rohlf 2000).

Reproductive Assays

Reproductive systems of fonio species were determined following the method described by Marshall and Brown (1974) and recently applied in various plants including *Hypericum perforatum* L. (Arnhold-Schmitt 2000) and *Paspalum rufum* Nees (Siena et al. 2008). This method involves progeny testing of known genotypes and assumes that auto-segregation is absent, and thus, all progenies derived from apomixis are identical to the mother plant. The sexual or asexual origin of the progeny plants was determined by comparing genotypic profiles of progenies to each other and to their maternal genotypes.

Twenty to 25 seedlings per progeny array were randomly selected and then genotyped. Isozyme electrophoresis (described later) was basically used for progeny plants (aliquot A) genotyping. Based on preliminary assays involving 8 enzymatic systems, three were selected for their reproducible and heterozygous allozyme profiles for maternal genotypes and assayed in progeny arrays analysis. The heterozygous profile of the maternal genotypes is essential to expect segregation in offspring. The three enzyme systems assayed were: Phosphoglucose isomerase (PGI, EC 5.3.1.9), Shikimate dehydrogenase (SKDH, EC 1.1.1.25) and Phosphoglucomutase (PGM, EC 5.4.2.2). In flowering plants, PGI is functionally dimeric while SKDH and PGM are known to be monomeric (Sosa and Lindstrom 1999). Besides, to explore more loci for further confirmation, the progeny arrays (aliquot B) were also AFLP-fingerprinted using the same six primer-pairs (see above).

In support to progeny test, seed set under self (bagged plants) and open (control plants) pollination conditions and pollen viability for all the six different parental genotypes were also determined. In seed set determination, three randomly selected panicles per plant were considered.

Mature spikelets on the panicles were counted and mean percentages of normal seeds (i.e. containing caryopsis) obtained under each pollination condition were estimated.

Pollen viability, used as potential indication of meiotic regularity in male organ, was estimated following the procedure of Pozzobon et al. (2006) in *Digitaria eriantha* Steud. Fresh mature pollen was collected by shaking several panicles from the flowered plants of the six maternal genotypes. The collected pollen were subsequently stained in propionic carmine at room temperature and examined using a light microscope (x100). The viability of pollen was scored according to staining level: the well stained grains were considered as viable while empty or very weakly stained ones were considered as sterile. From each plant, ca. 200 pollen grains were counted and the mean percentage of viable pollen grains per parental genotype was estimated from three replications.

AFLP Procedure

Total genomic DNA was extracted from freeze-dried leaf samples using CTAB (cetyl trimethyl-ammonium bromide) procedure of Doyle and Doyle (1990). In phylogeny analysis, bulked leaf material from 4 to 5 plants was used for the DNA extraction while individual plant leaf material was considered in progeny arrays test. The isolated DNA was purified (RNAse treatment) and further quantified using a Hoefer DyNAQuant™ 200 fluorometer (San Francisco, CA, USA). The AFLP analysis was conducted as described by Adoukonou-Sagbadja et al. (2007b) in genetic diversity analysis in fonio millets. Briefly, the purified genomic DNA (ca. 125 ng per sample) was double-digested with *Eco*RI and *Mse*I restriction enzymes. The restricted DNA fragments were ligated to specific adapters for both enzymes and subsequently pre-amplified by polymerase chain reaction (PCR) using primers that match the sequences of adapters but contain one additional selective base at the 3′ end. The PCR consisted of an initial denaturation of 94°C for 3 min, followed by 20 cycles of 94°C for 30 s (denaturation), 56°C for 30 s (annealing), 72°C for 1 min (extension), and 72°C for 5 min (final extension). The pre-amplified products were 10-fold diluted with 1xTE buffer (10 mM Tris-HCl; pH 8.0; 0.1 mM EDTA, i.e. ethylene diamine tetra-acetic acid) and used as template for selective amplifications based on the six selected primer-pairs to generate AFLPs. The PCR reaction mixture (total volume of 25 µl) consisted of 12.5 ng fluorescent dye-labeled *Eco*RI primer, 30 ng *Mse*I primer, 0.2 mM of each dNTPs, 2 µl PCR buffer, 0.5 U Taq-polymerase (Qiagen, Germany) and 5 µl of diluted pre-amplified PCR product in deionized distilled water. The following thermal program was used during the selective amplifications: one initial denaturation cycle of 3 min at 94°C; 12 touchdown cycles of 30 s denaturation at 94°C, 30 s annealing at 65°C (−0.7°C per cycle), 1 min extension at 72°C; and 22 cycles of 30 s denaturation at 94°C, 30 s annealing at 56°C and 1 min extension at 72°C; 5 min final extension cycle at 72°C. The final PCR products were then separated by electrophoresis on 0.25 mm thick polyacrylamide gels containing 8% acrylamide, 10% ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) in 1x TBE buffer using an automated Li-Cor IR^2 4200 DNA sequencer (Li-Cor Inc., Lincoln, NE, USA). AFLP bands were size-referenced by the standard 50–750 labeled DNA-ladder. Reliability of the analysis was assessed by the use of duplicated samples.

Isozyme Gel Electrophoresis

Isozyme extraction and gel electrophoresis were conducted following the procedure developed by Second and Trouslot (1980) on rice, except for minor readjustments described in Dansi et al. (2000). In brief, a crude tissue homogenate was produced by grinding pieces of fresh growing leaves (0.5–0.8 g) in a potassium phosphate buffer 0.5 M pH 7.0 containing 20% sucrose (w/v), 5% PVP-40, 0.5% Triton X-100 and 14 mM 2-mercaptoethanol. The homogenate, absorbed onto paper wicks, was loaded on a 12% starch (Sigma) gel and subjected to electrophoresis in a 0.153 M Tris/0.04 M citric acid (pH 8) electrode buffer system at a constant voltage/intensity of 120 V/30 mA for about 5 h or until the marker dye had migrated at least 8 cm from the origin. After electrophoresis, the gels were cut horizontally and incubated in the dark at 37°C for specific enzyme activity. All enzyme stains followed those described by Dansi et al. (2000).

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