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Genetic Characterization of Traditional Fonio millets (*Digitaria exilis, D. iburua* STAPF) Landraces from West-Africa:

Implications for Conservation and Breeding

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Dissertation

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by

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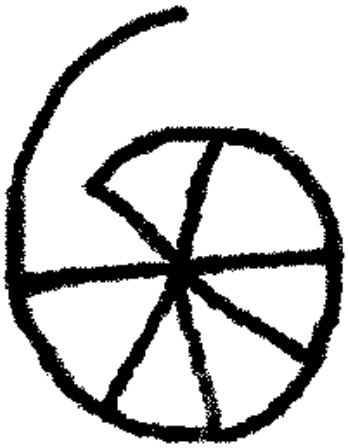
In memory of my beloved father Awo Edouard
and brother Laurent



Spiral of the birth of the Universe

"In the mythology of **Dogons** *(tribal group living the cliffs of Bandiagara in Mali), the grain of Digitaria exilis is the germ of the world, the central nucleus constantly ejecting other germs with increasing size, in conical spiral motion" (in Griaule and Dieterlen 1950)*

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ABSTRACT

Adoukonou-Sagbadja AH (2010) Genetic Characterization of Traditional Fonio Millets (*Digitaria exilis*, *D. iburua* STAPF) Landraces from West-Africa: Implications for Conservation and Breeding. PhD thesis, Justus-Liebig University, Giessen, Germany. With summaries in English, German and French, 107 pp.

Fonio millets (*Digitaria exilis*, *D. iburua*) are amongst the important indigenous cereal crops that greatly contribute to household food security in semi-arid and sub-humid drought-prone areas of West-Africa. Because of their complete scientific neglect, the potential of these crops for food and agriculture is not adequately exploited for improvement. This thesis therefore deals with the genetic characterization of fonio genetic resources with the overall objective to contribute to our knowledge on the biology and genetics of the crops. In the primary step of the study, a basic cytogenetic evaluation of fonio millets and some of their wild relatives was conducted. The genome size among these *Digitaria* taxa was variable, while its stability was evident within species. Besides, the longstanding hypotheses on cytological variability in fonio was not substantiated as the crops were found to be exclusively tetraploid with 2n=36 chromosomes. AFLP analysis supplemented by agro-morphological traits evaluation was further performed to quantify the genetic diversity in fonio crops and assess its population structure and geographical pattern of distribution. Globally, a relatively moderate to extremely narrow genetic background was detected in these crops, which need due attention from a conservation and breeding point of view. In *D. exilis*, the genetic diversity was structured and unequally distributed in the region. The genetic variability and phenotypic attributes were loosely correlated. Based on AFLP markers, the molecular phylogenetic relationships of fonio species with the wild relatives were also inferred. Previous view of direct domestication of *D. exilis* and *D. iburua* from the wild tetraploid *D. longiflora* and *D. ternata*, respectively, was confirmed. In the last step of the study, progeny analysis by both AFLP and isozymes, seed set determination and pollen viability test were conducted to determine the reproductive system in fonio millets. Apomixis was found to be the major (if not the absolute) mode of reproduction in these crops. The present work constitutes the first large scale genetic characterization of West-African fonio millets and substantially adds to the general scientific understanding of the crops. The diverse results obtained are relevant for conservation management and exploitation of fonio genetic resources in breeding that, ultimately, may boost fonio production in West-Africa.

KEYWORDS: *AFLPs, Breeding, Conservation, Cytology, Digitaria spp., Fonio, Genetic diversity, Isozymes, Phylogeny, Morphology, Reproductive system, West-Africa*

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ABBREVIATIONS

A	adenine
AFLP	Amplified Fragment Length Polymorphism
AMOVA	analysis of molecular variance
ANOVA	analysis of variance
ATAC	Atacora
bp	base pair
C	cytosine
°C	degree Celsius
1C DNA	DNA content in non-duplicated and reduced cell nucleus
2C DNA	DNA content in non-duplicated and non-reduced cell nucleus
cm	centimeter
CTAB	cetytrimethylammonium bromide
COPH	cophenetic
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediamine tetra-acetic acid
FAO	Food and Agriculture Organization of the United Nations
G	guanine
g	gram
GAT	Herbarium Gatersleben
G_d	genetic distance
GS_D	Dice genetic similarity
GTZ	Deutsche Gesellschaft für Technische Zusammenarbeit GmbH
H	Shannon index
H’	Nei gene diversity according to Lynch and Milligan (1994)
ha	hectare
IFZ	Interdisziplinäres Forschungszentrum, Giessen
IPGRI (SSA)	International Plant Genetic Resources Institute (South-Saharan Africa office), now Bioversity International
IRAG	Agriculture Research Institute of Guinea
IPK	Leibniz Institute of Plant Genetics and Crop Plant Research
INRAB	National Agriculture Research Institute of Benin
IRD	Institut de Recherche pour le Développement ; ex-ORSTOM
kg	kilogram
m	meter
mA	milliamper
Mbp	Mega (or million) base pairs
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimole
ng	Nanogram
NJ	Neighbor-Joining
NPK	Natrium, Phosphate, Kalium
ORSTOM	Institut français de recherche scientifique pour le développement en coopération ; currently IRD

PCR	polymerase chain reaction
PCA	principal component analysis
PCoA	principal coordinate analysis
pg	picogram
PGI	Phosphoglucoisomerase
PGM	Phosphoglucomutase
pH	Hydrogen proton
PI	Propidium iodide
PVP	Polyvinylpyrrolidone
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNAse	ribonuclease
SAHN	Sequential Agglomerative Hierarchical and Nested
SD	standard deviation
SE	standard error
sec	second
SIMQUAL	similarity for qualitative data
SKDH	Shikimate deshydrogenase
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat
T	thymine
t	ton
U	unit
UAC	University of Abomey-Calavi, Benin
UNIG	upper Niger
UPGMA	unweighted pair-group method with arithmetic average
vs	versus
USA	United States of America
V	volt
µl	microliter

Chapter I

GENERAL INTRODUCTION

Millets or small-seeded cereals are among the earliest domesticated crops used by humans (Baltensperger 1996). They form a diverse group including a dozen of crop species from different grass genera grown all over the world. Although minor in term of global food production, millets are crops of local importance in semi-arid regions, especially in marginal and drought-prone areas of Africa and Asia (Hilu 1994) where they constitute, along with sorghum, a principal source of energy, protein and vitamins (Wendorf et al. 1992). In Sub-Saharan Africa where the bulk of the production is achieved, over 130 millions of people daily depend on millets (Obilana and Manyasa 2002). Fonio millets (*Digitaria exilis* (Kippist) Stapf and *D. iburua* Stapf) represent a unique component of millet biodiversity traditionally grown in the savannah zone of West-Africa. They are crops showing high tolerance to drought, flooding and diseases. Due to their hardy nature, these traditional millets are regarded as priority crops in West-Africa, where they are essential to the diets of millions tribal people and deserve high value in their cultural traditions.

Fonio millets: a botanical overview

Fonio species belong to Poaceae family, sub-family of Panicoideae, tribe of Paniceae and the genus *Digitaria* Haller. This genus comprises 230-325 annual and perennial grass species with a wide geographic distribution in the tropics and subtropics (Henrard 1950; Clayton and Renvoze 1986). Fonio millets are the most economically important crops in this genus. *D. sanguinalis* L. has also been grown as millet in Eastern Europe from the Middle Age to the beginning of the 20th century while *D. crucita* Camus, domesticated at the late nineteenth century, and *D. compacta* Veldkemp (Raishan) are still grown in India (Nesbitt 2005). A number of wild species are valuable forage grasses throughout the tropics; many others have been harvested in the past for food in times of famine or food scarcity in Africa (Haq and Ogbe 1995, de Garine 2002, Adoukonou-Sagbadja et al. 2006).

Fonio species are C_4, free-tillering and annual herbaceous plants with erect, slender and glabrous culms. *D. exilis* is usually up to 80 cm tall while *D. iburua*, usually, can reach 1.5 m. Leaves are glabrous with a proximal sheathing base and distal strap-shaped blade. Their inflorescence is a finger-shaped panicle having 2-5 digitate (*D. exilis*) or 4-10 sub-digitate (*D. iburua*) racemes of 5-12 cm length. In *D. iburua*, the lowest raceme is somewhat distant from the remaining (Fig. 1). The spikelet contains two bisexual florets with the lower unfertile whilst the upper is fertile having three stamens with yellowish anthers, two lodicules and a pink or purplish stigma. The reproductive system in these species remains less understood. For some authors, fonio species are likely self fertilized crops (Watson and Dallwitz 1992, Sarker et al. 1993); however an outbreeding system has also been advocated (Fogg 1976, Hilu et al. 1997). Grains are extraordinary tiny (0.5-1mm diameter, 0.75-2mm length) with 1,000 weighting 0.5-0.6g. The caryopsis is tightly enclosed within two brown husks (lemma and palea). In *D. iburua*, the husks are intensively dark-brown; hence it is commonly named black fonio in contrast to *D. exilis* known as fonio or white fonio. Within each species, diverse varieties with a growth cycle varying from 60 to 130 days are recognized by farmers.

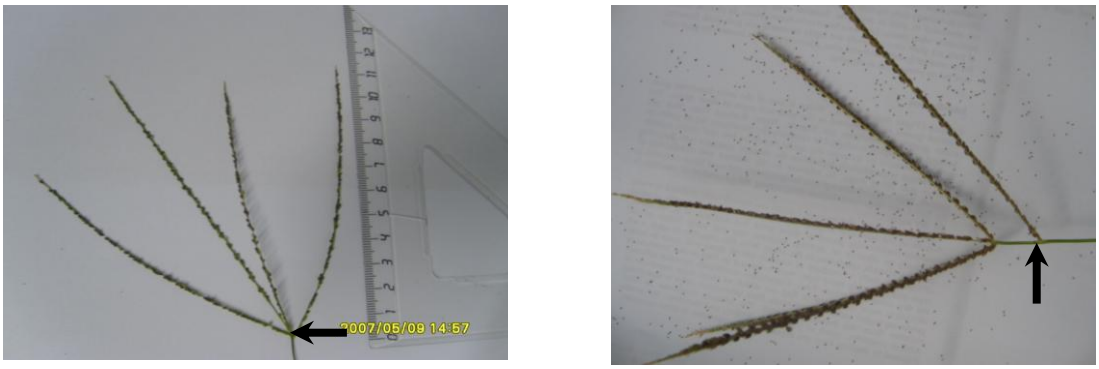

Figure 1: Differential disposition of racemes in a panicle of *D. exilis* (left) and *D. iburua* (right)

Origin and domestication of fonio millets

Fonio millets are both native to West-Africa with a cultivation history dating back to 5,000 BC (Murdock 1959). The cultivation of *D. exilis* scatters from Cape Verde in the West to the Lake Chad in the East, from the edge of the Sahara in the north to the beginning of the rain forest in the South (Fig. 2). *D. iburua* is currently much more limited in cultivation, being found only in Northern Nigeria, Togo and Benin that, almost certainly, represent a relic of formerly wider cultivation (Portères 1946, Haq

and Ogbe 1995). Unlike *D. iburua*, only *D. exilis* is reported to have reached the Dominican Republic in the 15th century (Deive 1974) but its cultivation as food crop is said to be very recent (Morales-Payán et al. 2002).

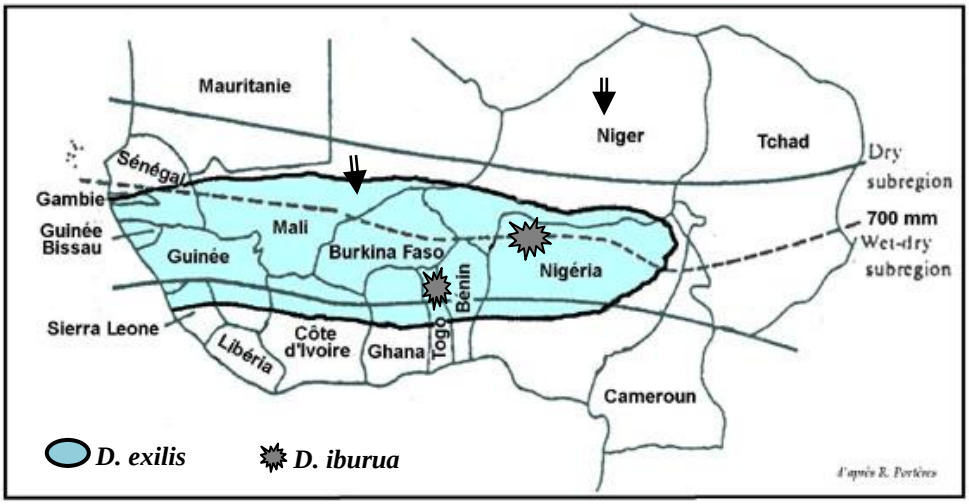

Figure 2: Fonio cultivation zone in West-Africa with proposed domestication area of *D. exilis* (left arrow) and *D. iburua* (right arrow) (modified from Portères 1976 by JF Cruz and completed)

While the West-African origin of the crops is well accepted, the number and precise areas of domestication of fonio millets in West-Africa are still in debate. Refering to historic, linguistic, varietal and ecological considerations, Portères (1959, 1976) but also Murdock (1959) located the earliest domestication of *D. exilis* in the vicinity of the central delta, in the upper Niger River basin. The authors explained that the vernacular name "*fonio*" or "*fonyo*" comes from Mande linguistic group living in the middle Niger, area where the largest landraces' diversity of the crop occurs. In a recent molecular study (RAPDs), Hilu et al. (1997) advocated the possibility of multiple domestications associated to different centers of diversification of this species. Regarding *D. iburua*, Portères (1946) related its local name "*iburu*" to the Haussa culture and indicated that its domestication may have been achieved in the Air montain (Northern Niger); the crop may have spread southward after the desertification of the Sahara.

Ancestral species or wild progenitors of fonio crops are not conclusively identified. Diverse wild species were proposed either as ancestors or close relatives based on their morpho-botanical affinities to cultivated fonio species (Table 1). Among these, *D. longiflora* Pers. and *D. ternata* Stapf are respectively the ones most botanically allied

to white and black fonio and largely admitted as their probable progenitors (Stapf 1915). In their molecular phylogenetic approach based on RAPD markers, Hilu et al. (1997) later confirmed the high genetic relatedness of *D. longiflora* and *D. ternata* to *D. exilis* and *D. iburua*, respectively, but revealed large genetic divergence of *D. fuscescens* to both cultivated species. These findings have provided up to date the clearest insight on the fonio origin and evolution. However, as suggested by the authors, other approaches such as cytological investigations and artificial crosses between taxa or exploration of other related species (e.g. *D. barbinodis* Henr.) are needed to exclude alternative hypotheses.

Table 1: Presumed wild relatives of cultivated fonio species

Species	Wild relatives	Authors	Characteristics
D. exilis	*D. longiflora**	Stapf (1915)	Annual and aggressive weed, widely distributed in the Tropics, well found in West-Africa
	D. barbinodis	Henrard (1950)	Annual, tropical Africa; present in fonio fields in Nigeria, Togo
	D. fuscescens	Henrard (1950)	Same section but rather closed to *D. longiflora*
D. iburua	*D. ternata**	Stapf (1915)	Annual and aggressive weed, hot regions of Africa and Asia
	D. barbinodis	Portères (1976)	As above mentioned
	D. tricostulata	Henrard (1950)	Botanically closed, but different geographical areas (North Kenya, South Africa)
	D. atrofusca	Haq & Ogbe (1995)	Botanically closed, but geographically more remote from the areas of diversity of the crops

* *Most probable progenitors*

Cytogenetics of *Digitaria* taxa

The genus *Digitaria* is cytologically variable with a basic chromosome number x = 9 that is typical for most genera of the Paniceae tribe (Hunter 1934). The genus is characterized by very small chromosomes and polyploidy is known to have played important role in its evolution. Karyologic analysis of various *Digitaria* species revealed a wide range of chromosome numbers / ploidy levels ranging from diploid (2n = 2x = 18) to dodecaploid (2n = 12x = 108) (Avdulov 1931, Hunter 1934, Gould 1963, Zeven and de Wet 1982, Wipff and Hatch 1994, Bennett et al. 2000, Caponio and Rua 2003). The vast majority of the species are polyploids with tetraploid and

hexaploid levels being the most commonly found. Some species like *D. cognata* subsp. *pubiflora* Wipff have more than one ploidy level (Wipff and Hatch 1994); a presence of B-chromosomes is also reported in *Digitaria eriantha* Steud (Pozzobon et al. 2006). In cultivated fonio, *D. exilis* is contradictory reported to be diploid, tetraploid or hexaploid (Hunter 1934, Zeven and de Wet 1982). Both tetraploid (Zeven and de Wet 1982) and hexaploid (Wanous 1990) levels have been proposed for *D. iburua*. The disparity in the reports and mainly the lack of unequivocal karyotypic information on these crops argue for the need of wide cytological reinvestigations for the effective use of fonio landraces in breeding. Except the chromosome number, little is known about other cytogenetic parameters of *Digitaria*. Marie and Brown (1993) and Bennett et al. (2000) reported the genome sizes in *D. setigera* Roth, *D. sanguinalis* L. and *D. ascendens* Rendle with 1C DNA content ranging from 1.2 pg to 2.3 pg, suggesting that *Digitaria* taxa may have a relatively small genome size.

Fonio cultivation and utilization in West-Africa

Production status and traditional uses

Fonio millets are small-scale farmers' crops and their production is still essentially at the subsistence level. The total production of fonio in West-Africa is not known as precise production statistics are lacking for many producing countries. According to Bezpaly (1984), approx. 300,000 ha are yearly devoted to the crop cultivation in the region. In 2008/2009 agricultural season, the available statistics indicate a total of 448,247 ha harvested with 480,227 tons of grains produced (FAOSTAT 2009). Most widely grown, white fonio furnishes the quasi-totality of the recorded production while black fonio accounts only for the negligible part (Ndoye and Nwasssike 1993). A survey of FAO production statistics the last two decades indicates that Guinea and Nigeria are the two leading fonio producers in the region, followed by Mali, Côte d'Ivoire and Burkina Faso (Fig. 3). Elsewhere, the production is minimal with somewhat in sensible decrease because of the tediousness of fonio cultivation and processing, strong competition from maize and other cash crops like cotton, absence of modern varieties, etc. (Sanou 1993, Adoukonou-Sagbadja et al. 2006). Productivity varies greatly across growing areas, years and is highly influenced by climate hazards. In general, the regional average yield oscillates between 0.6-0.9 t/ha with the best productivity reaching 1.5 t/ha. In the Sahelian zone, yields are extremely low and fall often under 0.2 t/ha.

Fonio is essentially produced for human consumption. It is an important household food security crop as the grains can be conserved many years without insect

damage. Fonio is well appreciated for its tasty and easily digestible grains and serves either as staple or co-staple food for several millions of tribal people. For instance in many tribal areas of Guinea, Mali, Togo and Nigeria, fonio can be consumed two to three times a day and is preferred to other cereals (Haq and Ogbe 1995). It is also the most prestigious and hence the first food choice reserved for guests or special occasions, e.g. ceremonies. Diverse biochemical investigations indicated that the nutritive value of fonio grain is favorably comparable with that of other cereals (Haq and Ogbe 1995). Fonio has excellent protein composition (9-12%) that is advantageously rich in methionine and cystine, two vital amino-acids almost deficient in the major cereals like sorghum, rice, wheat or barley (Vietmeyer et al. 1996). Traditionally, fonio is routinely consumed as stiff or thin porridge, couscous, and can be mixed with other flours to make breads. It is also popped or used to brew local alcoholic or non-alcoholic drinks. Nowadays, fonio foods are gaining importance in many urban centers particularly in Guinea, Mali and Nigeria while precooked products are timidly entering European market under the bio label.

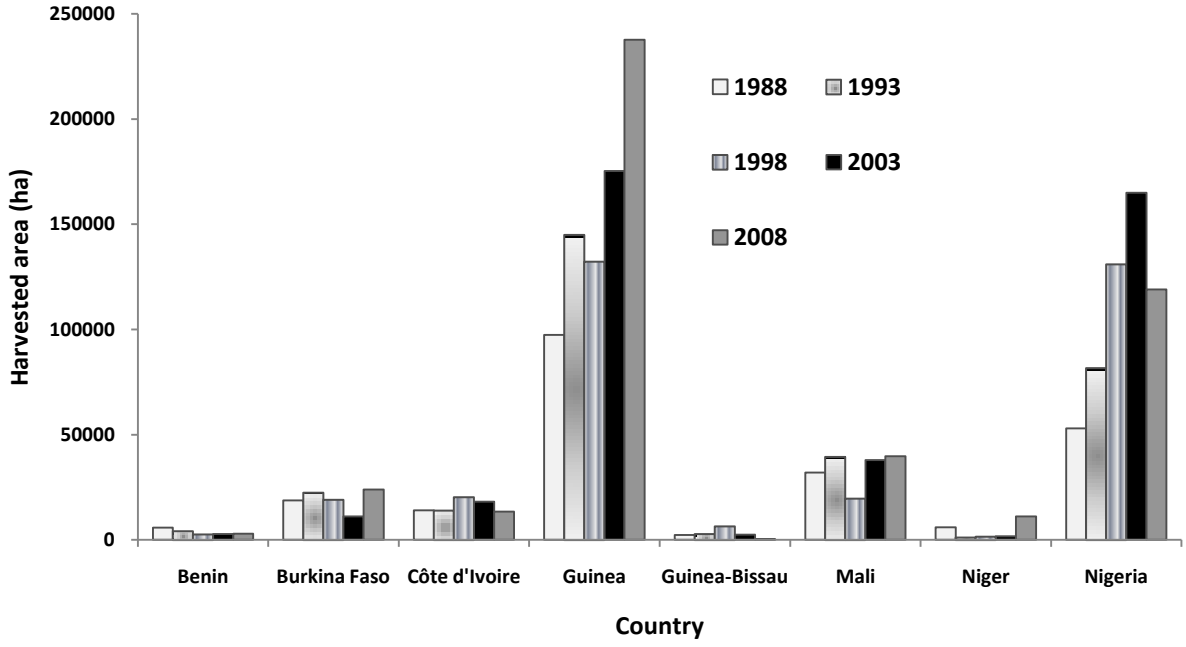

Figure 3: Land area devoted to fonio cultivation across selected years of the last two decades in different countries of West-Africa

Utilization of fonio grain as animal feed is not significant. However, the chaff and straw are important valued by-products widely used as livestock feed while the latter is often used by farmers in confecting mattresses, kitchen and barn roof. Fonio has also a number of folk medicinal values, for example, it is a useful diet for those

suffering from diabetes or for delivering women (Jideani 1999; Adoukonou-Sagbadja et al. 2006). Aside these usages, fonio is associated to the cultural and religious traditions of farmers. For instance, in the cosmology of Dogons (Mali), it is believed that the universe was born from a grain of *D. exilis* (Griaule and Dieterlen 1950).

Crop ecology, agricultural practices and seed system

In West-Africa, fonio millets are grown in traditional rain-fed farming system under a wide range of agro-climatic conditions. *D. exilis* is cultivated from sea level up to 1500 m altitude and mainly in areas receiving annually 700 to 1,000 mm rainfall; however the crop easily enters pluvial areas of critical rainfall deficiency with its current cultural limit at the annual isohyet of 150 mm whereas in general sorghum and pearl millet are limited by isohyets of 200-250 mm (Portères 1976). Southwards, the cultivation becomes rare when the annual rainfall reaches 2,000 mm (Diallo 2003). *D. iburua* is grown in similar but mostly in upland conditions (Portères 1976). Both crops are adapted to various soils including poor, sandy, degraded or marginal soils but heavy and saline ones are less suitable. In Fouta Djallon for instance, *D. exilis* copes well with acidic clays with high aluminium content, a combination often toxic to most food crops (Haq and Ogbe 1995). The optimal growing temperature range is 25-30°C with approx. 12 h daylight. In general, in contrast to black fonio, white fonio seems to be sensitive to day length (personal observation).

Fonio cultivation is fairly simple and remains exclusively manual. The crop is mainly grown in pure culture with rare associations with sorghum, pearl millet, guinea millet (*Brachiaria deflexa* Robyns), okra (*Hibiscus esculentus* L.), Roselle (*Hibiscus sabdariffa* L.), etc. Considered as a very low demanding crop, fonio occupies generally the last place in rotation systems after beans/groundnut and pearl millet/sorghum. Farm size is small and often below 1 ha. The sowing period varies among producing zones and depends on the onset of the rainy season. Soil preparation is minimal limiting to slight hoeing. Seeds are mainly broadcast-sown, at a seeding rate of ca. 10 to 30 kg of seed/ha. The weeding is performed manually two to three times from planting to heading (Fig. 4A). Pesticides and fertilizers are not applied by farmers and adequate information on the nutrient requirements of fonio is still yet lacking. At maturity, fonio is harvested by uprooting or cutting the straw. Harvesting is the most labor consuming activity, involving the farmer, his family and friends (Jideani 1990).Threshing is performed by beating or tramping the fonio sheaves (Fig. 4B). Grains are well storable (5-10 years) but their viability seems to decrease considerably after two years (Adoukonou-Sagbadja et al. 2006).

Farmers generally grow only one landrace but some rare households can grow two to three, depending on labor availability (Adoukonou-Sagbadja et al. 2006). In the entire cultivation zone, landraces are inherited from generation to generation. Fonio

seeds destined to be sown the next season are directly taken from the new harvested stock. In case of shortfall, farmers can obtain planting seeds from relatives or friends but buying from local market is not or less practiced because of possible seed mixture of different landraces.

Figure 4: (A) Weeding of fonio field by Wama women near Boukoumbé, northern Benin, **(B)** Farmers threshing fonio in Burkina Faso

Constraints to fonio productivity

Despite their importance in traditional agriculture, research efforts to improve fonio millets are still at a low level. In consequence, the crops remain primitive facing diverse agronomical and technological problems. First, fonio cultivation relies only on traditional landraces which are, despites their adaptability to marginal farming

system, less productive. In addition, traditional farming practices (e.g. systematic use of poor and eroded soils, poor husbandry, etc.) and frequent droughts occurrence, etc. may considerably affect the performance of the crops.

Lodging is a serious drawback in fonio cultivation because of the fragile shoot of the plant; it rends the harvest tedious and contributes notably to the yield lost. Besides, seed shattering at maturity, though limited in the crops, can become important if the harvest is delayed (up to 25% according to Vodouhè et al. 2003). While both fonio species have shown low susceptibility to pests and diseases, the fungi *Phyllachora sphearosperma* and *Helminthosporium spp.* have been seen to affect the crops. Fonio is also found to be susceptible to rust caused by *Puccinia cahuensis*. The parasitic *Striga*, particularly *S. rowlandi* known to abundantly occur in West-Africa, causes serious damage to the crops (Sanou 1993, Haq and Ogbe 1995). Besides, insect pests causing significant seed lost are also reported to occur occasionally.

The current low ranking of fonio millets in regional cereal production makes them less competitive than other major cereals like pearl millet, sorghum or maize and hampers their improvement through breeding, as the interest of breeders has been low. Progress in the genetic improvement of fonio has also been hindered by the biological characteristics of the crops and the fact that nothing is yet known on the inheritance of traits of agronomic relevance in fonio. The biological limitations among others include the miniature size of floral organs, the dearth of information on reproductive biology but also a poor knowledge of the level and organization of the genetic diversity present in the crops. Therefore, great efforts are needed to characterize and exploit fonio genetic resources for the improvement of these valuable but neglected crops in West-Africa.

Molecular markers as modern tools in plant genetics

Molecular markers are powerful genetic tools for investigating and characterizing genetic variability in any organism including plants. The use of molecular markers started with the discovery of biochemical markers (storage proteins, isozymes) in the 1960's (Lewontin and Hubby 1966). Along with the increase in knowledge on the genetic properties of DNA, numerous novel molecular techniques that detect directly polymorphisms at DNA level have evolved. The most commonly used DNA marker techniques in plant genetics are: Restriction Fragment Length Polymorphisms (RFLPs), Amplified Fragment Length Polymorphisms (AFLPs), Random Amplified DNA Polymorphisms (RAPDs), Inter Simple Sequence Repeats (ISSRs), Simple Sequence Repeats or microsatellites (SSRs) and Single Nucleotide Polymorphisms

(SNPs). These methods are used solely or as complementary tools to the traditional agro-morphological markers, known to be often subjected to environmental influences. In general, molecular methods differ in their principle, application, type and amount of polymorphism detected (reviewed by Semagn et al. 2006). Furthermore, each genetic marker system has its own benefits and drawbacks. Therefore, choosing the most appropriate marker system will depend on many factors such as the precise purpose, the desired levels of polymorphism, the availability of technical facilities, as well the efficiency in terms of costs and time requirements (Vendramin and Hansen 2005). Since isozymes and AFLP markers were used for this work, only these marker types are below in more detail described.

Isozymes

Isoenzymes or isozymes are the earliest molecular markers used to detect genetic variation in organisms. They are homologous enzymes differing in their amino acid sequences but share the same catalytic function (Markert and Möller 1959). Isozymes are expressed either by different alleles at the same locus (yet referred as allozymes) or by distinct loci. These differences may arise either from changes at the DNA level, which causes amino-acid substitutions and changes in charge of the protein or from post-translational modifications (e.g. glycozylation) which lead to changes in molecular weight. The ability to observe allelic variation at isozyme loci has revolutionized research in the fields of biochemical genetics, population genetics as well as in systematic and evolution studies (Hamrick 1989, Crawford 1989, Gao and Hong 2000). Isozymes have the advantages that their analysis requires no sophisticated equipment; they are usually co-dominant making them appropriate for heterozygocity estimates in genetic diversity studies. However, the main drawbacks to their use are the limited number of available enzyme systems (only 10 to 30 available for a given organism, reviewed by Avise 2004), the use of specific detection methods for each enzyme, and only genomic regions coding for expressed proteins can be analyzed resulting in low polymorphism (Lewontin 1991). Nowadays, isozymes are largely superseded by modern DNA-based approaches that are more informative and offer broader genome representation and higher prospects for selective neutrality. As the cheapest and quickest marker systems to develop, isozymes remain nonetheless an excellent choice for studies that only need to identify low levels of genetic variation, for instance in quantifying mating systems (Zeidler 2000).

Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) is among the most commonly used DNA-based molecular marker techniques and has been applied to a variety of

questions in plant biology, including genetic diversity and population genetics (e.g. Carr et al. 2003, Seehalak et al. 2006), molecular taxonomy and evolution (e.g. Bänfer et al. 2004, Milla et al. 2005), species/cultivar identification (e.g. Portis et al. 2004), genetic mapping and linkage analysis (e.g. Nissan-Azzouz et al. 2005), etc.

Originally developed by Vos et al. (1995), the essence of AFLP procedure lies in the combined use of two basic tools in molecular biology: the restriction endonuclease (Danna and Nathans 1971), which reduces the target genomic DNA into a pool of fragments; and the Polymerase Chain Reaction (PCR, Mullis et al. 1986), which allows amplification of a subset of these restriction fragments using primers with arbitrary selective extensions. In higher plants, fragment amplification is usually conducted in two steps: a pre-amplification and an amplification using primers with one and three selective nucleotides at their 3'-end, respectively. This allows a sequential reduction in complexity of the restricted patterns generated (i.e. to 1/16 and 1/4096 respectively). The presence or absence of the selective nucleotides in the genomic fragments being amplified and the restriction fragment size variation provide the basis for revealing polymorphism in AFLPs. This polymorphism can be due to differences in restriction sites, mutations around the restriction sites or inherent to insertions or deletions within the amplified restriction fragment (Bonin et al. 2005). The size and number of the resulting AFLP products make them ideally suitable for size-fragmentation and visualization as bands by polyacrylamide gel electrophoresis. In general, 20 to 150 polymorphic bands (markers) can be expected for any single assay, depending of the size and structure of the target species genome (Bonin et al. 2005). Their size range is typically between 50 to 500 bp. Successful AFLP analysis requires high quality DNA free of any contaminants that could otherwise alter the banding profiles. Besides, the choice of the restriction enzyme can be important. The two most commonly used enzymes in AFLP studies are *Mse*I / *Eco*RI (four-base / six-base cutter); *Taq*I / *Pst*I are the main respective alternatives found in the literature. In general, to increase the informativeness of the AFLP technique, different combinations of primer pairs leading to more polymorphic markers are usually used.

The AFLP fingerprinting technique offers several advantages compared to other molecular markers. It has the capacity to detect a higher number of polymorphic loci in a single assay than RFLPs or RAPDs (Powell et al. 1996), has a higher discrimination efficiency in comparison to RAPDs (Uptmoor et al. 2003, Wagner et al. 2005) and ISSRs (Archak et al. 2003), and produces highly reproducible results (Jones et al. 1997). However, like RAPDs and ISSRs, AFLPs show generally dominant inheritance which is the main detrimental aspect of the technique. The use of AFLP markers to study genetic diversity and population genetics in crops is promising because many polymorphic loci can be obtained fairly easily, in a relatively

short time and without any prior knowledge of the genome of the species under study (Vos et al. 1995). Therefore, they are found to be particularly attractive for the genetic diversity and differentiation studies, particularly in minor and neglected crops such as *Eragrostis tef* (Ayele et al. 1999), finger millet (Le Thierry d'Ennequin et al. 2000), proso millet (Karam et al. 2004), or African rice (Barry et al. 2006). The AFLP technique was also reported to work well for genetic relationships and phylogenetic studies in closely related species (Sharma et al. 1996, Le Thierry d'Ennequin et al. 2000, Bänfer et al. 2004) and found as efficient as microsatellites in parentage analysis and mating system determination (Gerber et al. 2000, Thomson and Ritland 2006).

In general, the estimation of allele frequencies and subsequently the population genetic parameters (e.g. number of alleles per locus, average heterozygocity or gene diversity, F_{ST}, G_{ST}, etc.) for dominant markers such as AFLPs present some statistical limitations because of the inability in distinguishing between homo- and heterozygote dominant genotypes (Lynch and Milligan 1994). These difficulties can be resolved by using indirect methods such as the Bayesian approach (Lynch and Milligan 1994, Zhivotovsky 1999) and/or alternative estimators like Shannon diversity index and Amova-based Φ_{ST} that rely on band frequencies (Shannon and Weaver 1949, Excoffier et al. 1992). On the other hand, for stable and biologically relevant results, Kimberling et al. (1996) suggested sampling a high number of loci as possible. In relation to this, Kremer et al. (2005) using AFLP markers, show that the monolocus estimation of genetic diversity has the potential to vary strongly with variations in the fixation index, but that the multilocus estimate is rather robust to deviations in Hardy-Weinberg equilibrium, because of the mechanistic effect of compensation between negative and positive biases of genetic diversity estimates for different AFLP loci exhibiting contrasting frequencies of the null homozygote.

Fonio genetic resources: current status and characterization in West-Africa

Fonio genetic resources are abundant in West-Africa. Hundreds of fonio landraces are recognized by farmers and still in use in the region. These landraces have been maintained for generations through traditional *in situ* on-farm conservation practices where farmers collect the seeds for raising the next season. They belong almost exclusively to *D. exilis*, the most widespread cultivated fonio species in the region. Fonio landraces exhibit some level of isomorphism, making them difficult to distinguish morphologically during the vegetative growth stage. Traditionally, farmers distinguish three groups of landraces, merely based on the growth cycle: precocious, intermediate and late maturing types. Precocious landraces mostly adapted to

drought abound in the dry agro-ecologies while the late ones are mainly grown in more wet conditions.

Through national / international germplasm collection initiatives (cf. Clément and Leblanc 1984, Kwon-Ndung et al. 1998, Adoukonou-Sagbadja et al. 2004, Clottey et al. 2006a), about 600-700 fonio accessions from diverse agro-ecological zones of West-Africa are yet maintained ex-situ by diverse National Agricultural Research Centers in West-Africa and IRD (ex-ORSTOM, France). These germplasms constitute important genetic resources but most of the material has to be evaluated or characterized properly.

The first insight into the characterization of fonio genetic diversity was the identification of racial groups based on morpho-botanical characteristics (Portères 1976). In *D. exilis,* four races were identified by the author and have been plotted according to their geographical localization: var. *gracilis*, *stricta*, *rustica* (including subvar. *clara* and *rubra*) located in the upper basin of the Niger River and the var. *densa* in northern Togo-Benin. Because of its much more restricted cultivation area, botanical varieties have not been described in the same way in *D. iburua* by the author who reported nonetheless the existence of two distinct black fonio varieties in Benin and Togo growing areas.

Sanou (1993) later characterized 54 ecotypes of white fonio from Burkina Faso and Mali while Clottey et al. (2006b) recently reported the characterization of thirteen Ghanaian fonio accessions. These works showed that significantly large variability exists regarding diverse agro-morphologic traits, e.g. grains mass, number of tillers produced, leaves length and wide, plant height and day to heading, panicle length, etc. However, the classification of these genotypes (ecotypes) evaluated did not support the racial grouping of Portères (1976).

Some rare attempts using RAPD markers to assess the molecular variability of fonio millets have been reported (Hilu et al. 1997, Kuta et al. 2005). Although involving very few fonio accessions (about 10) from geographically restricted areas (Togo and Nigeria, respectively), these preliminary studies demonstrated the existence of molecular polymorphism, but the global genetic diversity in these crops, its structure and also its pattern of distribution in West-Africa remain unknown.

Another important feature in characterizing the genetic resources of any crop is to consider its wild relatives as they are potential sources of disease resistance and stress tolerance genes (Ochatt et al. 2004). In crop breeding, they can be useful in broadening the genetic basis of the crops and assist in developing superior genotypes through inter-specific hybridizations. In general, closely related wild species, i.e. those belonging to the primary gene pools of crops, are commonly used.

Since the 1980's, crop improvement by genes introgression from distantly related and even non-related taxa (i.e. from secondary and tertiary gene pools) has become possible through genetic engineering (Meilleur & Hodgkin 2004). In consequence, crops' wild relatives should be collected, conserved and well characterized for future utilization in breeding. The only one study made to identify wild species closely related to cultivated fonio is that of Hilu et al. (1997) using RAPD markers and above referred to.

Thesis objectives and outline

Population growth and limited access to arable land worldwide, particularly in Sub-Saharan Africa, make it necessary to maintain and promote neglected traditional crops and increase their productivity. Fonio millets are among the important traditional cereal crops that significantly contribute to household food security in marginal areas of West-Africa. Despites their long cultivation history and importance in West-Africa, fonio millets remain the least studied among the cereal crops in general and millets in particular and, in fact, are ranked among the neglected, underutilized or the "lost" crops of Africa. The overall objective of this study was to contribute to the genetic knowledge of fonio that would enable efficient preservation and exploitation of its genetic resources in breeding programs. Cytogenetic, molecular and agro-morphological investigations were therefore conducted with the specific goals to shed light on fonio genome and genetic diversity. The study also aimed to evaluate fonio phylogeny and determine the reproductive system of the crops. Germplasm of both fonio species originally assembled from the main diversity centers was used in the study.

This dissertation is divided into five parts. After this introductory chapter dealing with generalities on fonio and the molecular tools employed in the study, the second chapter addresses the cytogenetic evaluation of fonio millets and some wild relatives, including their two presumed wild progenitors. This study documents their genome size using flow cytometry, explores the correlation of genome size variation with taxonomic/ancestral relationships between cultivated and wild gene pools as well as other geographic features. With a support from chromosome count, implications of the results for ploidy level considerations in fonio millets are also examined. In the third chapter, the genetic diversity analysis in fonio millets based on 1,065 AFLP markers supplemented by 16 agro-morphological traits is reported. The study estimates the extent of the genetic diversity, its population differentiation and geographical pattern of distribution in West-Africa. Correlations between genetic parameters and agro-morphologic features are also investigated considering the genotypes globally or the genetic groups identified in the germplasm under study.

The fourth chapter specifically deals with the reproductive system and molecular phylogeny of fonio species assessed using AFLP and isozyme markers. In the last chapter, a concluding discussion highlighting mainly the implications of the results for conservation and breeding of fonio millets in West-Africa are briefly presented.

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Chapter II

NUCLEAR DNA CONTENT AND FONIO GENOME*

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Flow cytometric analysis reveals different nuclear DNA contents in cultivated Fonio (*Digitaria* spp.) and some wild relatives from West-Africa

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Abstract. Nuclear DNA amounts of 118 cultivated fonio accessions representing 94 landraces collected from the major growing areas of West-Africa (Benin, Burkina Faso, Guinea, Mali and Togo) and eight accessions of four wild relatives were investigated by Laser flow cytometry. In cultivated species, average 2C-values ranged from 1.848 ± 0.031 pg for *Digitaria iburua* to 1.956 ± 0.004 pg for *D. exilis*. In *D. exilis* landraces the chromosome number was determined at 2n = 36. The closely related wild species *D. longiflora* and *D. ternata* showed similar 2C DNA contents of 1.869 ± 0.035 pg and 1.775 ± 0.070 pg, respectively. Distinctly larger genomes were identified for more distant species *D. lecardii* and *D. ciliaris* with 2.660 ± 0.070 pg and 2.576 ± 0.030 pg per 2C nucleus, respectively. Intra-specific variations were found to be slight and insignificant, suggesting genome size stability mainly within the cultivated gene pool. These results support the distance of cultivated fonio species *D. exilis* and *D. iburua* from *D. lecardii* and *D. ciliaris* as well as their close relationships with *D. longiflora* and *D. ternata*. Relevance of the results for ploidy level considerations in fonio millets is discussed.

Key words: Fonio, *Digitaria* spp., 2C-values, genome size, flow cytometry, chromosome number, West-Africa.

Introduction

The genus *Digitaria* Haller comprises 230–325 annual and perennial grass species with a wide geographic distribution in the tropics and subtropics (Henrard 1950, Clayton and Renvoize 1986). Many *Digitaria* species are important worldwide or regionally mainly as fodder but also as food crops. In West-Africa, *D. exilis* (Kipp.) Stapf and *D. iburua* Stapf are native millets cultivated as major staple food since five millennia BC (Murdock 1959). White fonio (*D. exilis*) is the most diverse

and widely cultivated species in the region. Conversely, *D. iburua* (black fonio) cultivation is restricted to northern Nigeria, Benin and Togo. In addition to this cultivated gene pool, there are a number of wild relatives that can provide potentially valuable resources for the improvement of fonio crops. They are aggressive weeds widely distributed in West Africa and some of them are considered by local farmers as "wild fonio" or "bird fonio" and were in the past harvested for food during long hunting trips or for fowl feeding (Adoukonou-Sagbadja et al. 2006). Based on botanical descriptions, several wild *Digitaria* species were proposed to be progenitors of cultivated fonio (for an overview ef. Haq and Ogbe 1995). However, using RAPD markers, Hilu et al. (1997) showed that only *D. longiflora* (Retz.) Persoon and *D. ternata* (A. Rich) Stapf were genetically closely related to white and black fonio, respectively.

According to Vietmeyer et al. (1996), fonio millets supply food to several millions of people. The special richness of their grains in methionine and cystine, two human-vital amino acids deficient in major cereals such as wheat, rice, maize, sorghum or barley, ranks fonio among the most nutritious of the grain crops (Jideani 1990). However, despite its important role in household food security the crop is still on a primitive production level and features many drawbacks, such as tiny seeds, poor yield, pests, diseases, plant lodging, laborious farming practices, difficult seed processing, etc. (Kwon-Ndung et al. 1998, Adoukonou-Sagbadja et al. 2006). During the last decade, important germplasm of fonio genetic resources was collected and conserved in the National Agricultural Research Centres of the main producing countries in West-Africa. For an efficient use of such germplasm in basic research and crop breeding programmes, information on chromosome numbers and genome size (DNA content) is very useful (Tuna et al. 2001). But, available information on ploidy level in fonio millets is still confusing. Hunter (1934) reported the unique chromosome count in *D. exilis* with $2n = 54$ chromosomes. Since the basic chromosome number of the *Digitaria* is thought to be $x = 9$, as in most of the Paniceae (Avdulov 1931, Hunter 1934), many authors assumed that this species is hexaploid with $2n = 6x = 54$ (Portères 1976, Wanous 1990, Haq and Ogbe 1995). In contrast, Zeven and de Wet (1982) suggested that *D. exilis* may be diploid with $2n = 2x = 18$ chromosomes or tetraploid having $2n = 4x = 36$ chromosomes. Both tetraploid (Zeven and de Wet 1982) and hexaploid (Wanous 1990) levels were proposed for *D. iburua*. To our knowledge, information on the nuclear DNA contents for both species does not exist until now. Furthermore, genome size documentation exists only for two species (*D. ascendens* Rendle and *D. sanguinalis* L.) in the genus *Digitaria* (Bennett et al. 2000).

Analysis of nuclear DNA content can be performed by microdensitometry or by flow cytometry. Nowadays, flow cytometry is the method of choice because of its ease, quickness, precision and accuracy in detecting small differences in DNA content (Rayburn et al. 1989). This technique has been successfully used in various ways in determining nuclear DNA content of major crop plants (Arumuganathan and Earle 1991), the ploidy level of grass species (Arumuganathan et al. 1999) or for taxonomical and evolutionary studies (Koopman 2000, Doležalová et al. 2002, Price et al. 2005).

In the present work, flow cytometric analysis was used to estimate the nuclear DNA content in a large and representative cultivated fonio germplasm and some wild related species. The study aims to investigate the possible correlations of genome size variations with taxonomic and ancestral relationships of these species or with ecological and geographic features. The study offers also a comparatively large and representative view on the ploidy level of fonio millets.

Materials and methods

Experimental material. One hundred and eighteen fonio accessions (six accessions of *D. iburua* and

112 accessions of *D. exilis*) representing 94 farmer-named landraces and eight accessions of wild *Digitaria* species originating from five West-African countries (Benin, Burkina Faso, Guinea, Mali and Togo) were used in this study (Table 1, Fig. 1). *D. exilis* and *D. iburua* accessions from Togo were collected from farmers' fields (Adoukonou-Sagbadja et al. 2004), while accessions from Benin were obtained from the Gene Bank of the Benin National Agricultural Research Institute based at Niaouli. Guinean, Malian, and part of Burkina Faso accessions were provided by the National Agricultural Research Institute of Guinea via the West and Central Office of Bioversity International (ex IPGRI) based at Cotonou, Benin. The second part of accessions from Burkina Faso came from Niaouli Gene Bank. Wild species recognized by local farmers as wild types of fonio were collected by the first author from different areas in these countries and taxonomically identified during the present study as *D. longiflora*, *D. ternata*, *D. ciliaris* (Retz.) Koeler (syn. *D. adscendens* (H. B. K.) Henrard), and *D. lecardii* (Pilg.) Stapf. Voucher specimens of the majority of the accessions analysed in this study are deposited in the Herbarium Gatersleben (GAT) at the Gene Bank of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK Gatersleben, Germany). All plants were grown under greenhouse conditions at approx. 22°C and 12 hours light.

Flow cytometry measurement of nuclear DNA content. Nuclear DNA content was determined at IPK with a FACSAria flow cytometer (Becton Dickinson, San Jose, CA, USA) using the WinMDI 2.8 analysis programme (Joseph Trotter 1993–1998, http://facs.scripps.edu/). *Glycine max* (2C value: 2.72 pg) or *Raphanus sativus* (2C value: 1.38 pg) were used as internal standards (Doležel et al. 1998). Nuclear suspensions were prepared and flow cytometry analysis was performed following Barow and Meister (2002). Approximately 50 mg of fresh and young leaf tissue was excised from individual plants and used for sample preparation. To release nuclei, leaf fragments of *Digitaria* and of the reference plant(s) were placed together in a pre-cooled Petri dish and chopped with a sharp razor blade in 1 ml ice-cold Galbraith's buffer (Galbraith et al. 1983) supplemented with 50 μg ml^{-1} propidium iodide (PI) and 50 μg ml^{-1} RNase (DNA-free). The suspension of isolated nuclei was filtered through a nylon mesh with a pore size of 35 μm and analysed immediately. If ever possible, four individual plants were separately analysed per accession, each of them was considered as one replicate. The mean DNA content per measurement was based on at least 10,000 scanned nuclei. The 2C DNA content of the sample was calculated as the sample peak mean, divided by the reference peak mean, and multiplied with the amount of DNA of reference plant ($2C_{Digitaria}$ = [$Peak_{Digitaria}$/$Peak_{reference}$] x $2C_{reference}$).

Statistics: Genomes size data were analysed using the SAS system for Windows software, release 8.02 (SAS Institute, Cary, NC, USA). Differences in DNA content were tested by one-way analysis of variance (ANOVA), and the Scheffé test was used to discriminate dissimilar groups within and between the studied species.

Chromosome counting. Fonio grains were germinated on moist filter paper at 24°C. About 1 cm long root tips were fixed in ethanol: acetic acid (3:1). After hydrolysis in 1N hydrochloric acid at 60°C for 15 min the roots were stained in Schiff's reagent according to the standard Feulgen method. Chromosome spreads were prepared in propion orcein. Because chromosomes could not be spread in one focus layer an epifluorescence microscope (Zeiss Axiophot) integrated into a Digital Optical 3D Microscope System (Schwertner GbR, Jena, Germany) was used to take image stacks to produce 3D images for chromosome counting. The image stacks were also used for karyogram establishment via the Ikaros software (MetaSystems GmbH, Altlussheim, Germany).

Results and discussion

The flow cytometric measurements yielded DNA histograms with standard deviations of DNA content measurements in most cases lower than 5%, regardless of the internal standard used. Histograms representing single plants of all species analysed are visible in Fig. 2. Table 1 shows the nuclear DNA contents of all 126 accessions of the six species investigated and Table 2 exhibits the results of Scheffé's test conducted on the average DNA contents by pairwise comparisons and the 1C genome sizes calculated for each taxon. Fig. 3 illustrates the major botanical characteristics of the spikelet of the six species investigated.

Table 1. Origin and DNA content of fonio landraces and wild species accessions (voucher numbers: Herbarium Gatersleben, GAT), with the number of determinations per accession (n), standard deviation (SD)

Acc. N°	Local name	Voucher	Origin			n	2C DNA content (pg)
			Country	District	Coll. site		Mean ± SD
D. exilis							
III-3	M'balia 2	—	Guinea	-	Unknown	4	1.660 ± 0.053
II-4	Kansambaran	GAT5304-5305	Guinea	-	Unknown	4	1.861 ± 0.086
I-7	Kokountèrè	GAT5268,5269	Guinea	-	Unknown	2	1.903 ± 0.179
III-2	Farmali	GAT5320	Guinea	Tougué	Tougué	2	1.911 ± 0.050
II-6	Litty	GAT5300,5301	Guinea	Mali district	Near Mali city	4	1.922 ± 0.163
IV-1	Siragbè	—	Guinea	-	Unknown	1	1.933
I-4	Fomba	GAT5274,5275	Guinea	-	Unknown	4	1.939 ± 0.089
III-1	Siragbé	GAT5321,5322	Guinea	-	Unknown	4	1.942 ± 0.111
IV-3	Siragbé	GAT5355,5356	Guinea	-	Unknown	4	1.942 ± 0.045
I-6	Foundelen	GAT5270,5271	Guinea	Lélouma	Lélouma	3	1.950 ± 0.104
I-2	Mamanden	GAT5278,5279	Guinea	-	Unknown	4	1.955 ± 0.055
II-7	Bassamba 2	GAT5298,5299	Guinea	Labé	Labé	3	1.960 ± 0.131
III-7	Fayahè	GAT5319	Guinea	Koundara	Koundara	1	1.961
IV-4	Konson	GAT5351-5354	Guinea	-	Unknown	4	1.970 ± 0.047
IV-6	Hothio 2	GAT5347,5348	Guinea	-	Unknown	4	1.972 ± 0.061
IV-11	Gblingbè	GAT5340,5341	Guinea	-	Unknown	4	1.972 ± 0.033
IV-12	Mora 2	GAT5336-5339	Mali Republic	Mopti	Unknown	4	1.983 ± 0.051
III-5	Momo	—	Guinea	Dalaba	Dalaba	1	1.992
II-8	Niougou	GAT5296,5297	Guinea	-	Unknown	4	2.002 ± 0.018
III-15	Dalaman	GAT5311,5312	Guinea	-	Unknown	4	2.005 ± 0.014
I-11	Mora	GAT5260,5261	Mali Republic	Mopti	Unknown	4	2.006 ± 0.016
I-16	Mossogbè	GAT5250,5251	Guinea	-	Unknown	4	2.013 ± 0.084
I-12b	Yaoukö	GAT5280,5281	Guinea	-	Unknown	4	2.019 ± 0.011
I-1	Konso	GAT5276,5277	Guinea	Mandiana?	Unknown	4	2.020 ± 0.033
I-3	Sèkètè	—	Guinea	Mali	Mali	4	2.022 ± 0.039
III-12	Dibon	GAT5285-5290	Guinea	-	Unknown	4	2.022 ± 0.013
II-10	Tobbhérè	GAT5282-5284	Guinea	Lélouma	Lélouma	4	2.024 ± 0.017
II-11	Koundara	—	Guinea	Labé	Labé	3	2.024 ± 0.027
III-16	Kouroussa	GAT5266,5267	Guinea	-	Unknown	4	2.026 ± 0.032
I-8b	Saara	GAT5264,5265	Guinea	-	Unknown	4	2.029 ± 0.030
I-8a	Saara	GAT5272,5273	Guinea	-	Unknown	4	2.031 ± 0.016

I-5	Werura	GAT5315,5316	Guinea	-	Unknown	4	2.034 ± 0.022
III-8	Raného	GAT5315,5316	Guinea	Tougué	Tougué	4	2.040 ± 0.036
I-10	Yéléboui	GAT5262,5263	Guinea	Kindia ?	Unknown	4	2.043 ± 0.035
I-12a	Yaoukö	GAT5254-5255	Guinea	Lélouma	Lélouma	4	2.058 ± 0.022
I-13	Hothio 1	GAT5252,5253	Guinea	Kita	Unknown	4	2.054 ± 0.018
IV-14	Oulè oulè	GAT5232,5233	Mali Republic	Bougouni	Dossola	4	1.871 ± 0.066
IV-8	Tama	GAT5346	Mali Republic	-	Unknown	4	1.974 ± 0.023
IV-13	Pon - Biré	GAT5334,5335	Mali Republic	Mopti	Biré	4	1.981 ± 0.073
IV-10	Kansambara	GAT5342,5343	Mali Republic	Kénieba	Kénieba	4	1.991 ± 0.033
II-9	Prépéazo	GAT5291-5295	Mali Republic	Kénieba	Kénieba	4	1.994 ± 0.022
IV-5	Dierry	GAT5349,5350	Mali Republic	-	Unknown	4	2.007 ± 0.021
IV-15	Pon - Madongon	GAT5330,5331	Mali Republic	Mopti	Madongon	4	2.025 ± 0.072
IV-9	Prépéazo 2	GAT5344,5345	Mali Republic	-	Unknown	4	2.031 ± 0.048
TAB 92a	Naman	GAT5481	Benin	Boukoumbé	Near Nadoba (Togo)	4	1.886 ± 0.014
BEN 38	Ipoya	—	Benin	Natitingou	Moupémou	1	1.925
BEN 34	Tontonga	GAT5427	Benin	Boukoumbé	Koutcheta	4	1.926 ± 0.063
BEN 21	Ipodapiah	GAT5430,5431	Benin	Boukoumbé	Koutchatahongou	4	1.941 ± 0.040
BEN 01	Ikantoni	—	Benin	Boukoumbé	Kouya	1	1.946
BEN 30	Ipodawon	GAT5428	Benin	Boukoumbé	Kountchéhégou	4	1, 948 ± 0.032
BEN 49	Ipoaga	—	Benin	Natitingou	Kouaba	4	1.950 ± 0.038
BEN 48	Afiyo	GAT5404,5405	Benin	Copargo	Koutchanti	4	1.951 ± 0.021
BEN 32	Iponi	—	Benin	Boukoumbé	Koutchatahongou	1	1.956
BEN 39a	Pei (precocious)	GAT5417,5418	Benin	Natitingou	Kotopounga	4	1.958 ± 0.042
BEN 43	Poigui	—	Benin	Tanguiéta	Hantanguéri	4	1.961 ± 0.036
BEN 13	Kpatinafa	GAT5436	Benin	Boukoumbé	Kouya	4	1.962 ± 0.015
BEN 16	Ikoumga	GAT5433,5434	Benin	Boukoumbé	Kountchougou	4	1.962 ± 0.055
BEN 103	Tamaou	—	Benin	Boukoumbé	Korontière	1	1.966
BEN 08	Tentenga	GAT5441,5442	Benin	Boukoumbé	Kountchougou	4	1.973 ± 0.062
BEN 15	Ipoda	—	Benin	Boukoumbé	Koutchagou	4	1.974 ± 0.031
BEN 05	Ipomoan	GAT5443,5444	Benin	Boukoumbé	Kouya	4	1.975 ± 0.024
BEN 40	Ipohaga (Ipoaga)	—	Benin	Natitingou	Tigniti	4	1.978 ± 0.056
BEN 22	Tentepera	GAT5429	Benin	Boukoumbé	Kountchougou	4	1.980 ± 0.014
BEN 11	Dipodawon	GAT5437,5438	Benin	Boukoumbé	Kounacogou	4	1, 985 ± 0.037
BEN 09	Ipodapieh	GAT5439,5440	Benin	Boukoumbé	Kodogou	4	1.995 ± 0.021
BEN 110	Iponouda	—	Benin	Boukoumbé	Koussétiengou	1	1.995
BEN 47	Cafera	—	Benin	Copargo	Koutchanti	4	1.997 ± 0.037
BEN 03	Tontonga	GAT5445,5446	Benin	Boukoumbé	Koudogou	4	1.998 ± 0.067

Table 1. (Continued)

Acc. N°	Local name	Voucher	Origin			n	2C DNA content (pg)
			Country	District	Coll. site		Mean ± SD
TKD 58	Djibiga	GAT5399	Togo	Doufelgou	Koré	4	1.833 ± 0.012
TKD 60	Fig'm	GAT5393,5394	Togo	Doufelgou	Baga	2	1.858 ± 0.004
TKD 75	Sèmbre	GAT5387	Togo	Doufelgou	Broukou	4	1.868 ± 0.032
TKK 85	Sèmbre	GAT5489,5490	Togo	Kéran	Ataloté	4	1.871 ± 0.011
TKD 59	Namba	GAT5395-5398	Togo	Doufelgou	Koré	4	1.887 ± 0.037
TSO 88	Ounfissa	GAT5491-5494	Togo	Oti	Gando-Djèbouri	4	1.892 ± 0.075
TKD 62	Tchabigô	GAT5390	Togo	Doufelgou	Amadi-Paha	4	1.896 ± 0.043
TKB 72	Fôlôm	—	Togo	Bassar	Koundoum	4	1.898 ± 0.035
TKB 74	Sèmbre	GAT5363-5365	Togo	Bassar	Didoudikpre	4	1.900 ± 0.042
TPA 26	Trikpa	GAT5499-5501	Togo	Amou	Mouna	4	1.901 ± 0.058
TKK 83	Ayôrô	GAT5487,5488	Togo	Kéran	Adjéidé	4	1.902 ± 0.071
TSO 86	Ounvonikpa	GAT5495,5496	Togo	Oti	Okparobôsso	1	1.902
TKD 61	Lanfig'm	GAT5391,5392	Togo	Doufelgou	Baga	4	1.907 ± 0.049
TKD 89a	Tchapionga	GAT5371,5372	Togo	Doufelgou	Massédéna	3	1.915 ± 0.039
TPW 42	Ougniva	—	Togo	Wawa	èkèto	2	1.916 ± 0.050
TKD 81	Yôlôm	GAT5373,5374	Togo	Doufelgou	Kadjalla	4	1.920 ± 0.029
TPW 32	Egniva	—	Togo	Wawa	Klabé-Akpéganmè	4	1.937 ± 0.032
TKK 69	Iporlapiah	GAT5486	Togo	Kéran	Warango	4	1.962 ± 0.032
TKD 56	Fig'm	GAT5400,5401	Togo	Doufelgou	Koka	4	1.966 ± 0.052
TKK 66	Kopordagou	GAT5482,5483	Togo	Kéran	Bassamba	2	1.969 ± 0.039
TKK 70	Itamali*	—	Togo	Kéran	Nadoba	1	2,012
TPW 52	Oufakpôh	—	Togo	Wawa	Yalla	1	2.048
TPW 54	Trikpa	—	Togo	Wawa	Kabanyi	1	2.048
TPA 27	Ezio	—	Togo	Amou	Mouna	1	2.049
TKB 71	Kiwo	—	Togo	Bassar	Koundoum	1	2.050
TPA 38	Ova	—	Togo	Amou	Amoutsi	4	2.060 ± 0.02
TPW 41	Dikaba	—	Togo	Wawa	Ekèto	1	2.071
TPA 23	Vitchi	—	Togo	Amou	Ougbo-Ali	4	2.074 ± 0.01
TPW 29	Vafoo	—	Togo	Wawa	Klabé-Akpéganmè	1	2.074
TPW 50	Gnimimbi	—	Togo	Wawa	Vhé-Nkougna	1	2.093
BUF 64	Fii (Cfv 533)	GAT5506	Burkina Faso	Orodara	Samogohiri	4	1.878 ± 0.028
BUF 74	Fomou (Cfv 413)	—	Burkina Faso	Banfora	Toumousseni	4	1.921 ± 0.052
BUF 69	Foni Femba	GAT5503	Burkina Faso	Nouna	Soin	4	1.942 ± 0.067

BUF 56	Foni (Cfv 453)	GAT5510	Burkina Faso	Nouna	Kouro	4	1.949
BUF 57	Pongwé (Cfv 411)	GAT5508,5509	Burkina Faso	Tougan	Séné	4	1.936 ± 0.041
BUF 65	Péri Maoulè	—	Burkina Faso	Nouna	Komanbira	1	1.957
BUF 66	Foni Maloulè	—	Burkina Faso	Nouna	Soin	1	1.960
IV-18	Feningué	GAT5324,5325	Burkina Faso	Orodara	Ouéléni	1	1.989
IV-19	CVF 107	GAT5323	Burkina Faso	-	Unknown	1	2.001
IV-16	Fonibâ	GAT5327-5329	Burkina Faso	Sideradougou	Degué	1	2.014
IV-17	Peri	GAT5326	Burkina Faso	Nouna	Towkorowi	1	2.024
BUF 70	Pogwôn	—	Burkina Faso	Titao	Ban	1	2.030
BUF 71	Peri	—	Burkina Faso	Nouna	Sanaba	1	2.045
BUF 67	Kiyu	—	Burkina Faso	Tibo	Fulse	1	2.080
D. iburua							
BEN 36b*	Péi (long cycle)	GAT5423-5426	Benin	Natitingou	Koudengou	4	1.792 ± 0.094
BEN 39b*	Péi (long cycle)	GAT5423-5426	Benin	Natitingou	Kotopounga	4	1.749 ± 0.054
BEN 40b*	Ipoaga	GAT5408-5411	Benin	Natitingou	Tigniti	4	1.836 ± 0.079
TKD 75b	-	GAT5380-5386	Togo	Doufelgou	Broukou	2	1.964 ± 0.041
TKD 63b	Tchibam	GAT5388,5389	Togo	Doufelgou	Défalé	2	2.002 ± 0.031
TKD 89b	-	GAT5367-5370	Togo	Doufelgou	Massédéna	4	1.856 ± 0.086
D. ternata							
BEN 36c*	Wild type	GAT5419-5422	Benin	Natitingou	Koudengou	4	1.775 ± 0.070
D. longiflora							
TAB 92b	Wild type	GAT5479,5480	Benin /Togo	-	Nadoba border	4	1.870 ± 0.035
D. ciliaris							
MAL 01	Wild type	GAT5523-5529	Rep. Mali	Sikasso	Niéno	4	2.472 ± 0.063(A) **
GUI 02	Wild type	GAT5511-5516	Guinea	Tougué	Kolé	4	2.593 ± 0.035(A) **
TKD 75c*	Wild type	GAT5378,5379	Togo	Doufelgou	Broukou	1	2.929 (B)**
D. lecardii							
SMB 06*	Wild type	GAT5456-5458	Benin	Matéri	Pingou	4	2.504 ± 0.172
STB 02	Wild type	GAT5459-5463	Benin	Toukountouna	Kouba	4	2.787 ± 0.036
SCB 08	Wild type	GAT5450,5451	Benin	Cobly	Touga	1	2.785

*indicates *Raphanus sativus* as internal standard, *Glycine max* was used for the other genotypes, **Scheffé's grouping in *D. ciliaris*; homogeneity was observed within the other species with more than one accession

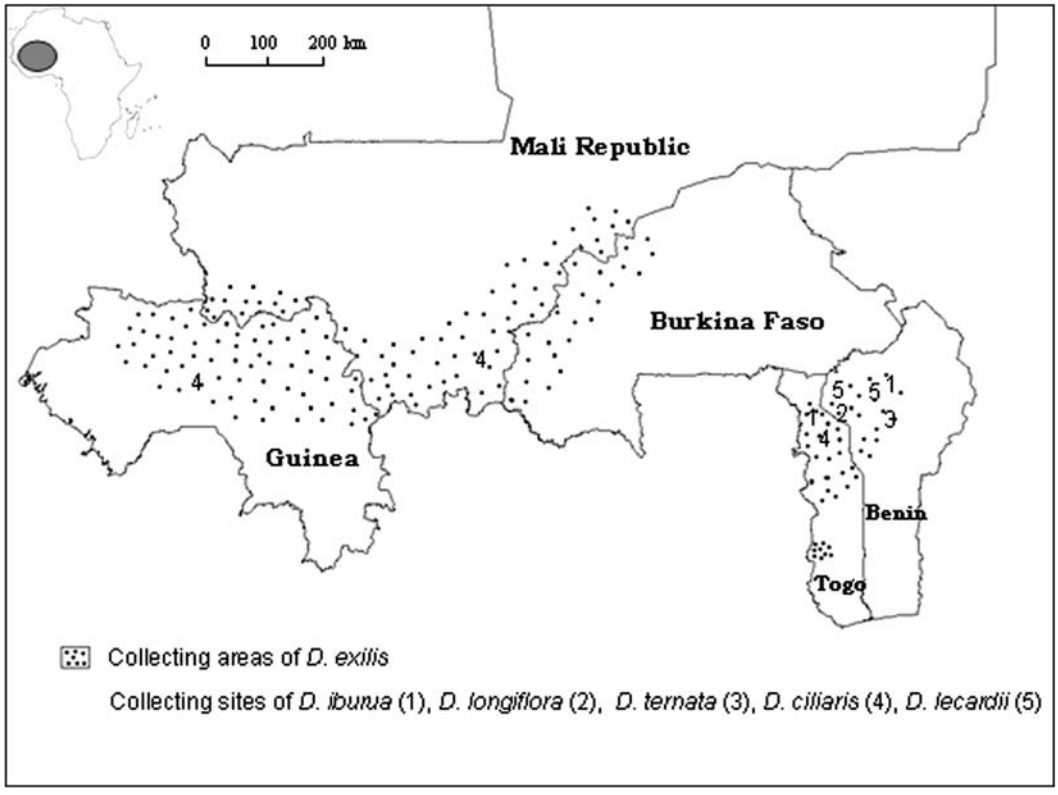

Fig. 1. Collecting areas/sites of fonio landraces and wild species in Benin, Burkina Faso, Guinea, Mali and Togo

In cultivated species, the nuclear DNA contents of *D. exilis* and *D. iburua* landraces were found to be very similar. In white fonio (*D. exilis*), the lowest mean 2C DNA content (1.660 pg) was documented for landrace M'balia 2 (III-3) collected from the Fouta-Djallon highlands in Guinea while the highest (2.093 pg) was observed in Gnimimbi (TPW 50), a landrace cultivated by the Akébou tribe in southern Togo. The DNA content of the six black fonio (*D. iburua*) accessions ranged from 1.792 pg in landrace Péi cultivated by Wama farmers in Benin to 2.002 pg in landrace Tchibam especially cultivated by Lamba tribe in northern Togo for brewing local beer (Adoukonou-Sagbadja et al. 2006). The overall average DNA content calculated for white fonio was 1.956 ± 0.004 pg while in black fonio a slightly lower average (1.848 ± 0.031 pg) was observed. The present results corroborate some morpho-botanical resemblance reported between the two cultivated fonio millets (Portères 1975, Haq and Ogbe 1985). However, evidence of genetic differentiation of the two species has been proven by molecular markers such as RAPDs (Hilu et al. 1997) and AFLPs (Adoukonou-Sagbadja, unpubl. res.).

Among the wild species, the nuclear DNA contents of *D. longiflora* and *D. ternata* were found very close to those of cultivated fonio species. In fact, mean 2C DNA content values of 1.869 ± 0.035 pg and 1.775 ± 0.070 pg, respectively, were observed in these two wild species that are far the most cited by local farmers as wild fonio types due to their high morphological resemblance with cultivated fonio. Botanically, *D. longiflora* and *D. ternata* resemble effectively in many ways white and black fonio,

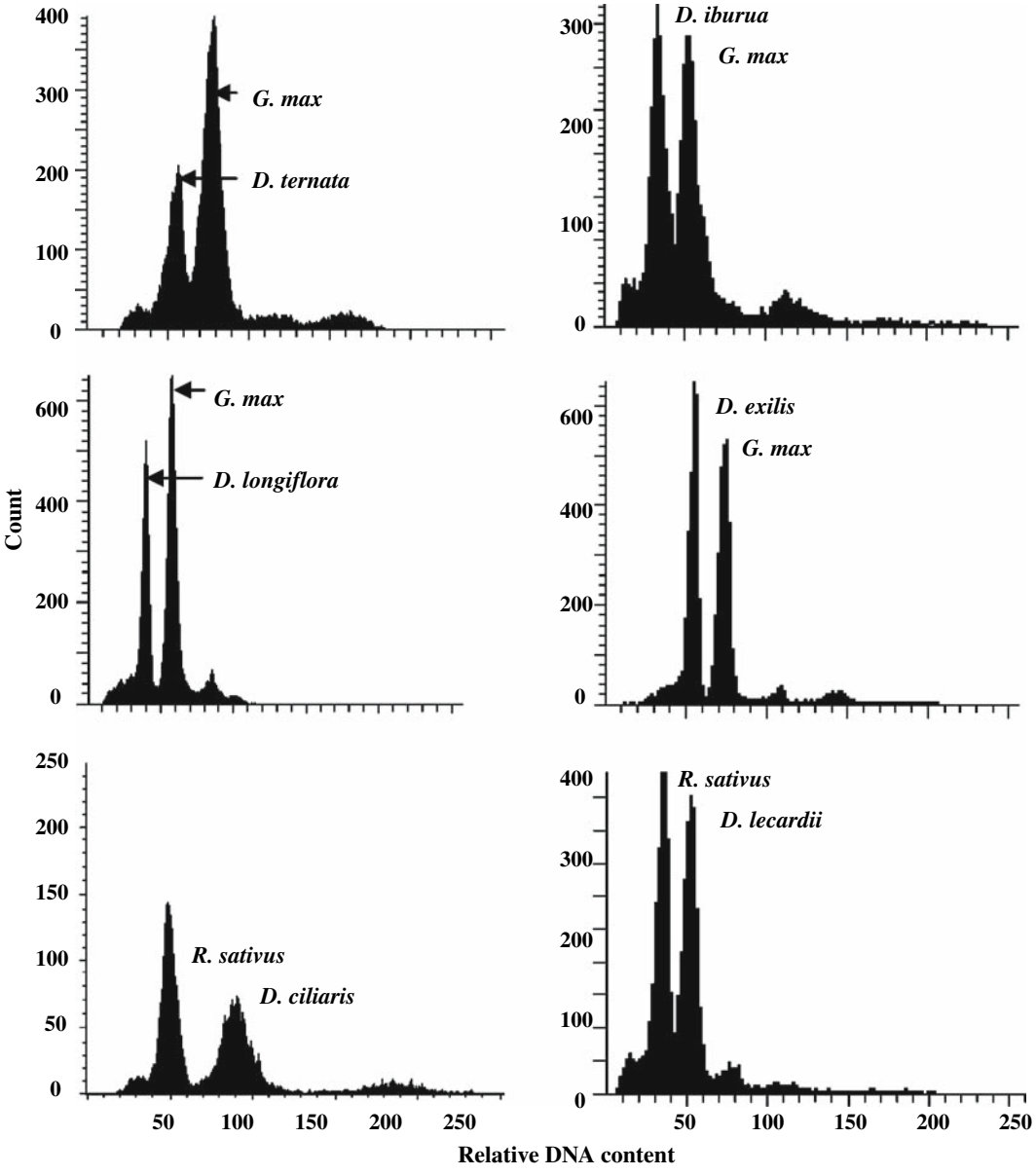

Fig. 2. Relative DNA content of different cultivated fonio and wild species in comparison to the reference plants *Glycine max* or *Raphanus sativus*

respectively, and were proposed by many scientists to be their probable progenitor(s) (Stapf 1915, Portères 1976). The relationships between *D. longiflora* and white fonio on the one hand and *D. ternata* with black fonio on the other were confirmed genetically by molecular studies using RAPD markers (Hilu et al. 1997). The convergence of their genome sizes with those of cultivated fonio millets, as arisen from this study, seems to support the trends on their ancestral relationships but does not agree with their classification in different taxonomic sections: *D. longiflora* and *D. ternata* in Verrucipilae and Clavipilae, respectively, but fonio species in Atrofuscae (Henrard 1950). This finding argues for the need for taxonomic revision and more emphasis on species relationships in the genus *Digitaria*, as

Table 2. Scheffé's grouping based on the general average 2C nuclear DNA content and calculated genome size (1C) of the cultivated and wild *Digitaria* species.

Species	Status	No. acc.[a]	n	2C nuclear DNA content (pg)			1C genome size (Mbp)*
				Mean Range	Average[b] ±SE	Scheffé $\alpha = 0.05$	
D. exilis	Cultivated	112 (92)	372	1.660 – 2.093	1.956 ± 0.004	B	956
D. iburua	Cultivated	6 (2)	18	1.792 – 2.002	1.848 ± 0.031	BC	904
D. longiflora	Wild	1	4	-	1.869 ± 0.035	BC	914
D. ternata	Wild	1	4	-	1.775 ± 0.070	C	868
D. ciliaris	Wild	3	9	2.472 – 2.929	2.576 ± 0.030	A	1260
D. lecardii	Wild	3	9	2.504 – 2.787	2.660 ± 0.070	A	1301

[a]Number of accessions investigated; in bracket, number of farmer-named fonio landraces used; n = number of measurements; [b]Average over all measurements (SE = Standard error); *conversion factor of 978 Mbp for 1pg of DNA (Doležel et al. 2003)

Fig. 3. Characteristics of the spikelets of the six *Digitaria* species investigated: spikelets with lower lemma (left) and upper glume and upper lemma (right); **1** *D. ternata* (BEN 36 c), **2** *D. iburua* (BEN 40 b), **3** *D. longiflora* (TAB 92 b), **4** *D. exilis* (TKD 56), **5** *D. lecardii* (STB 02), and **6** *D. ciliaris* (TKD 75 c) (Photo H. Ernst)

has been suggested by Haq and Ogbe (1995). Although the difference observed in the average DNA amounts of these four species was slight, it is nonetheless significant ($p<0.05$), indicating that genomic structure variability may be expected among them.

Conversely, the nuclear DNA amounts of *D. ciliaris* and *D. lecardii* were 1.3–1.4 fold higher (significant at $p<0.001$) than that observed for cultivated species and their closely related species. In fact, the overall average nuclear DNA content in *D. ciliaris* was 2.576 ± 0.030 pg while in *D. lecardii*, a value of 2.660 ± 0.070 pg was observed. In contrast to the first two wild species, important botanical divergences of *D. ciliaris* and *D. lecardii* with the cultivated species were reported by Henrard (1950). The differences observed in their genome sizes with fonio millets could then be explained by their phylogenetic distance and highly support their botanical classification in other sections of the genus *Digitaria*. These findings suggest a relationship between genome size and taxonomic distance in the genus, as was earlier reported for many other plant genera such as *Lactuca* (Koopman 2000, Doležalová et al. 2002) and *Pinus* (Hall et al. 2000).

Intra-specific DNA content variations detected in the species with more than one accession were found slight and insignificant, except in *D. ciliaris*. In this species, the statistically significant variation obtained was revealed by Scheffé test to be only due to a comparatively high 2C DNA content (2.929 pg, table 1) detected in the single plant available for analysis in the accession TKD 75c. Minor variations could be attributed to the experimental procedure (day to day variation, use of two different internal standards). But, heterochromatin polymorphisms, chromatin deletions or duplications known to induce small but significant DNA content differences among genotypes (Laurie and

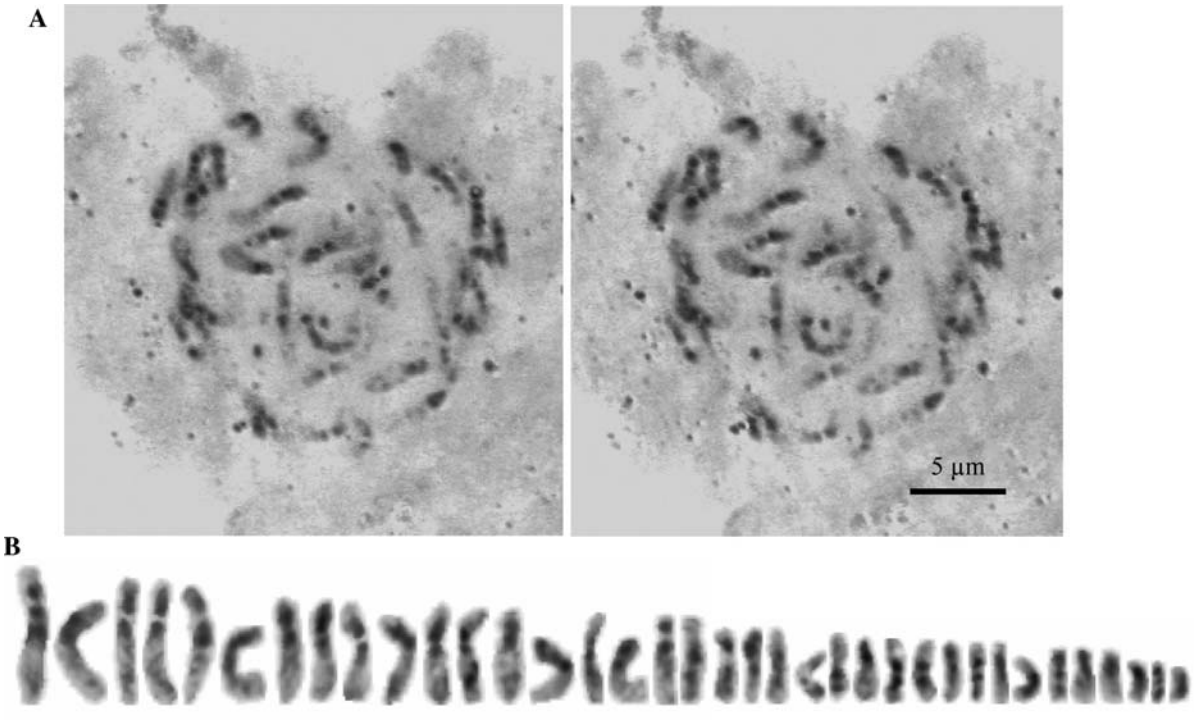

Fig. 4. *D. exillis* chromosomes (2n = 36) of landrace Iporlapieh (Ben21). **A** Spatial somatic metaphase cell (stereo pair can be observed with prism glasses or without glasses at a distance of about 30 cm); **B** Karyogram established from the same cell

Bennett 1985, Greilhuber 2005) could be responsible of the deviating 2C DNA observed in *D. ciliaris*. Although significant intra-specific differences have been reported within other grass species (Murray 2005), the present findings suggest genome size stability in the investigated species, mainly in the cultivated gene pool. Genome size uniformity has been prior proven in many crops such as soybean (Greilhuber and Obermayer 1997) and groundnut (Temsch and Greilhuber 2000). This is in line with a relatively low molecular genetic diversity observed in the cultivated species (Adoukonou-Sagbadja, unpubl. res.).

Fonio millets are grown through contrasting environments in the region and different farming systems (Portères 1976). In contrast to the results found for *Nerine* species (Zonneveld and Duncan 2006), in our study no significant correlation was identified between nuclear DNA content of fonio landraces and the climatic, agro-ecological features or geographic origins (altitude, latitude). These observations could be a consequence of low intra-specific variations detected in their 2C DNA content. Although accessions in *D. ciliaris* had also diverse origins, sample size is too small (only 3) for investigating adequately a possible correlation of 2C DNA variation with eco-geographic parameters. However, authors like Ohri and Pistrick (2001) had a more critical view on the ecological interpretation of genome-size variation in general.

As already reported for many grass genera (e.g. Avdulov 1931, Tuna et al. 2001), determination of chromosome number is difficult in fonio millets due to the small size of their chromosomes. In the investigated wild *Digitaria* species, chromosome counts were not possible since roots tips with good dividing cells were not found. The basic (haploid) chromosome number ($x = 9$) in the genus *Digitaria* and many other *Paniceae* has been reported first by Avdulov (1931) and confirmed by further studies (Hunter 1934, Wipff and Hatch 1994, Bennett et al. 2000). In the present study, $2n = 36$ chromosomes indicating tetraploidy were identified in somatic metaphase cells of *D. exilis,* as shown in Figure 4 established from landrace Iporlapieh (BEN21). The chromosomes are different in size and the mostly median centromeres are mainly surrounded by heterochromatin. Our findings are not in agreement with Hunter (1934) who reported hexaploidy ($2n = 6x = 54$) in *D. exilis*, but they partially support the report of Zeven and de Wet (1982) who suggested tetraploidy ($2n = 4x = 36$) with a possible existence of diploid forms. Although the germplasm analysed in the present study does not cover the whole fonio cultivation areas in West-Africa, it is considered to represent the majority of fonio landrace diversity that exists in the region since it was collected from the most important diversification centres (primary and secondary) where a broad genetic diversity is found (Portères 1976). If diploid and hexaploid types of fonio really exist, their occurrence may be low in comparison to the vast majority of tetraploids exclusively identified in our study. Just like white fonio, black fonio as well as the closely related species *D. longiflora* and *D. ternata* seem to be tetraploids. Expanding the investigations to landraces of Nigeria (another possible secondary centre), but also to minor producing countries like Senegal, Gambia, Côte d'Ivoire and Niger, could help to achieve a definitive conclusion on the existence of diploid or hexaploid fonio. Nonetheless, the present study offers a comparatively large and representative view on the ploidy level of fonio millets which is basic step towards the use of their landraces in fonio breeding programmes. Further investigations on the genomic composition of these crops, but also of the two closely related wild species, remain of practical interest.

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Chapter III

FONIO GENETIC DIVERSITY AND DIFFERENTIATION*

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ORIGINAL PAPER

Genetic diversity and population differentiation of traditional fonio millet (*Digitaria* spp.) landraces from different agro-ecological zones of West Africa

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Abstract Fonio millets (*Digitaria exilis* Stapf, *D. iburua* Stapf) are valuable indigenous staple food crops in West Africa. In order to investigate the genetic diversity and population differentiation in these millets, a total of 122 accessions from five countries (Benin, Burkina Faso, Guinea, Mali and Togo) were analysed by Amplified Fragment Length Polymorphisms (AFLPs). Genetic distance-based UPGMA clustering and principal coordinate analysis revealed a clear-cut differentiation between the two species and a clustering of *D. exilis* accessions in three major genetic groups fitting to their geographical origins. Shannon's diversity index detected in *D. iburua* was low ($H = 0.02$). In *D. exilis*, the most widespread cultivated species, moderate levels of genetic diversity (Shannon's diversity $H = 0.267$; Nei's gene diversity $H' = 0.355$) were detected. This genetic diversity is unequally distributed with the essential part observed in the Upper Niger River basin while a very low diversity is present in the Atacora mountain zone. Analysis of molecular variance (AMOVA) revealed that a large part of the genetic variation resides among the genetic groups (70%) and the country of origin (56%), indicating a clear genetic differentiation within *D. exilis*. Influence of mating system (inbreeding or apomixis), agricultural selection and ecological adaptations as well as founding effects in the genetic make-up of the landraces were visible and seemed to jointly contribute to the genetic structure detected in this species. The genetic variability found between the analysed accessions was weakly correlated with their phenotypic attributes. However, the genetic groups identified differed significantly in their mean performance for some agro-morphologic traits. The results obtained are relevant for fonio millets breeding, conservation and management of their genetic resources in West Africa.

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Introduction

Sub-Saharan Africa is known for its large diversity in various native crop plant species including many types of millet. Two of them, known as fonio millets, belong to the genus *Digitaria* Haller, i.e. white fonio (*D. exilis* Stapf) and black fonio (*D. iburua* Stapf). Fonio millets are small grained, C_4 metabolism cereals with a short life cycle and medium height (Haq and Ogbe 1995). Different hypotheses exist on their reproductive system, ranging from inbreeding (Watson and Dallwitz 1992; Sarker et al. 1993) to outcrossing (Fogg 1976; Hilu et al. 1997). Fonio species are previously reported to be diploid ($2n = 2x = 18$), tetraploid ($2n = 4x = 36$) or hexaploid ($2n = 6x = 54$) (Hunter 1934;

Zeven and de Wet 1982), but only the tetraploidy level has been recently confirmed (Adoukonou-Sagbadja et al. 2007).

Fonio millets are among the oldest cereal crops domesticated by West African farmers, at around 5 millennia BC (Murdock 1959). Besides others such as pearl millet, sorghum, etc., fonio crops have played a central role in the emergence and development of traditional agriculture in the West African savannah (Busson 1965; Haq and Ogbe 1995). In this region, fonio millets supply food for several millions of tribal people either as a staple or as a major part of the diet with a high value in the local food cultures (Vietmeyer et al. 1996; Adoukonou-Sagbadja et al. 2006). Dishes made with fonio (porridge, couscous, paste, etc.) are highly appreciated by traditional farmers, particularly in many tribes such as the Akposso and Lamba in Togo, the Peul and Malinké in Guinea or the Dogon in Mali. Fonio is ranked among the most nutritious grain crops due to its exceptional richness in the human-vital amino acids methionine and cystine, deficient in major cereals like wheat, rice, maize, and sorghum (Jideani 1990). As rain-fed and low input crops, they are highly adapted to marginal land farming, flourish well on poor soils and withstand drought and floods. Due to their large ecological adaptability these plants are believed to have a high potential as key-crops in future agriculture and food security supply in their traditional cultivation zones or beyond (Eyzaguirre and Thormann 1998; Adoukonou-Sagbadja et al. 2006).

Landraces or farmers' varieties constitute valuable resources for crop breeding and conservation of its genetic diversity (Kölliker et al. 2003). In the local agriculture of West Africa, hundreds of fonio landraces exist and derive from traditional selection. Unlike crops of worldwide importance, little effort has been made so far to improve these millets as no modern varieties are currently available. Despite the wide perspectives in utilisation of fonio genetic resources, the crops are still on a primitive production level and feature many drawbacks like poor yield, tiny grain size, seed shattering, plant lodging, pests and diseases, etc. (Kwon-Ndung et al. 1998; Adoukonou-Sagbadja et al. 2006). Additionally, the presence of high flavonoid content in the crude fonio grains with probably anti-thyroid properties (Sartelet et al. 1996) has been reported. Improving the efficiency in breeding strategies as well as conservation management of fonio genetic resources require adequate knowledge on the amount, distribution and structure of genetic diversity. Up to now, the genetic diversity present in these millets and the differentiation of landrace populations remain poorly understood.

Early efforts in studying the agro-morphological variability to assess genetic diversity indicate that fonio types are morphologically variable. In *D. exilis*, Portères (1976) identified a number of botanical varieties (with many cultivars each) based on morpho-botanical characters and geographic origin. However, Sanou (1993) reported divergent agro-morphological classification by studying some fonio ecotypes originating from Burkina Faso and Mali. Thus, it turns out that there is a limitation in using only morphological attributes for population characterization due to genotype × environment interaction and the complexity in genetic control of polygenic morphological and agronomic traits (Smith and Smith 1992). Such limitations have resulted in an early use of biochemical markers and the recently increased development of molecular approaches for assessing genetic diversity (Karp et al. 1997). The use of isozyme electrophoresis for genotype identification is very limited in fonio and no significant genetic variation has been detected (unpublished data). Molecular analyses allowing an accurate characterization of fonio accessions are quite rare. Recently, two molecular studies using Random Amplified Polymorphic DNA (RAPD) have been reported, but they are restricted to very small fonio germplasm samples originating from Togo (Hilu et al. 1997) or Nigeria (Kuta et al. 2005). This indicates the necessity of more comprehensive studies to consider patterns of genetic diversity in relation to its regional distribution.

In absence of SSR markers, Amplified Fragment Length Polymorphisms (AFLPs) have proven to be a powerful and efficient approach in population genetic and diversity analysis, molecular taxonomic classification, gene mapping and marker-assisted breeding in variable crops (Ayele et al. 1999; Carr et al. 2003; Uptmoor et al. 2003). Furthermore, AFLPs are a more stable and reproducible marker system compared to RAPDs (Rafalski and Tingey 1993). Therefore, AFLPs have been used in the present study to investigate the genetic diversity and population differentiation in a large collection of fonio landraces originally collected from diverse producing areas of West Africa. Additionally, landraces were evaluated morphologically in order to investigate the possible relationships of phenotypic variability to molecular attributes. The results are useful for defining strategies in fonio breeding and conservation management of the genetic resources of these indigenous millets in West Africa.

Materials and methods

Plant materials

A total of 122 accessions of *D. exilis* (118) and *D. iburua* (4) representing 89 farmer-named landraces of five West African countries, i.e., Togo (collected by the first author), Benin (partially provided by Niaouli Gene Bank, National Agriculture Research Institute of Benin—INRAB), Burkina Faso, Guinea and Mali (provided by National Agricultural Research Institute of Guinea—IRAG, via the West and Central Africa Office of Bioversity International, ex-IPGRI at Cotonou, Benin) were investigated (Table 1; Fig. 1). The

approximate collection areas of accessions of Burkina Faso, Mali and Guinea were reconstituted following the ORSTOM (actually, Institut de Recherche pour le Développement, France) catalogue (Clement and Leblanc 1984). This collection covers the major centres of diversification of fonio as identified by Portères (1976) and is assumed to be representative of fonio diversity in the region.

Assessment of molecular variability

DNA isolation and AFLP analysis

Fonio accessions were grown in the greenhouse (Plant Breeding Department, Giessen, Germany). In order to take into account possible genetic variability within each accession, total genomic DNA was extracted from bulked young leaves (100–200 mg per accession) of ten 4- to 5-week-old plants following the CTAB procedure according to Doyle and Doyle (1990). After RNAse treatment, DNA content was fluorometrically quantified (DynaQuant 200 Hoefer Scientific Instruments) and diluted to 25 ng μl^{-1} working solution.

AFLP analysis was performed according to Vos et al. (1995) by using the Invitrogen AFLP® Core Reagent Kit following the manufacturer's instructions. Here, 125 ng of genomic DNA (i.e., 5 µl of working solution) were digested using *Eco*RI and *Mse*I restriction enzymes, and generated fragments were ligated with double-stranded site-specific

Table 1 Countries of origin, local names and number (*N*) of fonio accessions used

Origin	Total	Local names (number of accessions)	
		D. exilis	*D. iburua*
Benin	35	Afiyo (1), Caféra (1), Ikantoni (1), Ikounga (1), Ipodawon/Dipodawon (5), Ipoeda/Ipoda/Ypoda (3), Ipohaga/Ipoaga (8), Iponi (1), Iporlapieh (2), Kpatinafa (1), Naman (1), Poigui (1), Tentepera (1), Tentenga (2), Tontonga (4)	Péi (2)
Burkina Faso	8	Feningué (1), Fii (1), Fomou (1), Foni (1), Foni Femba (1), Fonibâ (1), Péri (1), Pongwé (1)	–
Guinea	25	Dalaman (1), Dibon (1), Fannali (1), Fomba (1), Foundélin (1), Hothio (1), Kansambaran (1), Konso (1), Konson (1), Kokountèrin (1), Kouroussa (1), Mamanden (1), Mossogbé (1), Momo (1), Mora (1), Niougou (1), Ranéhô (1), Saara (1), Sèèkètè (1), Siguiridon (1), Siragbé (1), Tobbhéré (1), Werura (1), Yaoukô (1), Yéléboui (1)	–
Mali	8	Kansambahon (1), Pon-Madongon (1), Dierry (1), Tama (1), Oulè-Oulè (1), Pon-Biré (1), Prépézo (2)	–
Togo	46	Afiohoun (1), Ayôrô (1), Dikaba (1), Djibiga (1), Egniva (2), Eziô (1), Fig'm (3), Fôlom (1), Gnimimbi (1), Ipibim (1), Iporlapiah (2), Itamali (1), Kayara (1), Kiwo (2), Kopordagou (1), Lanfig'm (1), Namba (1), Ougniva (1), Ounfissa (1), Ounvoenikpa (2), Ova (7), Sèmbre (5), Tchabigô (1), Tchapionga (1), Trikpa (1), Vafoo (1), Vitchi (1), Yôlôm (1)	Tchibam (2)

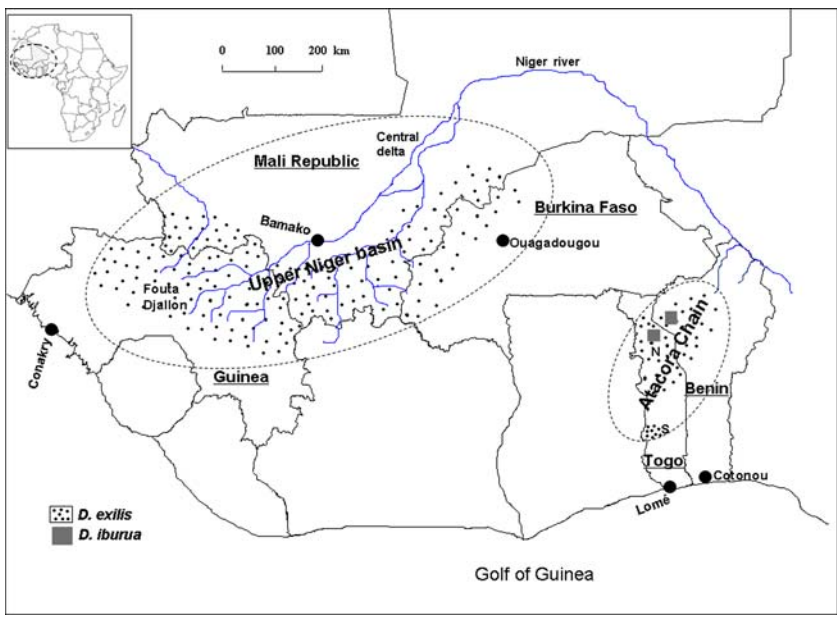

Fig. 1 Countries of origin and approximate collecting areas of the 122 accessions of *D. exilis* and *D. iburua* investigated in this study. *S* and *N* stand respectively for southern and northern growing areas of Togo

adapters using T4 DNA ligase. Ligation was followed by two pre-amplifications (+0, +1) prior to the final amplification phase performed by using primer combinations having three selective nucleotides. The selective amplification mixture (total volume of 25 µl) consisted of 7.5–12.5 ng fluorescent dye-labelled *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 pre-amplified PCR-product in deionised distilled water. Details of the PCR reactions programme were described by Scheurer et al. (2001). Twenty-four (Table 2) out of 72 primer combinations tested on a set of eight accessions were selected on the basis of their ability to generate informative data and further used for the total germplasm analysis. Selective amplification products were separated on 8% denaturing polyacrylamide gels using a Li-Cor 4200 DNA Analyzer. Fragments size was estimated in comparison to a 50–750 bp labelled DNA-ladder.

Scoring and analyses of AFLP data

AFLP fragments were detected using the RFLPScan 2.1 software package (Scananalytics, Fairfax, USA). Clear and unambiguous fragments were scored as present (1) or absence (0) to generate a binary data matrix. The total number of fragments scored, the number of polymorphic fragments and percentage of polymorphic fragments were determined for each primer pair used. Only polymorphic fragments were used for further data analysis.

Pairwise relatedness based on genetic similarity (Dice 1945) was estimated between all fonio accessions using the SIMQUAL module of NTSYS pc software version 2.20e (Rohlf 2000). UPGMA (unweighted pair-grouped method using arithmetic averages) cluster analysis was performed following GenDist and NEIGHBOR programs available in the software package PHYLIP 3.6 (Felsenstein 1985). Reliability and robustness of the clustering were based on 1,000 random re-sampling prior conducted on the datasets through the bootstrap procedure of this software package. The goodness of fit of the clustering compared to the basic data matrix was also tested by computing the co-phenetic correlation coefficient using normalized Mantel statistics *Z* test (Mantel 1967) via the COPH and MXCOMP procedures of NTSYS-pc version 2.20e (Rohlf 2000). Additionally, principal coordinate analysis (PCoA) was carried out

Table 2 AFLP primer-combinations, the number of DNA fragments generated and the number of uniquely identified accessions

No	Primer combination	Total markers	Polymorphic markers	Percentage polymorphism	No. of uniquely identified accessions
1	E-ACA/M-CCT[a]	82	53	64.6	65
2	E-AAT/M-CCG	78	49	62.8	54
3	E-CTC/M-GTC	55	36	65.4	36
4	E-CCT/M-GAC	48	30	62.5	41
5	E-CCC/M-GAC[a]	71	46	64.8	85
6	E-CGC/M-GGG	54	27	50.0	36
7	E-ACC/M-CAG	70	36	51.4	37
8	E-CCA/M-GCA	46	34	73.9	62
9	E-AGA/M-CGG[a]	59	31	52.5	36
10	E-AGG/M-CGT	50	32	64.0	37
11	E-CAT/M-GAG[a]	77	46	59.7	50
12	E-CTT/M-GTA[a]	79	45	57.0	55
13	E-ACC/M-CCC	61	38	62.3	66
14	E-AAG/M-CCA	103	67	65.0	47
15	E-CAG/M-GAC	49	28	57.1	52
16	E-ACT/M-CAA	101	63	62.4	34
17	E-AGG/M-CTG	91	58	63.7	43
18	E-CCT/M-GAA	51	33	64.7	43
19	E-CTC/M-GTA[a]	43	22	51.1	27
20	E-ATT/M-CTG[a]	106	76	71.7	85
21	E-ATG/M-CAC	74	49	66.2	56
22	E-AAT/M-CCC	70	46	65.7	29
23	E-ACA/M-CCA[a]	109	86	78.9	82
24	E-CAA/M-GAA	55	34	61.8	26
	Total	1682	1065	63.3	–
	Mean	70.1	44.3	–	–

[a] Indicates those primer-pairs that are uniquely able to differentiate the two fonio species

based on the pairwise genetic similarity matrix using the DCENTER and EIGEN procedures of NTSYS pc software package (Rohlf 2000).

Genotypic diversity was estimated using Shannon's phenetic index (Shannon and Weaver 1949) following Yeh et al. (1995) and Lacerda et al. (2001): $H = -\Sigma P_i \log_2 P_i/N$; where P_i is the frequency of a particular AFLP fragment and N is the total number of loci. Shannon's diversity index was used because it is recognized to be more insensitive to the bias related to the inability of differentiating heterozygous from homozygous loci when using a dominant marker system like AFLP (Dawson et al. 1995). Further on, Shannon's diversity index does not rely on prior knowledge of the mating system of the relevant species (Sun and Wong 2001) and is therefore well-suited for fonio millets since their mating system is not completely understood.

Analysis of molecular variance (AMOVA; Excoffier et al. 1992) was used to calculate variance components within and among the groups inferred from PCoA. Due to limiting information available on collection sites of fonio landraces originating from Burkina Faso, Mali and Guinea, genetic diversity among and within countries and specifically among and within the two producing areas (northern vs. southern agro-ecology) of Togo was estimated to reflect the geographic and agro-ecologic relevance in diversity shaping of *D. exilis*. Because *D. iburua* was only represented by four accessions, this species was excluded from AMOVA. The variance components' estimation was performed based on the presence/absence matrix using the software ARLEQUIN 3.0 (Schneider et al. 2000). The AMOVA-derived Φ_{ST} (Weir and Cockerham 1984) is analogous to Wright's F statistics differing only in their assumption of heterozygosity (Paun et al. 2006). Φ_{ST} provides an effective estimate of the amount of genetic divergence or structuring among populations (Excoffier et al. 1992). Significance of variance components was tested using a non-parametric procedure based on 1,000 random permutations of individuals using the software ARLEQUIN 3.0 (Schneider et al. 2000).

Genetic diversity (H') and population differentiation (F_{ST}) were examined in parallel following the method of Lynch and Milligan (1994) based on allelic frequencies using the software package AFLP-SURV 1.0 (Vekemans et al. 2002). Estimates of alleles' frequencies were performed using the Bayesian approach for dominant data types such as AFLP markers developed by Zhivotovsky (1999). A non-uniform prior distribution of allelic frequencies was assumed with its parameters derived from the observed distribution of fragment frequencies (Zhivotovsky 1999). Because of hints that fonio seems to be a self-pollinating species (further discussed below), estimates were made by using an hypothetical inbreeding (F_{is}) value of 0.9, assuming thus deviation from Hardy–Weinberg genotypic proportions. The between populations differentiation level was tested by using pairwise F_{ST} distance comparisons with 1,000 random permutations of individuals among populations using the software package AFLP-SURV 1.0 (Vekemans et al. 2002).

Phenotypic evaluation and data analysis

In order to investigate the relationship of molecular attributes with phenotypic traits, all accessions were morphologically evaluated in the 2005 growing season at the Laboratory of Genetics and Biotechnology Experimental field, University of Abomey-Calavi (Benin). This location is 6N24 and 2E20 at 15 m above sea level with ferralitic soil and has received 1,160 mm rainfall in 2005. Each fonio accession was grown in a one-row plot (five plants per plot) in a randomised complete block design with three replications under traditional rain-fed conditions supplemented by moderate occasional irrigations when needed. Approx. 30 kg/ha NPK fertilizer was applied. Row and plant spacing was 40 and 20 cm, respectively. Phenotypic data were recorded on 16 different traits (Table 6) and averaged across three plants per plot.

To access trait variability and significance levels, an analysis of variance (ANOVA) followed by a multiple means' comparison was performed using the SAS system for Windows software, release 8.02 (SAS Institute, Cary, NC, USA). Principal component analysis (PCA) was carried out in order to reveal a two-dimensional grouping pattern of fonio accessions using the software package PC-ORD 4.41 (McCune and Mefford 1999). Prior to PCA computation, z-transformation of the variables was performed to reduce the effects of different scales. Associations of phenotypic traits with molecular attributes of fonio accessions were tested by evaluating the relatedness between genetic and Euclidean distance matrices, pairwise computed for all accessions based on AFLP data and morphological variables, respectively. Genetic distance (G_d) was calculated as $G_d = 1 -$ Dice's similarity. Mantel statistics (Mantel 1967) calculated with NTSYS pc 2.20e (Rohlf 2000) was used to test the goodness of fit and significance was tested by 1,000 permutations. Furthermore, the identified genetic groups were additionally compared by ANOVA to determine whether there were any significant differences regarding the mean of the 16 evaluated traits. Pairwise linear contrasts between the genetic groups were calculated and tested at 0.05 level of significance using the Bonferroni correction.

Results

AFLP polymorphisms

The 24 selected AFLP primer-pairs yielded a total of 1,682 scorable bands of which 1,065 (63.3%) were polymorphic

(Table 2) ranging in size from 50 to 460 bp. The number of polymorphic bands generated by each primer-combination varied from 27 (E-CGC/M-GGG) to 86 (E-ACA/M-CCA) with a mean of 44.3. The level of polymorphism ranged from 50% (E-CGC/M-GGG) to 78.9% (E-ACA/M-CCA). All the AFLP primer-combinations used were suitable to fingerprint the 122 accessions while the number of individual accessions uniquely identified by a given primer combination ranged from 27 (E-CTC/M-GTA) to 85 (E-ATT/M-CTG). Marker specificity to a single accession was rare (6.2% of polymorphic bands) and most of the polymorphic bands appeared to be either frequently or infrequently present in all accessions (Fig. 2). However, high marker-specificity was detected at the species level: 27 AFLP markers were strictly associated with black fonio while 557 were specific to white fonio; the remaining 481 bands (45.2%) were shared between both species. Out of the 24 selected primer-pairs, eight were found efficient to uniquely differentiate between the two species (Table 2).

Genetic relationships and cluster analyses

Dice genetic similarity (GS_D) for all accessions under investigation varied from 0.41 (landraces Dibon vs. Oulè-Oulè) to 1.00 (two accessions of landrace Ova) with an overall mean of 0.79 (data not presented). At the species level, mean GS_D values of 0.77 (0.41–1.00) and 0.97 (0.96–0.99) were observed between accessions within *D. exilis* and *D. iburua*, respectively. In general, the lowest GS_D in the whole germplasm analysed was estimated between white and black fonio landraces indicating interspecific divergence between the two fonio species. Within species, GS_D values were generally much lower between accessions from different origins compared to within estimates.

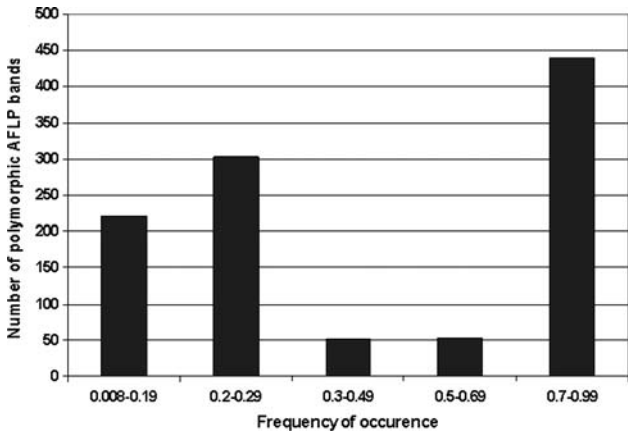

Fig. 2 Number of AFLP polymorphic markers in relation to their frequencies of occurrence in all fonio accessions

UPGMA cluster analysis revealed the genetic relatedness among the fonio accessions (Fig. 3). In general, the main clusters of fonio genotypes were supported by high bootstrap values, indicating the reliability and stability of the relationships as well as the robustness of the AFLP dataset. The high co-phenetic correlation coefficient obtained ($r = 0.91$) confirmed also this trend. The dendrogram revealed four main clusters (Fig. 3). The first node separated *D. iburua* accessions (cluster I) from *D. exilis* ones that split afterwards into three clusters fitting largely to the geographic origin of the fonio accessions. Cluster II comprised the vast majority of Guinean accessions (24 of 26 analysed) and four Malian accessions (landraces Tama, Dierry, Kansambahon, Pon-biré). Cluster III concerned essentially the eight accessions of Burkina Faso and the remaining four accessions from Mali (two Prépéazo, Pon-Madongon and Oulè-Oulè) and two Guinean landraces (Mora, Yeleboui). Cluster IV essentially comprised Benin and Togo accessions differentiated in two sub-groups (A, B) independent of the accessions' origin (country) or their agro-ecologic cultivation area. Focussing on each sub-group, further separation is visible with the landraces from the southern agro-ecologic area clustering distinctly from those of the northern area. Regarding each cluster, accessions grouped very closely at a mean similarity coefficient ranging from 0.84 to 0.95. Associations of minor sub-clusters of the introduced major clusters to particular morphological traits such as plant habit, plant cycle, grains' colour, etc., traits mainly used by farmers in traditional classification and naming system, were in general weak. However, many accessions with the same (Ova, Prépéazo, etc.) or equivalent names (Fig'm/Kiwo, Egniva/Vitchi/Vafoo, etc.) clustered almost together, supporting the hypothesis of their identity or common genetic origin.

Further on, the results of a principal coordinate analysis (PCoA, Fig. 4) support the ones of UPGMA cluster analysis (Fig. 3). The PCoA revealed that the first two axes, explaining almost 59% of the total variation, clearly separated the black and white fonio gene pools and strictly differentiated the white fonio accessions into three major genetic groups, i.e., groups 1, 2 and 3 corresponding to the UPGMA clusters II, III and IV, respectively. Geographically, the genetic groups 1 and 2 reflect approximately the same areas, i.e. a zone covering the whole Upper basin of Niger River from Guinea (predominance of group 1) to Burkina Faso (exclusively group 2). Conversely, the group 3, containing Beninese and Togolese accessions, is geographically isolated from the first ones and covers the cultivating areas along the Atacora Massif Chain which crosses the two countries from Northern Benin to Southern Togo. They are hereafter referred to as Upper Niger group 1 (UNIG 1), 2 (UNIG 2)

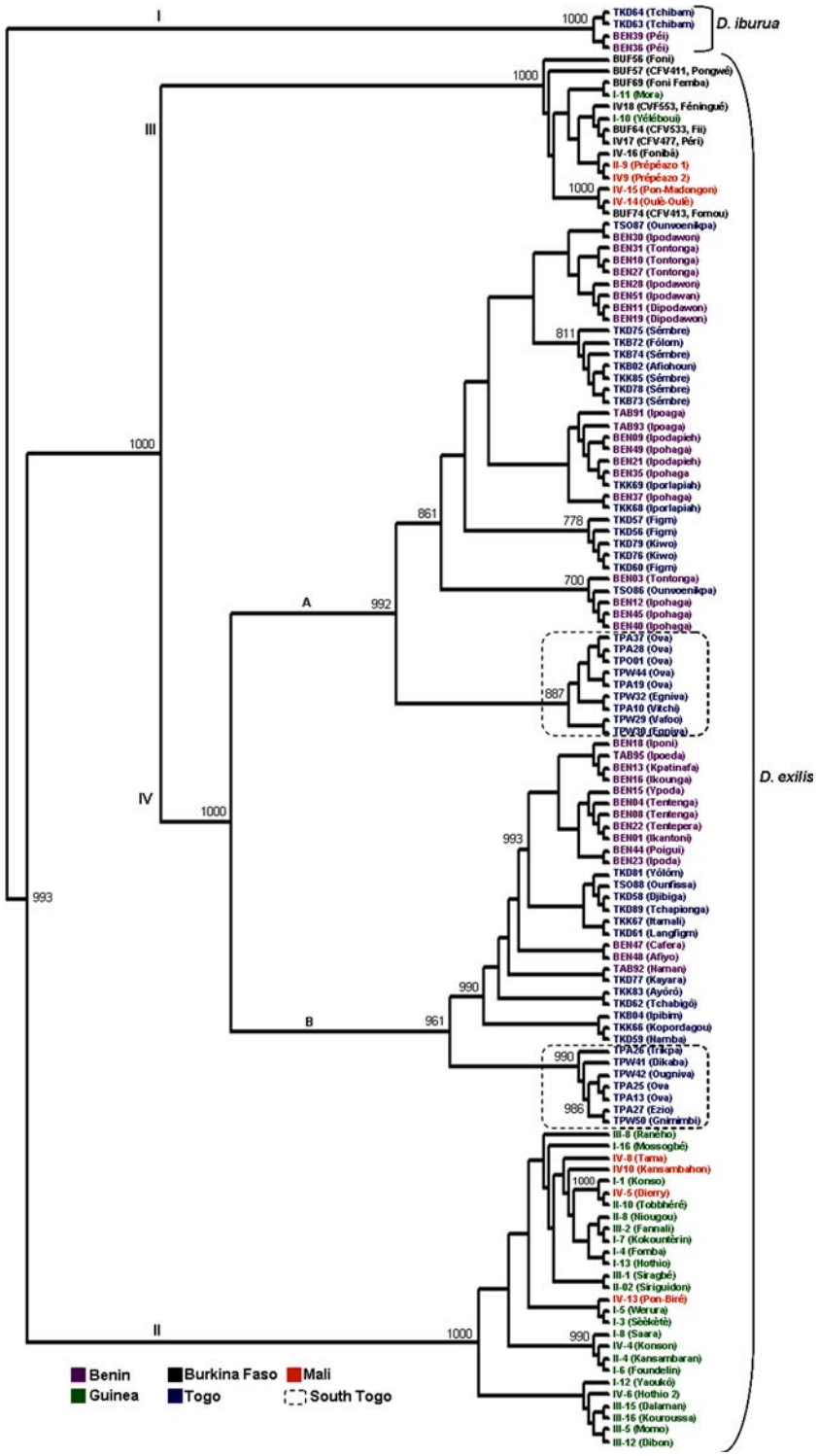

Fig. 3 UPGMA dendrogram showing relationships among 122 accessions of fonio millets based on 1,065 AFLP markers. Bootstrap values obtained from 1,000 re-samplings higher than 70% are indicated. The local names of landraces preceded the accession numbers. I, II, III, IV, A and B indicate landrace clusters and sub-clusters; circled landraces originated from southern agro-ecologic zone of Togo

and Atacora group (ATAC), respectively. Basically, all fonio accessions of the same origin clustered in identical group, with the exception of Guinean and Malian accessions, which split in two different groups (UNIG 1 and 2).

Genetic diversity and differentiation

Estimates of Shannon's index of phenetic diversity (*H*), calculated for each species as well as the different *D. exilis* populations are summarized in Table 3. The total genotypic

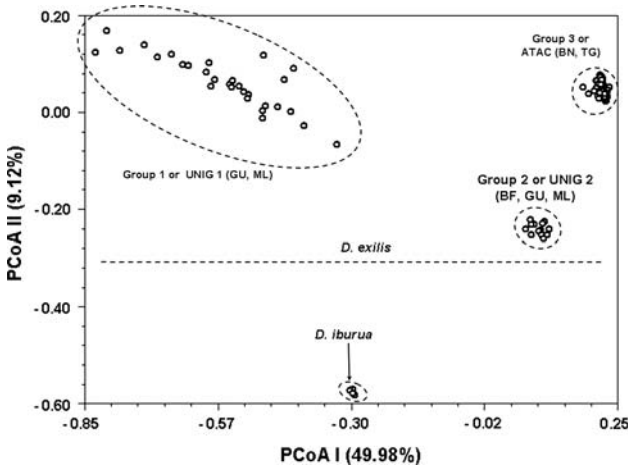

Fig. 4 Diagram showing the relationships among 122 accessions of fonio millets based on Principal Coordinate Analysis (PCoA) using AFLP markers. *D. exilis* accessions formed three genetic groups occupying broadly two geographic areas: UNIG (Upper Niger basin) groups 1 or 2; ATAC (Atacora group); BN (Benin), TG (Togo), GU (Guinea), ML (Mali) and BF (Burkina Faso)

diversity of *D. exilis* was estimated at $H = 0.267$ while a diversity of $H = 0.020$ was detected in the four *D. iburua* accessions. Regarding the *D. exilis* populations, the highest diversity ($H = 0.172$) was observed within UNIG 1, which held also the highest number of polymorphic loci (80.9%) and specific AFLP fragments (159). Conversely, a lower genetic diversity was found in UNIG 2 and ATAC groups with $H = 0.083$ and 0.060, respectively. The mean pairwise genetic similarities within the groups varied in a similar manner from UNIG 1 ($GS_D = 0.84$) to UNIG 2 and ATAC ($GS_D = 0.94$ and 0.95, respectively). At the country level, fonio diversity was found to be concentrated in Guinea ($H = 0.205$) and Mali ($H = 0.203$) followed respectively by Burkina Faso ($H = 0.076$), Togo ($H = 0.060$) and Benin ($H = 0.050$). Average GS_D values between landraces were 0.69, 0.74, 0.93, 0.94 and 0.96 for Guinea, Mali, Burkina Faso, Togo and Benin, respectively. The number of innate loci also varied among origins with the highest present in Guinean producing areas. For both genetic groups and countries of origin, Nei's genetic diversity (H') obtained according to Lynch and Milligan (1994) was ordered in the same way as Shannon's index estimates with slightly different values (Table 3).

AMOVA estimates revealed that most of the genetic variation was attributable to differences among genetic groups ($\Phi_{ST} = 0.71$) or among origins ($\Phi_{ST} = 0.56$); the remaining at each level (29 and 44% of total variation, respectively) being found within populations (Table 4). The variance components for each source of variation were highly significant ($P < 0.001$). Significant differentiation ($P < 0.001$) with approx. 33% of the total variation ($\Phi_{ST} = 0.33$) was also detected among the two Togolese agro-ecologies, attesting that within countries substantial differentiation may exist among diverse agro-ecological areas.

Allele frequency-based F_{ST} estimates based on Bayesian approach under the hypothesis of high self pollination were in the same order of magnitude as those of AMOVA Φ_{ST}, though always somewhat lower (Table 4). The pairwise F_{ST} test (Table 5) revealed significant differentiation ($P < 0.001$) among all pairs of genetic groups with ATAC and UNIG 2 less differentiated ($F_{ST} = 0.52$) than other pairwise comparisons ($F_{ST} = 0.69$ for ATAC/UNIG 1 and 0.64 for UNIG 1/2), fitting then well to the results of the UPGMA cluster analysis. Considering the geographic ori-

Table 3 Genetic variability within each fonio species and *D. exilis* populations (genetic groups, country of origin) based on AFLP data

Populations	*N*	P_{loci}	S_{loci}	GS_D	*H*	*H′*
D. iburua	4	44	28	0.97	0.020	–
D. exilis	118	1021	557	0.77	0.267	0.355
Upper Niger group 1 (UNIG 1)	27	862 (80.9)	159	0.84	0.172	0.231 ± 0.005
Upper Niger group 2 (UNIG 2)	14	603 (56.6)	6	0.94	0.083	0.088 ± 0.004
Atacora group (ATAC)	77	268 (25.2)	28	0.95	0.060	0.058 ± 0.004
Benin	33	175 (16.3)	7	0.96	0.046	0.040 ± 0.020
Burkina Faso	8	210 (19.7)	5	0.93	0.076	0.070 ± 0.040
Mali	8	599 (56.2)	49	0.74	0.203	0.210 ± 0.130
Guinea	25	921 (86.5)	111	0.69	0.205	0.240 ± 0.140
Togo	44	238 (22.3)	21	0.94	0.060	0.055 ± 0.030
Northern Togo	28	233 (21.9)	13	0.95	0.050	0.046 ± 0.022
Southern Togo	16	141 (13.2)	8	0.95	0.050	0.043 ± 0.022

[a] By assuming self pollination rate of 0.95; in brackets, proportion to total diversity in *D. exilis*

Calculations were additionally computed for each agro-ecology of Togo. *N* number of different genotypes, P_{loci} number of polymorphic loci, S_{loci} number of specific loci, GS_D mean genetic similarity (Dice 1945), *H* Shannon index, *H′* Nei's gene diversity (Lynch and Milligan 1994)

Table 4 Analysis of molecular variance (AMOVA) and *F*-statistics (Lynch and Milligan 1994) for the 118 *D. exilis* accessions assembled from five West-African countries (origins) and assigned to three genetic groups (see text) based on 1,065 AFLP fragments

Source of variation	AMOVA estimates						*F*-statistics [b]	
	df	Sum of squares	Variance components	% of variance	Φ_{ST}	*P*-value[a]	F_{ST}	Std
Among groups	2	7,478.845	123.117	70.66	0.71	<0.001	0.64	0.09
Within groups	115	5,879.087	51.122	29.34		<0.001		
Among origins (countries)	4	7,343.225	80.813	56.55	0.56	<0.001	0.44	0.10
Within origins	113	7,234.663	62.367	43.45		<0.001		
Among agro-ecology (Togo)	1	322.917	11.7854	32.8	0.33	<0.001	0.27	0.05

[a] Probability of obtaining a more extreme random value computed from non-parametric procedures (1,000 permutations)

[b] Under self pollination rate $F_{is} = 0.90$ (conclusions do not change significantly when assuming 0.80 (1.3% decrease) or complete inbreeding (5.77% increase) as compared with $F_{is} = 0.9$); Std standard deviation

Estimates were additionally computed for the two Togolese agro-ecologies

Table 5 Pairwise genetic differentiation (below diagonal) and Nei's genetic distance (above diagonal) between *D. exilis* populations (groups and origins) based on Bayesian approach (Lynch and Milligan 1994) by assuming self pollination rate of $F_{is} = 0.90$ using AFLPsurv 1.0

Genetic groups				Origins					
	UNIG 1 (*x*)	UNIG 2 (*y*)	ATAC (*z*)		Benin (1)	Togo (2)	Burk. Faso (3)	Mali (4)	Guinea (5)
x	–	0.42	0.46	1	–	0.04	0.09	0.26	0.33
y	0.64[a]	–	0.09	2	0.07[a]	–	0.09	0.26	0.33
z	0.69[a]			3	0.52[a]	0.50[a]	–	0.19	0.28
–	–	–	–	4	0.53[a]	0.52[a]	0.42[a]	–	0.01
–	–	–	–	5	0.58[a]	0.57[a]	0.51[a]	0.03[ns]	–

[a] Values are all significant at $P < 0.0001$; ns not significant

gins, all pairwise differentiations were found to be significant ($P < 0.001$), except between Guinea and Mali (Table 5). The lowest level of pairwise differentiation was observed for Guinea/Mali and Benin/Togo while other combinations indicated a higher level of differentiation. Nei's genetic distances (Lynch and Milligan 1994) highlighting the genetic relationships between the three groups or five origins are also presented in Table 5.

Morphological variation, relationship with molecular data and comparison of genetic groups

ANOVA of 16 quantitative traits revealed large and significant morphological differences between the 122 fonio accessions (Table 6). The phenotypic relationships among fonio accessions as assessed by the first two principal components (PCs) are presented in Fig. 5. Contrary to the AFLP data, *D. iburua* accessions could not be clearly differentiated from *D. exilis* accessions. Furthermore, no meaningful morphological groups were formed within *D. exilis* accessions (Fig. 5) in contrast to the results obtained at the molecular level.

The relationship of morphological traits with AFLP markers was assessed by the Mantel matrix correspondence test. This test revealed no correlation between Euclidean and genetic distances matrix ($r = 0.04$, $P > 0.05$). However, pairwise linear contrasts between the genetic groups for phenotypic traits followed by Bonferroni correction for multiple testing indicated significant differences for nine of the 16 traits used (Table 6). Among them, dry biomass, panicle and grain yields were found to be particularly useful to discriminate the genetic groups. On average, landraces from UNIG 1 showed a significantly better performance regarding these traits as compared to ATAC and UNIG 2. Furthermore, the mean number of grains per cm of raceme and fresh biomass significantly differentiated UNIG 1 and UNIG 2. Leaf morphology (length of the leaf and leaf sheath) and panicle length significantly discriminate ATAC accessions from UNIG 1 and UNIG 2. The harvest index, days to heading, days to maturity, plant height, number of internodes and tillers seemed to be non-distinctive traits for discrimination of genetic groups. In summary, these results indicate that genetically differentiated groups are obviously characterised by distinct phenotypic characteristics.

Table 6 Morphological characteristics showing the mean traits for all genotypes (including *D. iburua*) and mean traits differences (linear contrasts) among the three genetic groups of *D. exilis*

Traits	Total genotypes		Group means			Linear contrasts between pairs of groups (*P*-values)		
	Mean	SD	ATAC (1)	UNIG 1 (2)	UNIG 2 (3)	1 vs 2	1 vs 3	2 vs 3
Days to 50% heading	82.9[a]	7.7	82.6	84.7	80.2	0.370	0.584	0.181
Days to physiological maturity	108.2[a]	9.4	107.6	111.6	104.8	0.121	0.597	0.076
Plant height (cm)	97.9[a]	14.9	97.6	103.2	89.5	0.106	0.410	0.682
Number of internodes	8.8[a]	1.6	8.8	9.0	8.3	0.648	0.339	0.255
Number of tillers	171.3[a]	54.9	167.7	179.0	176.9	0.272	0.437	0.153
Leaf length (cm)	17.0[a]	3.0	17.3	16.2	17.1	***<0.001***	***0.001****	0.500
Flag leaf sheath (cm)	15.7[a]	2.9	16.5	14.1	14.0	***<0.001***	***<0.001***	0.606
Fresh biomass weight (g/plant)	128.6[a]	22.2	132.4	123.7	116.8	***0.003***	0.685	***0.015***
Dry biomass weight (g/plant)	39.5[a]	5.8	38.9	41.6	38.9	***0.017***	***<0.001***	***0.025***
Panicle exertion (cm)	14.6[a]	4.4	14.0	16.1	15.3	0.507	0.511	0.914
Panicle length (cm)	12.3[a]	2.0	12.7	11.7	11.5	***<0.001***	***<0.001***	0.880
Panicles yield/plant (kg/ha)	1705.9[a]	234.1	1684.2	1803.9	1650.4	***0.006***	***<0.001***	***0.001***
Grains yield/plant (kg/ha)	874.3[a]	162.5	861.2	953.6	804.5	***<0.001***	***<0.001***	***0.006***
Harvest index	25.5[a]	2.7	25.5	26.3	24.2	0.06	0.213	0.799
Mean number of grains/cm of raceme	9.0[a]	1.5	9.1	9.2	8.1	0.141	0.017	***0.002***
1,000-grains mass (mg)	822.8[a]	189.0	793.7	865.4	905.4	***<0.001***	***<0.001***	0.118

[a] Indicates significant mean difference ($P < 0.05$) between genotypes

* Bold italic indicates significant mean differences on an experiment-wise confidence level of significance of 0.05 (after Bonferroni Correction) between pair of groups

Discussion

This study represents the first approach that comprehensively investigates the genetic diversity within a large collection of fonio millets and assesses its structure and regional pattern of distribution based on AFLP markers. Despite its drawback of being dominant markers, the major advantage of AFLPs is their capacity to generate a large number of markers comparative to other molecular marker systems, making it an important tool for population genetic investigations. In the present study, AFLP analysis offered the possibility for screening a large number of polymorphic loci (Table 2) allowing an adequate assessment of the genetic diversity of fonio millets and particularly detecting highly informative genetic population structure within *D. exilis*. In the absence of SSR markers as is still the case in fonio millets, AFLPs appear to be highly suitable for genetic diversity studies of these crops.

Genetic diversity and pattern of distribution

Unlike *D. exilis*, the most widely cultivated fonio species in the region, *D. iburua* is actually grown by very few farmers in some restricted areas of Northern Benin, Togo and Nigeria. Four *D. iburua* accessions from Benin and Togo were included in this study. The diversity indices estimated in this sample were found to be very low (Shannon index $H = 0.02$, $GS_D = 0.96–0.99$), suggesting less genetic diversity and differentiation within this species. Although further conclusions are avoided due to the small number of accessions analysed, this result is in concordance with the residual state of this crop and the low varietal diversity known for this species in its growing areas (Portères 1946; Adoukonou-Sagbadja et al. 2004).

Regarding all 1,065 polymorphic loci investigated, the total genetic diversity estimated in *D. exilis* (Shannon index $H = 0.267$, Nei's gene diversity $H' = 0.355$, $GS_D = 0.41–1.00$) is quite moderate when considering the comparably small effort invested into the breeding of landraces and the large area of origin that comprises the main centres of fonio diversity in West Africa (Portères 1976). However, the level of diversity detected in this species is comparable to that reported in Tunesian and West African (Nigeria, Ghana, Mali, Mauritania) pearl millet cultivars ($H = 0.283$, Ibrahima et al. 2005) but higher than that found in *Eragrostis tef*, an African millet endemic to Ethiopia with a mean GS_D of 0.89 (Ayele et al. 1999), African and Indian finger millet ($GS_D = 0.64–0.92$, Hilu 1995) or at regional level for Southern African Sorghum landraces ($H = 0.169$, Uptmoor et al. 2003). Regarding its geographical distribution, our results reveal that the genetic diversity in *D. exilis* is concentrated in the Upper Niger basin with an eastwards

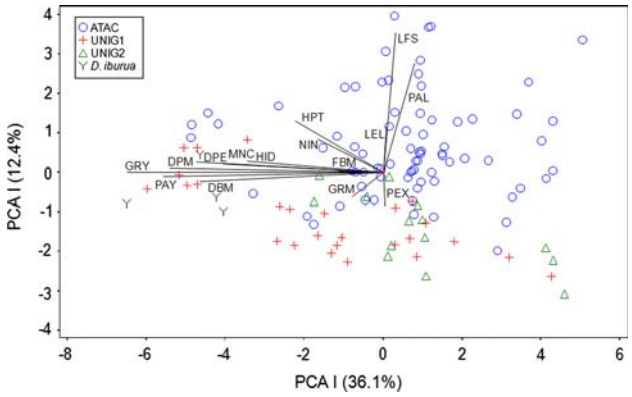

Fig. 5 Diagram showing the relationships among 122 accessions of fonio millets based on Principal Component Analysis (PCA) using phenotypic data. ATAC (Atacorian group), UNIG (Upper Niger group), GRY (grain yield), PAY (panicle yield), DPM (days to physiological maturity), DPE (days to 50% heading), DBM (dry biomass), MNC (mean number of grains per cm of racemes), HID (harvest index), HPT (plant height), NIN (number of internodes), FBM (fresh biomass), GRM (grain mass), LEL (leaf length), LFS (flag leaf sheath), PAL (panicle length), PEX (panicle exertion)

decrease from Guinea/Mali to the Atacora Montain zone, i.e. the Benin and Togo growing areas, where the genetic diversity was found to be particularly low (Table 3). These findings strongly confirm the Upper Niger basin as a major centre of white fonio genetic diversity in the region as described by Portères (1976) based on the varietal distribution pattern. The low genetic diversity found in the Atacorian zone may therefore be related to a narrow genetic make-up of the founding genotypes introduced in this area. However, focussing on Togolese germplasm, our results contrast the report of Hilu et al. (1997) who detected approximately similar range of genetic variability (0.27–0.88, RAPD) in only ten accessions of *D. exilis* compared to the present study with 122 accessions of diverse origin. Because our Togolese *D. exilis* material was directly collected from farmers in the entire growing area (Adoukonou-Sagbadja et al. 2004), it is assumed that the diversity detected in the present study is representative for Togo.

Inter-specific differentiation and genetic structure within *D. exilis*

Morphologically, *D. iburua* resembles *D. exilis* in many ways (Portères 1976; Haq and Ogbe 1995). The 16 morphological traits used in this study seem to confirm this observation because no clear-cut separation of both species was possible. The morphological discrimination of the two species is mostly based on limited discrete morpho-botanical traits mainly related to the structure of their inflorescence and spikelet (Stapf 1915; Henrard 1950). In the present study, UPGMA and PCoA based on AFLP data revealed a clear separation of the two species demonstrating their high genetic differentiation at the DNA level. This finding supports the results of a previous molecular investigation by Hilu et al. (1997) using RAPD markers and confirms the botanical distinction of the two species as described by Stapf (1915) and Henrard (1950). Furthermore, regarding the high number of species-specific markers detected in *D. iburua* (28) and *D. exilis* (557), the AFLP technique appears to be a tool to strengthen the resolution of morpho-botanical approaches (cf. Stapf 1915; Henrard 1950; Portères 1976; Sanou 1993; and the present study) for fonio species' identification and should be useful for taxonomic investigations in the genus *Digitaria*.

The relatedness of the two species as observed in this study seems to be substantial. Out of 1,065 AFLP markers used, 480 (i.e. 45%) were shared by the two species. This high degree of shared genome, supported by their almost substantial morphological resemblance (Portères 1976; Haq and Ogbe 1995), suggests a relatively late evolutionary separation of the two species. Further on, recent work revealed a tetraploid genome of white and black fonio landraces with very similar genome sizes (Adoukonou-Sagbadja et al. 2007). The genetic relatedness between the two species observed here is slightly higher than that reported by Hilu et al. (1997) but the discrepancy may be due to the much larger number of loci and genotypes investigated in the present study.

In this study, a large phenotypic variability was detected among *D. exilis* accessions. However, no distinct morphological group could be ascertained. Although morpho-botanical (racial) groups have been identified by Portères (1976), it has been observed that these races have no geographic unity and grade morphologically one into another (Zeven and de Wet 1982), supporting thus the present findings. The absence of clear racial differentiation was already reported for many grass species like Kodo millet (*Paspalum scrobiculatum* L., de Wet et al. 1983) and saltgrass (*Distichlis spicata* L., Ram et al. 2004). Lem and Lallemand (2003) stated that the lack of reliable and highly discriminative traits is common in many grass species and hamper their morphological characterisation and discrimination. Traditionally, farmers classify and name their fonio landraces merely based on the growth cycle but also on other traits such as plant habit, coloration of shoot or seeds, organoleptic characteristics, etc. (Adoukonou-Sagbadja et al. 2006). In general, our results of AFLP analysis did not conform to the morphological classification. The clustering of white fonio accessions into three main genetic groups (Figs. 3, 4) follows mostly the geographic origin with two groups overlapping in the Upper Niger basin and the last isolated in the Atacora Mountain. The substantial genetic structure obtained after UPGMA and PCoA demonstrates the high degree of differentiation within this species, probably due to limited gene flow primarily restricted by the

mating system. This finding is further supported by AMOVA which shows that the vast proportion of AFLP variation detected in *D. exilis* was present rather among than within groups (Table 5). Furthermore, an important part of the total variation was found among origins (countries), indicating genetic divergences among landraces across the growing areas of *D. exilis* as evidenced by the substantial differentiation detected between the two agro-ecologies in Togo.

The reproductive system is one of the important life-history characteristics that strongly influence genetic variability (Clegg et al. 1992). In fonio millets, the mating system is not well understood and the available information is mostly speculative. Fogg (1976) as well as Hilu et al. (1997) stated that the *Digitaria* millets are probably cross-pollinated plants. Conversely, describing the reproductive organisation in the genus *Digitaria*, Watson and Dallwitz (1992) indicated that *Digitaria* species are inbreeding. Similarly, regarding the tiny size of their florets, Sarker et al. (1993) assumed that small millets including fonio millets are self-fertilized crops. In their review, Hamrick and Godt (1989) reported that, in contrast to outcrossing species, selfing species have most of their genetic diversity partitioned among populations. The estimated Φ_{ST} values obtained either among genetic groups ($\Phi_{ST} = 0.70$) or among origins ($\Phi_{ST} = 0.56$) are consistent with this pattern. Furthermore, the F_{ST} estimated under high inbreeding hypothesis ($F_{is} = 0.9$) using the approach of Lynch and Milligan (1994) strongly supports these results. Moreover, assuming either predominant (0.8) or complete (1.0) inbreeding values had very little effect on the results (a difference of 1.3% decrease or 5.8% increase respectively, as compared with $F_{is} = 0.9$) and did not affect the general conclusions. Comparable results were reported for rice species such as *Oryza glumaepatula* Steud. (Buso et al. 1998; $F_{ST} = 0.67$, RAPDs) and *Oryza sativa* ssp. *japonica* (Yu et al. 2005; $F_{ST} = 0.746$, RAPDs), known as predominantly autogamous plants. However, Heywood (1991) stated that the genetic architectures observed for predominantly autogamous species are frequently comparable to those of taxa that reproduce by a mixture of apomixis and sexual outcrossing (facultative apomicts). Apomixis prevents sexual recombination within a population and gene flow by pollen among populations, resulting in low genetic variation within but a high differentiation between populations. Because apomixis is common in grass species (Clayton and Renvoize 1986), this would be a different conceivable mating system of fonio which is in accordance to the population structure found. Nonetheless, this hypothesis deserves further specific investigations for finding a definitive conclusion.

In crop plants, farmers and traders play an important role in gene flow between growing areas. In this study, except Burkina Faso, the lowest differentiation was observed between countries within the UNIG basin or the ATAC zone (Table 5), indicating that germplasm exchange may occur frequently rather within than between geographic zones. Although such pattern apparently suggests an isolation by distance process (Wright 1946), this may have an incidental effect since it is obvious that landraces from the ATAC zone are more closely related to those of UNIG 2 landraces than the latter are with their sympatric UNIG 1 landraces (Fig. 1; Table 5). A possible explanation of limitation in seed exchange between these two zones could be due to traditional seed management systems, limited market development towards and within Togo and Benin, marginal fonio cultivation and progressive abandonment of the crop in the Atacora zone (Adoukonou-Sagbadja et al. 2006). Since most of the diversity is present between genetic groups, the differentiation pattern of Burkina Faso landraces from those of other countries in the basin is essentially caused by their clear clustering in one group, i.e. UNIG 2. Because the growing areas of Burkina Faso and Mali are conspicuously linked (Fig. 1) and may allow seed exchange between farmers, a narrow sampling background of the eight accessions originating from Burkina Faso can be hypothetised.

Portères (1976) proposed that the earliest domestication of *D. exilis* occurred in the UNIG basin in the vicinity of central delta. Refering to the large genetic divergence observed in their study, Hilu et al. (1997) speculated that multiple domestication events associated with the different diversity centres may have occurred in *D. exilis*. Similarly, a local domestication of some landraces has been stated by certain Togolese farmers (Adoukonou-Sagbadja et al. 2006). This seems unlikely to be the case considering the low genetic diversity detected in the ATAC group and its close genetic relationship with the cluster UNIG 2 (Table 5). The geographic distribution of the genetic diversity as detected in this study strongly supports the origin of white fonio in the UNIG basin (Portères 1976; Munson 1976). Consequently, the differentiation of two sub-clusters within the ATAC group as detected by UPGMA can be better explained as the result of a secondary diversification (Portères 1976) or of multiple introduction events from the UNIG basin (Adoukonou-Sagbadja et al. 2006). It can be hypothesised that the genetic groups identified represent the major evolutionary groups differentiated over time during the cultivation and dispersal history of the crop. Based on their genetic relatedness and level of diversity, UNIG 1 may well represent the most anciently cultivated fonio group in the basin. From this ancestral group may have evolved UNIG 2 from which the ATAC group differentiated almost recently after the introduction of fonio to Benin and Togo.

Population genetic structures are determined by joint effects of many factors including mating system, selection

and adaptation, mutation, migration and dispersal mechanism, drift, founding effects, etc. (Hamrick and Godt 1989). Most likely, the large differentiation observed within *D. exilis* may be due to strong selection associated with differences in traditional agricultural practices and adaptation of fonio landraces to the contrasting ecological conditions where they have been grown since centuries (Portères 1976; Hilu et al. 1997; Adoukonou-Sagbadja et al. 2006). The substantial differentiation observed among countries and within the Togolese agro-ecological sites as well as the presence of many specific AFLP bands (Tables 4, 5) support this hypothesis. Due to this and because of its geographic isolation, the ATAC group may well represent an ecologically specialized deme. Phenotypically, this group shows more significantly divergent traits from UNIG groups than found between the latter, providing further support of local adaptation (Table 6). Such conclusion may be made with caution for UNIG 1 and 2, though these groups seem nonetheless to be more geographically predominant in southern (humid savannah) and northern (dry savannah) parts of the basin, respectively. Therefore, the possibility that these genetic groups correspond to fonio ecotypes remains an attractive hypothesis that needs further investigation.

In applied breeding, phenotypic or genetic distances between genotypes are expected to provide predictors for high heterosis effects and performance of their hybrids. In the present study, weak correlations were found between genetic and morphological distances of fonio accessions. Discrepancy between molecular and phenotypic distances seems to be a widespread phenomenon in plants (Gerdes and Tracy 1994; Portis et al. 2004, etc.). This is expected to be related to the environmental effects on morphological traits while molecular markers such as AFLPs are neutral and not necessarily linked to genes underlying morphological traits. Furthermore, the number of informative AFLP loci (1,065) compared to the limited number of quantitative traits investigated (16) may also have contributed to this discrepancy. Nevertheless, the statistically significant differences observed between *D. exilis* groups regarding some morphological traits (Table 6) are indicative of genetic determinism. However, additional experiments such as mapping studies are needed to identify specific genes or genomic regions that have an influence on the phenotypic variation observed. Since the germplasm was evaluated in only one location, a more comprehensive view on phenotypic plasticity and adaptive potential of the germplasm has to be developed.

In conclusion, our results on genetic relationships and diversity within some West African fonio accessions revealed a clear separation between the species and the existence of three highly differentiated genetic groups in *D. exilis*. Although essentially present between groups, the genetic diversity observed in *D. exilis* is nonetheless substantial. Many landraces performed well in the field trial and a great variability was observed for most of the agro-morphological traits considered. Detailed knowledge of the genetic population structure, e.g. based on AFLP markers, and its linkage to important agronomic traits will be very useful for future fonio breeding efforts in the region. In addition, the results of this study are relevant for developing effective management and conservation strategies for fonio genetic resources in their traditional growing areas. For this purpose, i.e. breeding efforts and conservation programmes, definitive knowledge on the reproductive system in these valuable but neglected West African native millets is urgently needed.

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Chapter IV

REPRODUCTIVE SYSTEM AND FONIO PHYLOGENY*

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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 arid and 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 evolution of fonio, contributing to the knowledge on their biology, and thus providing useful subsides for future genetic improvement of these valuable crops.

Key words: AFLPs, *Digitaria spp.*, Fonio, Isozymes, Phylogeny, Pseudogamous apomixis

List of 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
SKDH	Shikimate deshydrogenase
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 [14, 29]. This large specific 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 [28]. Henrard [29] 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 confuse requiring further detailed studies to delineate boundaries [34]. 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 important native 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) [3].

Fonio millets are among the most suitable native cereals for production in marginal 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 yearly devoted to its cultivation in the region [11]. While the crops are well 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 [2, 27]. 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 (personal observation) and AFLP [4] 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 [26]. The genus *Digitaria* presents different reproductive systems: outbreeding, inbreeding, intermediate and vegetative reproduction [34, 63, 64]. 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 [49]; however the possibility of outbreeding system has also been advocated [25, 31]. In a recent study focusing on genetic diversity and population differentiation in West-African fonio millets, Adoukonou-Sagbadja et al. [4] confirmed the self-oriented mating behavior but noted that apomixis can be a conceivable alternative mating system to inbreeding in fonio millets since the genetic architecture observed for predominantly autogamous species are frequently comparable to those of facultative apomictic taxa that reproduce by a mixture of apomixis and sexual outcrossing. Apomixis or agamospermy is an asexual mode of reproduction in which the ovule develops into a seed without involving meiosis and fertilization [41]. 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 [38, 54].

A number of investigators have speculated on the origins and evolution of the domesticated fonio millets. Based on botanical affinities, Stapf [57] and Dalziel [17] 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 [29] 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 [27]. Regarding *D. iburua*, Stapf [56] proposed the wild *D. ternata* Stapf while Portères [44] 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 [27]. Using random amplified polymorphic DNA (RAPD) markers, Hilu et al. [31] 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 [3]. 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 [43]. Other important traits like resistance to lodging and especially big seed size critically useful to fonio breeding are also expected to be found in wild *Digitaria* species [36]. 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. 8, 21], molecular taxonomy and phylogeny investigations [e.g. 9, 19, 39], as well as mating system determination [e.g. 32, 33, 35], 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 [31]. 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 [59]. Amplified Fragment Length Polymorphism (AFLP) [62] analysis is currently a method of choice in molecular studies and has been, as in many other crops [e.g. 8, 9, 51], applied with success in genetic diversity and population differentiation analysis in West-African fonio germplasm [4]. 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 [9].

As part of ongoing comprehensive characterization of West-African fonio genetic resources [3, 4, 16, 31, 37, 48], 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 [4]. To confirm the close phylogenetic relationships of these species, accessions of four 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 reproducibility values, calculated as the percentage of bands that were identical in the two repeats, are very high and range from 97% to 100% for the six primer-pairs (Table 1).

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*

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 (IIa) and on the other hand *D. sanguinalis* accessions (IIb). 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 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 well 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).

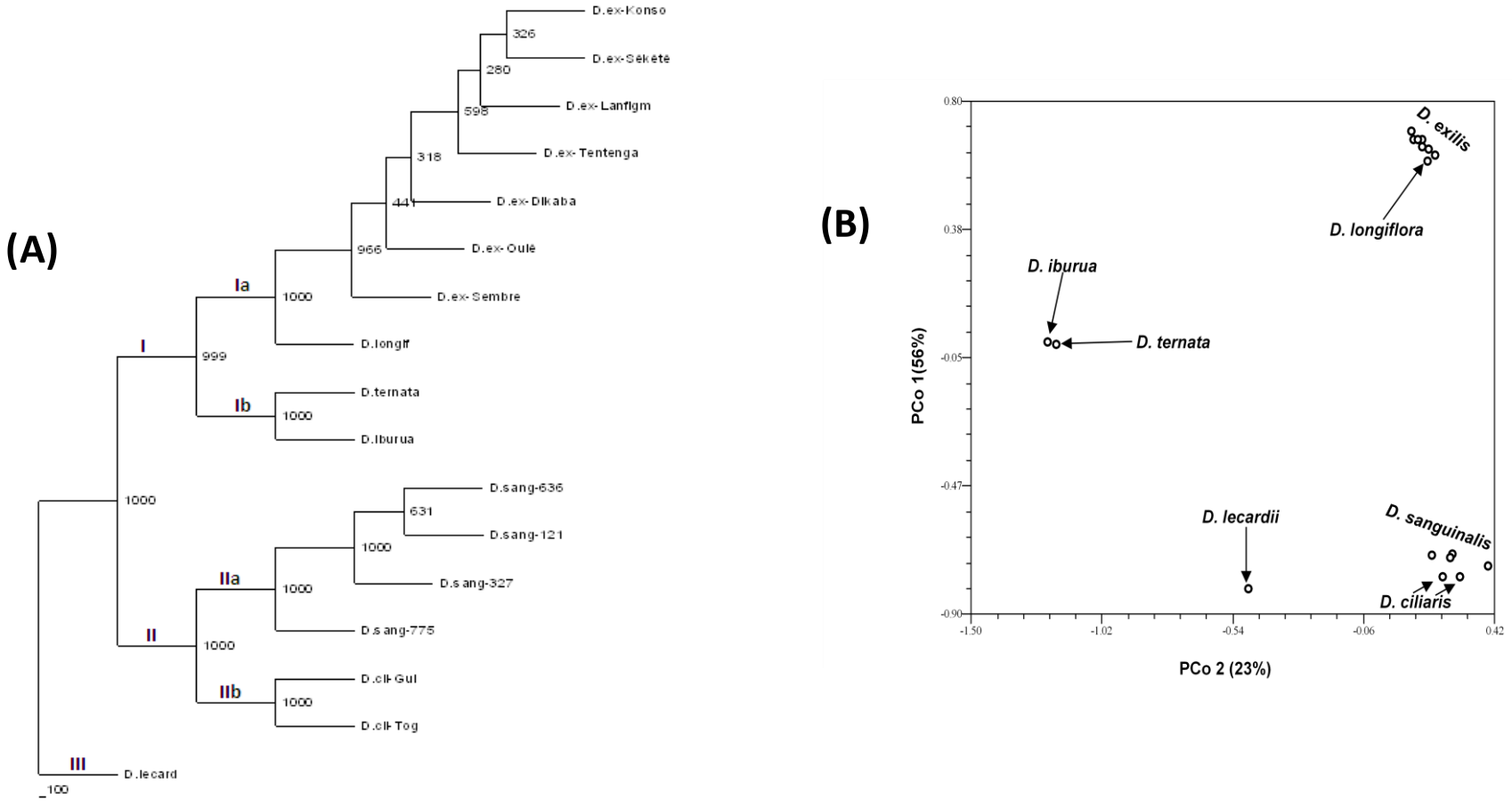

Figure 1 Phenetic analysis of 17 accessions of 7 *Digitaria* species based on 371 AFLP markers: **(A)** Consensus neighbor-joining (NJ) tree showing the phylogenetic relationships of investigated accessions, **(B)** Principal Coordinate Analysis (PCoA) illustrating the general grouping of the different 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).*

Intra-specific variability in AFLPs was evident in both cultivated and wild species in which more than 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.

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* [4].

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 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. [31] 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 well 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. [4] 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

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	Nb. of analyzed progenies	Nb. 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, Nb. of progenies analyzed with AFLPs, others are by isozymes*

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) well attest 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 [17, 29, 57], RAPD [31], and recent cytological data [3] 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 confine this wild species from the crops [13]. In contrast, the only divergent traits of *D. longiflora* and *D. ternata* from the cultivated species are the fine pubescence of their spikelets and in some instance 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 attests the view of direct domestication of fonio crops from these two wild tetraploid species. However, in contrast to Hilu et al. [31], 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 [4]. However, more throughout sampling of both *D. longiflora* and *D. ternata* will be required to confirm the 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 important differences in morphology [29] and cytology [3] 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 [31] and AFLP [4] studies and supports the view of differences in genomic composition of these crops [3]. 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 [27,

44]. 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 resemblance as has already been reported in *Echinochloa* millets [30]. It is notwithstanding to note here that the genetic relationships between the representative *D. exilis* accessions observed in the present phylogenetic tree (Fig. 1A) do not follow that previously described between their genetic groups of origin [4]. 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, indicating 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 wild species has been controversially discussed [29, 23]. They are morphologically similar and lie at the center of a complex of somewhat intergrading and weedy species sometimes difficult to be distinguished [53] despite their different origins: *D. ciliaris* is a pantropical species while *D. sanguinalis* is from temperate regions [29]. 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 well supported by their morpho-botanical divergence [29, 60].

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 commonly well known feature in crops [e.g. 21]. Crop domestication is a relatively recent (about 10,000 years) evolutionary process from a few wild ancestral populations. In 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 utilize 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 [15]. 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 [7]. The tetraploid level of both fonio species [3] is then concordant with their apomictic reproduction. Apomixis has been previously described in other *Digitaria* taxa such as *D. arenicola* Beetle and *D. cognata* Pilger [64] and is known to be a widespread evolutionary phenomenon within the Poaceae, particularly in Chlorideae and Paniceae [41]. In many well characterized apomictic species, apomixis is often associated with hybridization and allopolyploidy [7]. 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. [52] 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. 5, 10, 35, 58]. 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 [4]. The

percentage of sexual seedlings detected in *D. exilis* is clearly lower than that reported in many facultative apomicts [42, 35, 58, etc.]. Since the experiments were only conducted under greenhouse conditions, further investigations are 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* [45]. 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. 1, 61]. Since hand emasculation is often unsuccessful due to the miniature size of fonio florets, the effect of outbreeding on seed set could not be accessed. 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 [12], 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 [64]. 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 [31] and cytological [3] 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. In 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 [31]. 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 [3, 4, 16, 31, 37, 48] can yet 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. [3, 4]. Samples of *D. exilis* were carefully selected to cover the three gene pools previously identified in West-African fonio germplasm based on AFLP markers [4]. Because of the extremely low genetic diversity detected in *D. iburua* germplasm [4], 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. [4] 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 [20] which is equivalent to equation 21 of Néi and Li [40]. 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 [19]. Phylogenetic phenograms were generated by the unweighted pair group method with arithmetic average (UPGMA) [55] and the neighbor-joining (NJ) [47] clustering procedures using PHYLIP 3.6 software package [24]. 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 [46].

Reproductive assays

Reproductive systems of fonio species were determined following the method described by Marshall and Brown [38] and recently applied in various plants including *Hypericum perforatum* L. [6] and *Paspalum rufum* Nees [54]. 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: Posphoglucose 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 [56]. 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. [45] 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 [22]. In phylogeny analysis, bulked leaf material from 4-5 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^{TM} 200 fluorometer (San Francisco, CA, USA). The AFLP analysis was conducted as described by Adoukonou-Sagbadja et al. [4] 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 25µl) consisted of 12.5 ng fluorescent dye-labeled *Eco*RI primer, 30 ng *Mse*I primer,

0.2 mM of each dNTPs, 2µl 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 [50] on rice, except for minor readjustments described in Dansi et al. [18]. 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. [18].

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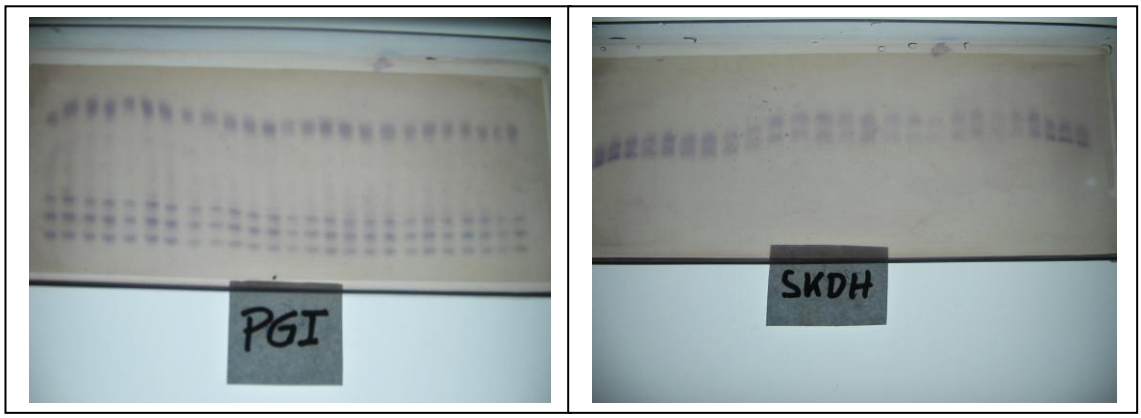
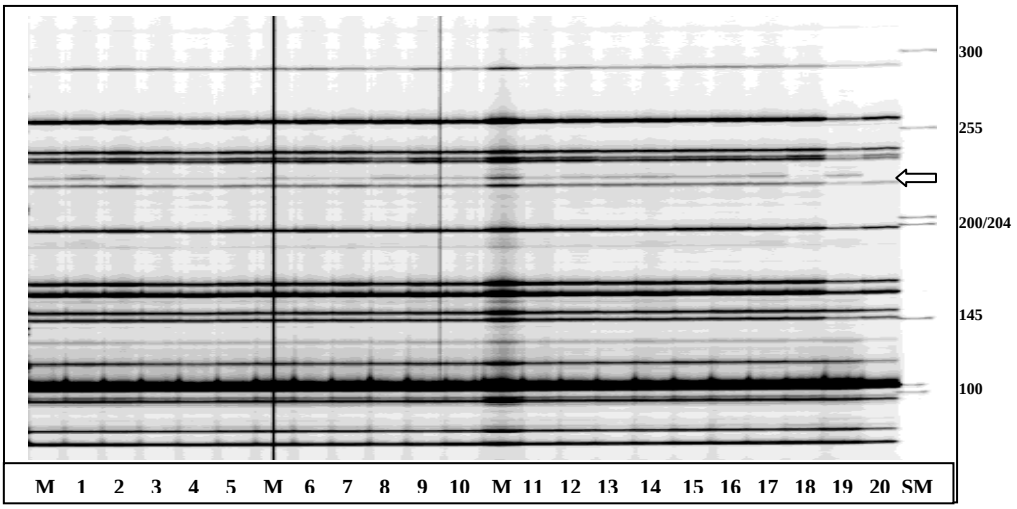
Supplementary table 1: List of accessions of cultivated fonio and wild *Digitaria* species analyzed, their geographic origin and ploidy status

N°	Species	Acc. n° / names	Geographic sources	Ploidy Status[c]
1	*Digitaria exilis*	I 3 / Sèkètè	Guinea	4X
2	*Digitaria exilis*	I1 / Konso	Mali	4X
3	*Digitaria exilis*	IV 14 / Oulè oulè	Guinea	4X
4	*Digitaria exilis*	TKB 74 / Sémbre	Togo	4X
5	*Digitaria exilis*	TPW 41 / Dikaba	Togo	4X
6	*Digitaria exilis*	BUF 69 / Foni Femba	Burkina Faso	4X
7	*Digitaria exilis*	BEN 08 / Tentenga	Benin	4X
8	*Digitaria iburua*	BEN39b / Péi	Benin	4X
9	*Digitaria longiflora*	TAB 92b	Benin/Togo border	4X
10	*Digitaria ternata*	BEN36c	Benin	4X
11	*Digitaria ciliaris* [a]	GUI 02	Guinea	6X
12	*Digitaria ciliaris*	TKD 75c	Togo	6X
13	*Digitaria lecardii*	STB 02	Benin	6X
14	*Digitaria sanguinalis* [b]	DIG 327	unknown	4X
15	*Digitaria sanguinalis*	DIG 775	China	6X
16	*Digitaria sanguinalis*	DIG 121	unknown	4X
17	*Digitaria sanguinalis*	DIG 636	Poland	4X

[a] *Syn. D. adscendens ;* [b] *accessions of this species are provided by GAT Genebank (IPK, Gatersleben); other accessions belong to West-African research collection maintained at the Laboratory of Genetics and Biotechnology, Univ. Abomey-Calavi, Benin;* [c] *following Adoukonou-Sagbadja et al. [3], except D. sanguinalis accessions determined alongside this study by flow cytometry at IPK using the landrace Iporlapiah (BEN 21) as internal reference.*



Supplementary figure 1 Photographs of starch gels showing the complete absence of variation (fixed heterozygous pattern) in PGI (Phosphoglucoisomerase, slow migrating locus) and SKDH (Shikimate desydrogenase) profiles in the offspring obtained after self pollination of the landrace Sèkètè. Results are similar for other *D. exilis* landraces as well as that of *D. iburua* involved in the study. The PGM (phosphoglucomutase) profile is not shown.



Supplementary figure 2 AFLP fingerprints of the maternal genotype (M) and 20 progenies obtained with the primer-pair E-ACA / M-CCA. The arrow indicates the AFLP locus (~230 bp) where the individuals 18 and 20 differ from the profile of M; SM = size markers.

Chapter V

CONCLUDING DISCUSSION

The overall objective of the work presented in this thesis was to characterize fonio genetic resources, to contribute to their effective conservation and to facilitate their exploitation in breeding. This has been achieved by using diverse cytological, agro-morphological and molecular approaches. From these investigations, important information on fonio biology and genetics has been gained. This work constitutes the first large-scale genetic characterization of traditional West-African fonio millets and has substantially added to the general knowledge on these neglected crops. This concluding chapter reviews and synthesizes the key findings of the study presented in this thesis and discusses their implications for breeding and conservation of fonio genetic resources in West-Africa. Finally, some perspectives for future research are proposed.

Key findings

The major key findings of this study can be summarized as follows:

1. Fonio millets are tetraploid plants (2n=36 chromosomes) having stable and relatively small genome sizes (1C = 956 Mbp and 904 Mbp for *D. exilis* and *D. iburua*, respectively) (Chapter II). These crop plants have evolved by traditional selection from the wild tetraploid species *D. longiflora* and *D. ternata,* respectively (Chapters II, IV) through a single domestication event (Chapters III, IV) and are both allopolyploids (Chapter IV). These genomic characteristics documented here for fonio crops are concordant with those previously described for many small millets such as tef or finger millet (Ayele et al. 1996, Hilu 1995).

2. The two crops are genetically different (Chapters III, IV) and may have a distinct genomic composition (Chapters II, IV). Based upon our observations, the genomic structure can be illustrated as follows: AABB for *D. exilis* and CCDD for *D. iburua*. This anotation still holds true for their respective putative wild progenitors *D.*

longiflora and *D. ternata*. Further cytogenetic and molecular phylogenetic studies can help identifying the diploid genome contributors to the genomic constitution of these cultivated/wild progenitor complexes.

3. Globally, fonio crops have a relatively narrow genetic background (Chapter III). The lack of genetic diversity is common for small millets (Hilu and Johnson 1992) and can be explained by their domestication from a subset of the wild species (domestication bottleneck) and by the subsequent limited cross pollination between wild and domesticated gene pools because of their self-oriented mating system (Chapters III, IV). An additional bottleneck may be imposed by the dispersal of a few accessions from the area of domestication/diversification to other growing areas and human selection for particular superior cultivars further augmenting the loss of genetic diversity. The wide phenotypic variability observed in fonio millets is then mainly due to their adaptations to the diverse agro-ecological conditions in which these crops are grown.

4. *D. exilis* is the most diversified fonio species (Chapter III). Its genetic diversity is unequally distributed through the region with a hotspot in the UNIG basin. Besides, significant population differentiation was also observed within this species, with the largest variation detected among populations.

5. Fonio millets are apomicts: ranging from highly (*D. exilis*, ~2% residual sexuality) to obligate (*D. iburua*) self-compatible pseudogamous apomictic behaviour (Chapter IV). These observations suggest that mutations and residual sexuality through occasional outcrossings are the essential sources of genetic variations in fonio. This may explain the extremely low genotypic diversity (H=0.02) in *D. iburua* (only mutations) and the relatively moderate genotypic diversity (H=0.267) observed in *D. exilis* (both mutations and occasional outcrossings).

6. AFLP appears to be a useful molecular tool for genetic studies in *Digitaria*. In the present study, this method was found to be efficient in genetic diversity and differentiation analysis of fonio crops and in reconstructing their phylogenetic history and assessing their reproductive system (Chapters III, IV).

Genetic characterization and implications for fonio breeding strategies

Fonio millets are important staple crops for small scale farmers in semi-arid areas of West-Africa. Because of their low productivity, these crops have to be improved in order to make them as competitive as other widely cultivated cereals in the region such as pearl millet, sorghum and maize. Target goals in fonio breeding include yield increases, improved lodging and pathogen resistance, and enhanced environmental

adaptability without impairing the nutritional quality of the grains (Kuta et al. 2003). Genetic characterization is the primary step in the utilization of crop genetic resources in breeding (Fikiru et al. 2007). For instance, knowledge on the genetic variations within crops and their phylogenetic relationships with wild relatives is an important consideration in designing breeding strategies and assists breeders in the process of decision-making (Badr et al. 2002). Besides, reproductive system as well basic cytogenetic data such as nuclear genome size, chromosome number and ploidy level information play also a crucial role in the success of any crop breeding (Tuna et al. 2001, Djè et al. 2004). Thus, the diverse inferences obtained through this study are greatly instructive for fonio improvement.

For exploiting the genetic diversity occurring within a crop species for breeding, information on both its level and structure is important. In contrast to *D. iburua*, the moderate genetic diversity detected in *D. exilis* in the present study (Chapter III) suggests that selection will be possible in this crop. The wide range observed for most of the plant traits, i.e. plant height, number of productive tillers per plant, panicle and grain yields, days to physiological maturity, etc. already suggests flexibility that could be exploited for selection and the development of cultivars. Besides, the AFLP analysis could be extended to mapping and linkage analysis in fonio, as molecular markers linked to desirable traits are useful tools for the early generation selection of genotypes with desirable traits in any breeding programs. In view of the significant correlation of many agronomic traits to molecular genetic structure detected in *D. exilis* (Chapter III, Tab. 6), the potential for improving fonio through marker-assisted breeding is promising. Besides, exploring the genetic variation patterns in *D. exilis* with respect to environmental factors or connecting these patterns to the disease resistance patterns in fonio may result in grouping of accessions with similar adaptability or disease resistance and thus would facilitate the search for unique genotypes from the collection.

The identification of heterotic groups is essential in modern programs for genetic improvement of many crops, as it allows for selection of only those crossings expressing the maximum heterosis potential, which permits a more efficient use of germplasm (Morales et al. 2010). In this study, *D. exilis* landraces analysed can be divided into three distinct genetic groups based on AFLP markers. Furthermore, these groups differ significantly in many important agro-morphological traits, including panicle length, panicle and grain yields, 1,000-grains mass, etc. (Chapter III, Tab. 6). These divergent groups correspond to possible germplasm pools. However, it still needs to be shown, whether these germplasm pools are heterotic groups in the sense that crosses among them would give a positive heterotic response for important traits. For quantitative characters such as yield, heterotic response is expected to increase with the parental genetic distance (Melchinger 1999). Following

this assumption, maximal heterosis could be expected by crossing UNIG 1 landraces with genetically distant UNIG 2 or ATAC landraces. The genetic characterization of landraces' populations using DNA-based molecular markers such as the AFLPs used in this study could be a useful pre-screening step in a breeding program to fully exploit existing genetic diversity and to maximize heterosis in hybrid cultivars. The fact that all fonio landraces are of the same ploidy level (Chapter II) shows that chromosome number is no barrier to gene exchange in fonio.

In general, the relatively narrow genetic background of fonio crops requires broadening for successful breeding. This is particularly crucial for *D. iburua* in which an extremely low genetic diversity (H=0.02) was detected. As earlier envisaged for small millets by Sarker et al. (1993), one strategy could be the use of induced mutagenesis to generate novel genetic variations. Another but most promising option is to exploit, through inter-specific hybridizations, the untapped genetic variations in wild relatives of these crops, especially those closely related such as their two putative wild progenitors. As in many other crops, problems of pests and diseases can also be targeted following this approach for the release of resistant fonio varieties. Besides, the seed size can be improved through wide crosses with *D. sanguinalis* in which larger seeds can be observed. This may be a breakthrough in fonio breeding as it could help overcoming the technological limitations related to fonio processing and may then help to considerably improve the quality of the food products.

The presence of apomixis in fonio crops is advantageous for their breeding in the sense that its utilization can lead to the fixation of desirable agronomic characteristics. However, genetic recombination and cross breeding are difficult to achieve in apomictic crops (Asker and Jerling 1992, Hovmaln et al. 2004). Despites its high level of apomixis, *D. exilis* was found to still have 2% residual sexuality (Chapter IV). This study also showed that dominant molecular markers like AFLPs can help identifying occasional sexual tetraploid individuals that can be utilized as female parents in crossing; any landrace has the potential to function as male parent since fonio species are all pollen fertile. Because of the obligate nature of apomixis in *D. iburua*, biotechnological approach like somatic hybridization by means of protoplast fusion, yet available for modern plant breeding, could be adopted as a way to overcoming this natural reproductive barrier.

Diverse researches have revealed that the genomes of grasses (Poaceae) are remarkably similar as indicated by extensive conservation not only of genes but also of entire linkage groups that represent the chromosomes (Cook 1998, Mahalakshmi and Ortiz 2001). This implies that the huge research activities on the genome of major cereals such as maize, rice, sorghum or barley, etc., can benefit any other

cereal, particularly the neglected crops such as fonio millets. A comparative analysis of the small foxtail millet genome (1C=450 Mbp), a member of Paniceae tribe which also includes fonio millets, with rice (1C=400 Mbp) revealed a simple relationship between the chromosomes of the two species (Devos and Gale 2000). The relatively small genome size of fonio (which represents only 2.4 times that of rice) is a favourable feature for the development of genomic tools/resources and should be easily amenable for analysis and mapping at the molecular level. Adopting such strategy could accelerate the breeding efforts to provide the first improved varieties to enhance fonio production in West-Africa.

Conservation considerations and priorities

Although being minor crops, the contribution of fonio millets, principally *D. exilis*, to food and nutrition security of many tribal communities living in the semi-arid and sub-humid drought-prone areas of West-Africa remains substantially important. The neglected nature of fonio millets and the increasing abandonment of their cultivation in many growing areas such as those of Benin, Burkina Faso, Mali and Togo, etc. (Sanou 1993, Adoukonou-Sagbadja et al. 2004 & 2006), make the conservation of these crops a high priority. Besides providing useful indications for breeding, the information obtained from the genetic diversity and differentiation analysis in these crops (Chapter III) are also instructive for making practical conservation actions.

The observation that most of the AFLP variation is partitioned among rather than within *D. exilis* populations suggests that a conservation plan should integrate a large number of populations of this species. One major strategic action in a conservation plan is the identification of populations which truly contribute to the overall genetic diversity of the species of concern (Gilbert et al. 1999). In this study, the largest genetic diversity of this fonio species is located in the UNIG basin populations (Chapter III, Table 3), suggesting that this zone should be more attractive for the conservation actions. Nonetheless, attention should also be paid to the zone of low diversity but of distinctness like that of the ATAC growing area for preventing the loss of unique genetic variants. Such action may be relevant for any other isolated populations not included in the present study such as that of the Nigerian growing area. Since in general landraces' genetic differentiation is low between countries within each zone (Chapter III, Table 5), a strategy of minimal sampling per country should be adopted. If applicable as in the case of Togo in this study, all existing agro-ecological areas within the target country should be considered. In contrast to *D. exilis*, *D. iburua* requires priority in conservation actions due to its current residual status in its present growing areas (Portères 1946, Adoukonou-Sagbadja et al. 2006). Although the genetic diversity detected in *D. iburua* is extremely low (Chapter

III, Table 3) this does not mean that sampling a single population would be enough to ensure the maintenance of the available diversity in this crop. AFLP-based genetic relationships revealed by UPGMA clustering showed black fonio accessions from Benin well separated from those from Togo (Chapter III, Fig. 3). This observation suggests that all known populations of this species should be sampled to maintain the integrity of its genetic diversity.

Substantial efforts for germplasm collections and ex situ maintenance have been made over the past decades (cf. Clément and Leblanc 1984, Kwon-Ndung et al. 1998, Adoukonou-Sagbadja et al. 2004, Clottey et al. 2006). Establishing a regional core subset (see Brown 1989) of such germplasm maintained ex situ can considerably enhance the conservation management and utilization of fonio germplasm in West-Africa given the technical limitations and resources restrictions at the individual national level. The present germplasm assembled from five countries and characterized during this study can serve as a starting collection to address this issue. This can further be completed by future germplasm collections through the entire growing areas of the crops, particularly in countries in which no collection yet exists or has completely disappeared. Because of increasing abandonment of fonio cultivation in many growing areas, future efforts should be directed to in situ conservation as complementary approach. For such actions, tribal areas where fonio remains a staple food crop may be targeted. Combining these two approaches would make the conservation efforts well efficient and more sustainable.

Further research priority

The work presented in this thesis has considerably contributed to the understanding of fonio in many aspects of its biology and genetics. However, there is still more work required to complement the present findings:

1. This study focused essentially on the primary and secondary centers of diversity as defined by Portères (1946, 1976). Therefore, extending the cytological and genetic diversity analyses to other areas not included in the present study such as those of Nigeria (for both species), Niger, Côte d'Ivoire, etc. is a priority. Although extremely low genetic diversity is expected as in the ATAC zone, this may nonetheless help to handle the unique alleles agronomically or ecologically useful to be conserved for future use. Special focus on fonio grown in the Dominican Republic (Morales-Payán et al. 2002) is also of great importance and may help understanding the evolution of fonio beyond its traditional growing region. Besides, germplasm collection and characterization of the genetic diversity in closely related wild species, particularly the two putative wild progenitors are also of prime importance. The diverse methodological approaches experienced in this study

(flow cytometry, agro-morphological traits, AFLP markers, etc.) could be useful for such studies.

2. The molecular phylogenetic investigations also need to be extended. They may target the diploid wild *Digitaria* species in West-Africa and beyond to determine the diploid genome contributors to *D. exilis*/*D. longiflora* and *D. iburua*/*D. ternata* complexes. It is also interesting to confirm the genomic constitution above proposed for fonio millets and verify whether allopolyploidy is of the segmental (involving two closely related diploid species) or the genomic (genetically different diploid parents) type. In this regard, molecular cytogenetic techniques such as fluorescent and genomic in situ hybridizations (FISH, GISH) can be of great help.

3. Until now, the genetic variations in fonio crops have been assessed only using dominant markers such as RAPDs (Hilu et al. 1997) and AFLPs in the present study. Since the genetic variation detected by isozymes in fonio crops is extremely limited, it would seem appropriate to develop and utilize SSR markers in future molecular studies of fonio millets. This may help to obtain estimates of allele frequencies, allowing a more efficient description of genetic population parameters.

4. The mechanisms of apomixis, in particular its inheritance in fonio crops, need further investigations. Diverse methods including classical cytoembryological and molecular tools widely used in many other grass species (i.e. in Martinez et al. 2007) can well be used for such purpose. Achieving these issues could help managing and better exploiting apomixis in fonio breeding.

5. It would be interesting to extend this characterization to the nutritional aspects since the presence of anti-nutritional molecules in the crude fonio grains have been reported (Sartelet et al. 1996). This could help for the development of cultivars with enhanced quality food products.

Final remark

Fonio millets remain crops that show promise for agriculture in less arable and arid regions of West-Africa, particularly during the current and prospective climate change. They are well adapted to hard stressed conditions and even to flooding. Furthermore, the potential for agriculture beyond the traditional cultivation region is also encouraging with regard to the current success of *D. exilis* as food crop in Dominican Republic (Morales-Payán et al. 2002). Nowadays, fonio is increasingly utilized as food and drink because of its perceived nutritional and health values. There is now a need to focus on producing concrete results in exploiting the potential of the crops. The present study has substantially increased the knowledge on these species by exploring diverse aspects of fonio biology and genetics, including their cytology, genetic diversity, phylogeny, as well their reproductive system. The work

provides the foundation for comprehensive conservation and efficient utilization of fonio genetic resources, thus contributing to their improvement indispensable to boost fonio production in West-Africa. Considerable research efforts are still required before the knowledge on fonio crops can be brought up to the same standard as that of the major cereals. Undoubtedly, concerted local, regional and international scientific networks are necessary if all these objectives for the crops are to be met.

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SUMMARY

Genetic Characterization of Traditional Fonio millets (*Digitaria exilis, D. iburua* STAPF) Landraces from West-Africa: Implications for Conservation and Breeding

Fonio millets are important indigenous minor cereals grown for centuries in semi-arid and sub-humid drought-prone areas of West-Africa. The crops are traditionally valued and serve either as staple or a major part of the diet for millions of tribal people. Fonio includes two cultivated species, both falling in the genus *Digitaria*: the white fonio (*D. exilis* Stapf), the most widespread cultivated species in West-Africa, and the black fonio (*D. iburua* Stapf), only grown in some restricted areas. A large number of fonio landraces are cultivated in the region; these are well adapted to marginal local conditions and traditional farming systems. Because of their complete scientific neglect, the potential of these crops for food and agriculture is not adequately exploited for improvement. An important initial step towards efficient preservation and exploitation of crop genetic resources is detailed genetic characterization. In this work, an important set of fonio landraces' accessions (globally 160) originally collected from different growing areas in West-Africa (Benin, Burkina Faso, Guinea, Mali, and Togo) was characterized and diverse aspects of fonio biology and genetics were investigated. The work aimed at contributing to the general knowledge on these millets and facilitating a better management and an optimal utilization of their genetic resources in breeding.

A basic cytogenetic analysis of the fonio collection and some wild relatives was first conducted using flow cytometry supported by chromosome count. The nuclear genome sizes documented among these species are variable. Average 2C values estimated for the cultivated *D. exilis and D. iburua* were respectively 1.956 and 1.848 pg. Approximately similar genome sizes were documented for their closely related wild species *D. longiflora* (2C = 1.869 pg) and *D. ternata* (2C = 1.775 pg). In contrast, larger genome sizes (2C = 2.576-2.660 pg) were obtained for other taxonomically distant species investigated, i.e. *D. lecardii* and *D. ciliaris*. Intra-specific variations in DNA content were slight and statistically not significant, suggesting genome size stability within species. All fonio landraces investigated were found to be tetraploid with 2n = 4x = 36 chromosomes, contrasting some previous considerations on diploid or hexaploid level of fonio millets.

Amplified Fragment Length Polymorphism (AFLP) analysis combined with an agro-morphological evaluation was further conducted to evaluate the extent of fonio genetic diversity, assess its gene pool structure and geographical distribution pattern. Cluster analyses (UPGMA and PCoA) conducted on AFLP data revealed a clear differentiation between the two fonio species' accessions and structured *D. exilis* genotypes into three major distinct groups that largely fit to their geographic origins. The first two groups, overlapping in the Upper Niger (UNIG) basin, included accessions from Guinea, Mali and Burkina Faso. The last group, geographically isolated, and covering the Atacora Mountain (ATAC) zone, comprised accessions from Benin and Togo. In each inferred group, landraces were mostly close-related substantiating a relatively narrow genetic background of fonio species. Shannon diversity index estimated in *D. iburua* was extremely low (H = 0.02) and could be related to the current residual status of this crop. In *D. exilis*, the genetic diversity detected was moderate (Shannon index H = 0.267, Nei's gene diversity H' = 0.335) and unequally distributed among populations. The essential part of this diversity was found in UNIG basin countries (H = 0.07- 0.135) while very low diversity was detected in the ATAC zone (H = 0.05-0.06), confirming the UNIG basin as the major centre of white fonio genetic diversity/diversification in the region. AMOVA analysis revealed that the large part of the genetic variation in *D. exilis* is attributable to differences among genetic groups (70%) or origins (56%), indicating that substantial genetic differentiation has occurred over time during the cultivation and dispersal history of this crop. Diverse factors e.g. mating system, agricultural selections, and ecological adaptations as well as founding effects may have jointly contributed to the observed genetic structure in this species.

Large phenotypic variations were detected between fonio accessions for the 16 agro-morphological traits investigated. In contrast to molecular data, neither clear separation was observed between the two species nor could meaningful groups be ascertained in *D. exilis*. Furthermore, no correlation was detected between the overall AFLP genetic- and phenotypic-based distances (r= 0.04, P>0.05). However, the genetic groups identified differed significantly in the mean performance of nine phenotypic traits in which dry biomass, panicle and grain yields were found to be particularly discriminative for all inferred groups. The ATAC group is more phenotypically divergent from the UNIG groups, providing further support of local adaptations in the shaping of the genetic diversity.

AFLP markers were further employed to assess the phylogenetic relationships among cultivated fonio species and the wild relatives and examine proposed hypotheses on fonio ancestry. A strong genetic affinity (over 92% similarity) was detected between the cultivated *D. exilis* and *D. iburua* and the wild tetraploids *D. longiflora* and *D. ternata*, respectively. These results, in combination with cytological data, provide additional evidences supporting the previous view of direct domestication of fonio millets from these two wild species. High genetic divergence was, expectedly, found between fonio species and the other

taxonomically distant *Digitaria* taxa investigated. The results also confirmed *D. ciliaris* and *D. sanguinalis* as separate species but sharing close ancestry.

Selfing experiments and subsequent progeny analyses were further conducted using three isozyme systems (PGI, PGM, SKDH) supplemented by AFLP markers. The results obtained, in conjunction with previously observed population genetic structure in fonio germplasm, let us to conclude to apomixis as the major reproductive system in these species: *D. iburua* was found to be obligate apomict while *D. exilis* was highly apomictic with nearly 2% residual sexuality. Additional data documented on seed set and pollen viability suggested that apomixis in fonio would be of the pseudogamous type. The results also revealed fonio crops as highly self-compatible species and of allopolyploid origin.

This study provides new insight on fonio genome, its origin and evolution. It is also the first attempt that comprehensively investigates the genetic diversity and reproductive system of fonio millets. The information data provided by this study is important and can be of immediate use in initiating efficient programs in fonio breeding. It can also be used for sustainable conservation management of the genetic resources of these neglected but valuable traditional cereals in West-Africa.

ZUSAMMENFASSUNG

Genetische Charakterisierung von traditionellen Fonio (Digitaria exilis, D. iburua STAPF) Landsorten aus West-Afrika: Auswirkungen für die Erhaltung und Züchtung

Fonio-Hirsen sind wichtige einheimische Cerealien, die seit Jahrhunderten in den halbtrockenen bis semihumiden Gebieten Westafrikas, in denen Wassermangel häufig auftritt, angebaut werden. Diese Getreidearten werden traditionell geschätzt und dienen entweder als Grundnahrungsmittel oder stellen wenigstens einen Hauptteil der Diät für Millionen von Angehörigen verschiedener Stämme dar. Der Name „Fonio" bezeichnet zwei Kulturarten der Gattung *Digitaria*: Weißer Fonio (*D. exilis* Stapf), die am weitesten verbreitete Kulturarte in Westafrika, und Schwarzer Fonio (*D. iburua* Stapf), der nur in einigen Gebieten angebaut wird. In Westafrika wird eine große Zahl von Fonio-Landsorten kultiviert, die an die lokalen Umweltbedingungen und traditionelle Landwirtschaftssysteme gut angepasst sind. Wegen ihrer generellen Vernachlässigung in Wissenschaft, Forschung und Entwicklung wird das Potenzial dieser Getreidearten für die Verbesserung der landwirtschaftlichen Produktion und der Ernährungssituation nicht entsprechend genutzt.

In der vorliegenden Arbeit wird über erste Schritte für eine effiziente Erhaltung und Nutzung von Fonio berichtet. Hierfür wurden u.a. molekular-genetische Methoden für eine genetische Charakterisierung des vorliegenden Materials eingesetzt. Dafür wurden zunächst wichtige lokale Fonio-Landsorten (insgesamt 160) in verschiedenen Anbaugebieten Westafrikas (Benin, Burkina Faso, Guinea, Mali und Togo) gesammelt. Zur Charakterisierung der Fonio-Landsorten wurden morpho-physiologische und genetische Untersuchungen durchgeführt. Damit verfolgt die Arbeit das Ziel, zu einer allgemeinen Erweiterung unserer Kenntnis über Fonio-Hirse beizutragen sowie ein besseres Management bei der Erhaltung und einen optimalen Einsatz moderner genetischer Werkzeuge in der Züchtung zu ermöglichen.

Zunächst wurde eine grundlegende zytogenetische Analyse der Fonio-Kollektion und einiger wilder Verwandter durchgeführt; hierfür kam die *Flow cytometry*, unterstützt durch Chromosomzahlbestimmungen zum Einsatz. Die bei den genannten *Digitaria*-Arten dokumentierten Kerngenom-Größen sind variabel. Die geschätzten, durchschnittlichen 2C-Werte für die Spezies *D. exilis* und *D. iburua* betragen 1,956 bzw. 1,848 pg. Ähnliche

Genom-Größen wurden für die nahe verwandten Wildarten *D. longiflora* (2C = 1,869 pg) und *D. ternata* (2C = 1,775 pg) dokumentiert. Im Gegensatz dazu wurden bei anderen, taxonomisch entfernteren Arten, d. h. *D. lecardii* und *D. ciliaris*, größere Genome (2C = 2,576-2.660 pg) festgestellt. Die intraspezifische Variation im DNA-Gehalt war gering und statistisch nicht signifikant, so dass von einer hohen Stabilität der Genom-Größe innerhalb der Arten gesprochen werden kann. Im Ergebnis kann festgestellt werden, dass es sich bei allen untersuchten Fonio-Landrassen um tetraploide Genotypen (2n = 4x = 36) handelt. Damit werden frühere Befunde, in denen von diploiden und hexaploiden Fonio-Landsorten berichtet wurde, relativiert.

Neben agro-morphologischen Untersuchungen wurde eine molekulargenetische Analyse mittels der AFLP-Technik (Amplified fragment length polymorphism) durchgeführt. Damit sollte das Ausmaß der genetischen Diversität in Fonio-Landsorten charakterisiert sowie die populationsgenetische Struktur und das geografische Verteilungsmuster aufgeklärt werden. Anhand der AFLP-Daten wurden Cluster-Analysen (UPGMA und PCoA) durchgeführt, die klare Unterschiede zwischen den zwei Fonio-Arten und den strukturierten *D. exilis* Genotypen offenbarten. Hier zeigen die Ergebnisse drei verschiedene Hauptgruppen, die größtenteils mit dem jeweiligen geografischen Ursprung übereinstimmen. Die ersten beiden Gruppen, die sich im Oberen Niger-Becken (ONIG) überlappen, beinhalten Landsorten aus Guinea, Mali und Burkina Faso. Die letzte, geografisch isolierte Gruppe, die der Zone des Atacora-Bergs (ATAC) zugehört, beinhaltet Sorten aus Benin und Togo. Die große Ähnlichkeit der Landrassen innerhalb jeder abgeleiteten Gruppe deutet auf einen relativ engen genetischen Hintergrund der Fonio-Arten hin. Der geschätzte Shannon-Ungleichheitsindex in *D . iburua* war äußerst niedrig (H = 0.02) und wird mit dem gegenwärtigen Residual-Status dieses Getreides erklärt. Bei *D. exilis* war die genetische Diversität (Shannon Index H = 0.267, Nei's Index H' = 0.335) eher gering und in den einzelnen Populationen verschieden. Der größte Teil dieser Diversität wurde in der ONIG-Region gefunden (H = 0.07-0.135), während in der ATAC-Zone eine sehr niedrige Diversität (H = 0.05-0.06) festgestellt wurde. Diese Befunde bestätigen die Hypothese, dass das ONIG -Becken das Hauptzentrum der genetischen Diversifikation bei Weißem Fonio darstellt. Eine AMOVA-Analyse zeigte, dass der Großteil der genetischen Variation bei *D. exilis* zwischen genetischen Gruppen (70%) bzw. Herkünften (56%). Dies deutet darauf hin, dass die wesentliche genetische Differenzierung im Laufe der Zeit während der Kultivierung und Verbreitungsgeschichte dieses Getreides erfolgt ist. Verschiedene Faktoren, wie z.B. das Fortpflanzungssystem, landwirtschaftliche Präferenzen und ökologische Anpassungen sowie „Foundation" Effekte dürften gemeinsam zu der beobachteten genetischen Struktur in dieser Art beigetragen haben.

Für die 16 untersuchten agro-morphologischen Charakteristika wurden eine große phänotypische Variation zwischen den Fonio-Herkünften festgestellt. Im Gegensatz zu den

molekulargenetischen Daten wurden jedoch weder eine klare Trennung zwischen den beiden Arten noch signifikante Gruppierungen in *D. exilis* nachgewiesen. Außerdem wurde praktisch keine Korrelation zwischen den ermittelten genetischen (AFLP) - und phänotypbasierten Distanzen ($r = 0.04$, $P>0.05$) festgestellt. Jedoch manifestierten sich die genetischen Gruppenunterschiede deutlich in der mittleren Leistungsfähigkeit; insbesondere die Merkmale Biomassetrockensubstanz, Rispen- und Korn-Ertrag erwiesen sich als besonders zwischen den Gruppen diskriminierend. Die ATAC-Gruppe ist phänotypisch besonders von den ONIG Gruppen differenziert, was die Annahme lokaler Anpassungen und der Entstehung genetischer Strukturen weiter unterstützt.

Im Weiteren wurden AFLP-Marker verwendet, um die phylogenetischen Beziehungen unter den Fonio-Kulturarten und den wilden Verwandten zu bewerten und vorgeschlagene Hypothesen bzgl. der Herkunft von Fonio zu untersuchen. Eine starke genetische Ähnlichkeit (mehr als 92%) wurde zwischen den Kulturarten *D. exilis* und *D. iburua* und den wilden tetraploiden Spezies *D. longiflora* und *D. ternata* aufgedeckt. Diese Ergebnisse, in Kombination mit zytologischen Daten, stellen zusätzliche Beweise dar, welche die gängige Hypothese einer direkten Domestizierung der Fonio-Hirse ausgehend von diesen beiden Wildarten unterstützen. Eine große genetische Abweichung wurde dagegen erwartungsgemäß zwischen den Fonio-Arten und den anderen, taxonomisch weiter entfernten *Digitaria*-Arten gefunden. Die Ergebnisse bestätigten auch *D. ciliaris* und *D. sanguinalis* als getrennte Arten, die aber eine gemeinsame Abstammung teilen.

Ferner wurden Selbstbefruchtungen- und nachfolgende Nachkommenschafts-Analysen anhand von drei Isozym-Systemen (PGI, PGM, SKDH), ergänzt durch AFLP-Marker durchgeführt. Die Ergebnisse, in Verbindung mit früheren populationsgenetischen Untersuchungen in Fonio, lassen vermuten, dass Apomixis das Hauptfortpflanzungssystem in diesen Arten darstellt: *D. iburua* wurde als obligater Apomikt identifiziert, während *D. exilis* als hochgradig apomiktisch mit residualer Sexualität (ca. 2%) gelten kann. Zusätzliche Daten bezüglich Pollenvitalität und Samenansatz deuten an, dass es sich bei der Apomixis in Fonio um den Pseudogamous-Typ handeln dürfte. Ferner zeigen die Ergebnisse auch, dass Fonio-Getreide hochgradig selbstkompatible, allopolyploide Spezies darstellen.

Die vorliegende Studie gewährt neue Einblicke zum Fonio-Genom, seinem Ursprung und seiner Evolution. Es handelt sich hier um den ersten Versuch einer umfassenden Beschreibung der genetischen Diversität und des Fortpflanzungssystems von Fonio-Hirse. Die durch diese Studie zur Verfügung gestellten Informationen und Daten sind von unmittelbarem Nutzen für die Gestaltung effizienter Programme zur Konservierung und Züchtung von Fonio. Damit kann ein wichtiger Beitrag zur nachhaltigen Verbesserung und Nutzung dieser vernachlässigten, aber wertvollen traditionellen Cerealien in und für Westafrika geleistet werden.

RESUME

Caractérisation génétique des variétés locales du fonio (Digitaria exilis*, *D. iburua* STAPF*) cultivé en Afrique de l'Ouest: implications pour la conservation et l'amélioration

Les millets fonio sont des céréales locales d'importance cultivées depuis des millénaires dans les aires semi-arides et subhumides d'Afrique de l'Ouest. Ces cultures sont traditionnellement d'une grande valeur et servent d'aliment de base pour des millions de personnes vivant dans plusieurs aires tribales. La biodiversité du fonio comprend deux espèces cultivées issues du genre *Digitaria*: le fonio blanc (*D. exilis* Stapf), le plus cultivé dans la région, et le fonio noir (*D. iburua* Stapf), cultivé en petite échelle seulement au Nigeria, Bénin et Togo. Les variétés cultivées sont nombreuses et bien adaptées aux conditions locales difficiles et aux systèmes traditionnels de culture. Malgré les perspectives encourageantes de ces cultures pour une agriculture durable, les ressources génétiques du fonio sont largement négligées et peu exploitées pour son amélioration. Un pas important vers une meilleure préservation et une exploitation efficiente des ressources génétiques du fonio passe par une caractérisation génétique détaillée des variétés locales utilisées par les paysans. Dans ce travail, une collection importante et représentative d'accessions de variétés locales (160 au total) originellement collectée dans des aires culturales différentes à travers l'Afrique de l'Ouest (Benin, Burkina Faso, Guinée, Mali, Togo) a été caractérisée et divers aspects sur la biologie et la diversité génétique du fonio ont été documentés. Ce travail se propose de contribuer à une meilleure connaissance de ces céréales traditionnelles afin de faciliter une gestion efficiente et une exploitation durable de leurs ressources génétiques en amélioration.

Dans la première étape de cette étude, une caractérisation cytogénétique de la collection du fonio avec certaines espèces sauvages apparentées utilisant la cytométrie en flux appuyée par un comptage chromosomique a été conduite. La taille du génome nucléaire documentée pour ces espèces était variable. Les valeurs 2C moyennes estimées pour *D. exilis* et *D. iburua* étaient respectivement 1.956 and 1.848 pg. Des tailles de génome approximativement similaires ont été documentées pour les espèces sauvages apparentées

D. longiflora (2C = 1.869 pg) et *D. ternata* (2C = 1.775 pg). Par contre, des tailles plus larges du génome (2C = 2.576- 2.660 pg) sont documentées pour les espèces taxonomiquement distantes analysées (*D. lecardii* et *D. ciliaris*). Les variations intra-spécifiques en quantité d'ADN sont mineures et non significatives, indiquant la stabilité du génome des espèces étudiées. Toutes les variétés analysées sont tétraploïdes ayant 2n = 4x = 36 chromosomes, contrastant les considérations préalables sur l'existence de fonio diploïde ou hexaploïde.

Une analyse AFLP (Amplified Fragment Length Polymorphism) combinée avec une évaluation agro-morphologique a été ensuite conduite pour évaluer l'étendue de la diversité génétique, sa structuration en pool génique et sa distribution géographique. Des analyses de classification basées sur UPGMA et PCoA utilisant les données de l'AFLP ont montré une nette différentiation des accessions des deux espèces et un regroupement des cultivars de *D. exilis* en trois groupes génétique majeurs largement concordant avec leurs origines géographiques. Les deux premiers groupes, sympatriques dans le bassin supérieur du Niger (BSN), incluaient les accessions de la Guinée, du Mali et du Burkina Faso. Le dernier groupe, géographiquement isolé, et couvrant les chaines de l'Atacora (ATAC), comprenaient les accessions du Bénin et du Togo. Dans chacun des groupes obtenus, les variétés locales sont très liées indiquant une base génétique très étroite des espèces de fonio. L'index de diversité de Shannon estimé pour *D. iburua* était extrêmement bas (H = 0.02) et serait dû à l'état résiduel actuel de cette espèce. La diversité génétique détectée chez *D. exilis* était modérée (index de Shannon H = 0.267, diversité génique de Nei H' = 0.335) et inégalement répartie entre les populations. Les pays du BSN présentaient les plus larges diversités (H = 0.07-0.135) alors que ceux de l'ATAC étaient les moins diversifiés (H = 0.05-0.06), confirmant BSN comme le centre majeur de diversification de *D. exilis* dans la région. L'analyse de variances moléculaires (AMOVA) a révélé que la plus grande partie de la diversité de *D. exilis* était liée aux différences entre groupes génétiques (70%) ou entre origines (56%), indiquant qu'une différentiation génétique substantielle s'est opérée dans le temps lors de la culture et la dispersion de cette espèce. Des facteurs divers comme le mode de reproduction, les sélections culturales, les adaptations écologiques et les effets de fondation ont conjointement contribué à cette structure génétique observée.

Les variations phénotypiques détectées entre les accessions du fonio pour les 16 traits agro-morphologiques étudiés étaient larges. Contrairement aux données moléculaires, aucune séparation nette entre les deux espèces n'a été observée, de même aucun regroupement significatif n'était observable au sein des accessions de *D. exilis*. Aussi, les distances génétiques basées sur l'AFLP et celles phénotypiques sont très peu corrélées (r= 0.04, P>0.05). Néanmoins, les groupes génétiques identifiés diffèrent significativement dans les valeurs moyennes de neuf traits phénotypiques au nombre desquels la biomasse sèche, les rendements en panicules et en grains sont les plus discriminants. Le groupe ATAC est

plus phénotypiquement divergent des groupes UNIG, confirmant les adaptations locales dans la structuration de la diversité génétique.

Les marqueurs AFLP sont aussi utilisés pour analyser les relations phylogénétiques entre fonio cultivé et les espèces sauvages apparentées et évaluer les diverses hypothèses sur son origine. Les résultats ont révélé une très grande affinité génétique (plus de 92% similarité) des espèces cultivées *D. exilis* et *D. iburua* avec les formes sauvages *D. longiflora* et *D. ternata*, respectivement. Ces données AFLP en combinaison avec celles cytologiques confirment les hypothèses d'une domestication directe des fonio cultivés de ces deux espèces sauvages. Comme attendu, des divergences génétiques majeures sont observables entre les fonio cultivés et les autres espèces taxonomiquement distantes étudiées. Les résultats ont par ailleurs montré *D. ciliaris* et *D. sanguinalis* comme des espèces différentes mais partageant un ancêtre commun.

Des essais d'autofécondation et des analyses subséquentes des progénitures ont été conduits en utilisant trois systèmes isozymiques (PGI, PGM, SKDH) complétés par des marqueurs AFLP. Les résultats obtenus, en conjonction avec la structure génétique décrite plus haut, révèlent que l'apomixie est le majeur mode de reproduction des fonio cultivés : *D. iburua* s'est révélé un apomict absolu alors que *D. exilis* est hautement apomictic avec 2% de sexualité résiduelle. Des données additionnelles obtenues sur la production de grains et la viabilité des pollens suggéraient que l'apomixie chez le fonio est de type pseudogamique. Les données ont aussi révélé que les fonio sont auto-compatibles et d'origine allopolyploïde.

Cette étude a apporté des informations nouvelles sur le génome du fonio, son origine et son évolution. C'est aussi la première étude qui a évalué en détail la diversité génétique des millets fonio et leur mode de reproduction. Les résultats obtenus et présentés dans cette thèse sont importants et d'utilité immédiate pour la mise en œuvre de programmes concrets d'amélioration génétique de ces cultures. Ils peuvent aussi aider à la conservation des ressources génétiques de ces cultures négligées mais d'importance capitale en Afrique de l'Ouest.

APPENDICES

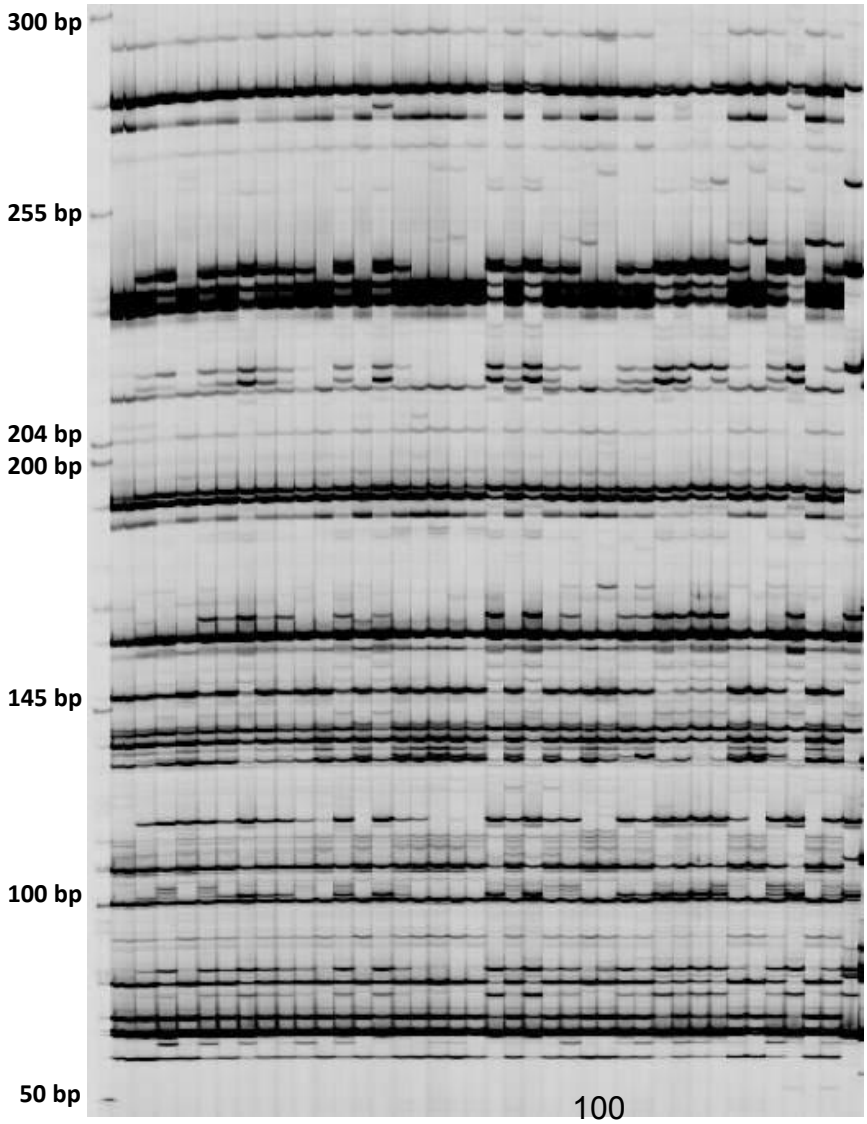
Appendix 1. Recapitulation of fonio accessions that vary between cytogenetic and genetic diversity analyses. Those accessions (113) not referenced here (cf. Table 1, Chapter II) were simultaneously analyzed in both studies.

Ac. N°	Local name	Country	Cytology	Diversity analysis
Digitaria exilis				
BEN 49	Ipoaga	Benin	**x**	
BEN 32	Iponi	Benin	**x**	
BEN 39a	Pei	Benin	**x**	
BEN 43	Poigui	Benin	**x**	
BEN 44	Poigui	Benin		**x**
BEN 103	Tamaou	Benin	**x**	
BEN 31	Tontonga	Benin		**x**
BEN 10	Tontonga	Benin		**x**
BEN 27	Tontonga	Benin		**x**
BEN 05	Ipomoan	Benin	**x**	
BEN 19	Dipodawon	Benin		**x**
BEN 28	Ipodawon	Benin		**x**
BEN 51	Ipodawon	Benin		**x**
BEN 40	Ipohaga	Benin	**x**	
BEN 09	Ipodapieh	Benin	**x**	
BEN 110	Iponouda	Benin	**x**	
BEN 38	Ipoya	Benin	**x**	
BEN 34	Tontonga	Benin	**x**	
TKK 70	Itamali	Togo	**x**	
TKK 67	Itamali	Togo		**x**
TPW 52	Oufapôh	Togo	**x**	
TKW 54	Trikpa	Togo	**x**	
TKB 71	Kiwo	Togo	**x**	
TPA 38	Ova	Togo	**x**	
-	7 other Ova	Togo		**x**
TPA 23	Vitchi	Togo	**x**	
III-3	M'balia 2	Guinea	**x**	
II-4	Kansambaran	Guinea	**x**	
II-6	Litty	Guinea	**x**	
IV-1	Siragbè	Guinea	**x**	
IV-3	Siragbé	Guinea	**x**	
I-2	Mamanden	Guinea	**x**	
II-7	Bassamba 2	Guinea	**x**	
III-7	Fayahè	Guinea	**x**	
IV-6	Hothio 2	Guinea	**x**	
IV-11	Gblingbè	Guinea	**x**	
IV-12	Mora 2	Guinea	**x**	
II-11	Koundara	Guinea	**x**	

Appendix 1. Recapitulation of fonio accessions (Cont.)

Ac. N°	Local name	Country	Cytology	Diversity analysis
Digitaria exilis				
IV-9	Prepeazo	Guinea		**x**
BUF 65	Péri Maoulè	Burkina Faso	**x**	
BUF 66	Foni Maoulè	Burkina Faso	**x**	
IV-19	CVF 107	Burkina Faso	**x**	
BUF 70	Pogwôn	Burkina Faso	**x**	
BUF 71	Peri	Burkina Faso	**x**	
BUF 67	Kiyu	Burkina Faso	**x**	
Digitaria iburua				
BEN 40b	Ipoaga	Benin	**x**	
TKD 64	Tchibam	Togo		**x**
TKD 75b	-	Togo	**x**	
TKD 89b	-	Togo	**x**	
Wild relatives	cf. Tab. 1, Chap. II; suppl. Tab. 1, Chap. IV		**x**	

Appendix 2. Partial view of AFLP profile of 39 fonio accessions using the primer-combination E-ATT/M-CTG. The first lane shows the size markers.



Appendix 3. UPGMA groups identified in West-African fonio germplasm using AFLPs

Group	Landraces
I	TKD 64/Tchibam
	TKD 63/Tchibam
	BEN 39/Péi
	BEN 36/Péi
II	III-8/Ranèho
	I-16/Mossogbé
	IV-8/Tama
	IV-10/Kansambahon
	I-1/Konso
	IV-5/Dierry
	II-10/Tobbhéré
	II-8/Niougou
	III-2/Fannali
	I-7/Kokountèrin
	I-4/Femba
	I-13/Hothio
	III-1/Siragbé
	II-02/Siriguidon
	IV-13/Pon-Biré
	I-5/Werura
	I-3/Sèèkètè
	I-8/Saara
	IV-4/Konson
	II-4/Kansambaran
	I-6/Foundelin
	I-12/Yaoukô
	IV-6/Hothio 2
	III-15/Dalaman
	III-16/Kouroussa
	III-5/Momo
	III-12/Dibon
III	BUF 56/Foni
	BUF 57/Pongwé
	BUF 69/Foni Femba
	I-11/Mora
	IV-18/Féningué
	I-10/Yéléboui
	BUF 64/Fii
	IV-17/Péri
	IV-16/Foniba
	II-9/Prépéazo 1
	IV-9/Prépéazo 2
	IV-15/Pon-Madongon
	IV-14/Oulè-Oulè
	BUF 74/Fomou

Group	Landraces
IV-A	TSO 87/Ounvoenikpa
	BEN 30/Ipodawon
	BEN 31/Tontonga
	BEN 10/Tontonga
	BEN 27/Tontonga
	BEN 28/Ipodawon
	BEN 51/Ipodawan
	BEN 11/Dipodawon
	BEN 19/Dipodawon
	TKD 75/Sémbre
	TKB 72/Fôlom
	TKB 74/Sémbre
	TKB 02/Afiohoun
	TKK 85/Sémbre
	TKD 78/Sémbre
	TKB 73/Sémbre
	TAB 91/Ipoaga
	TAB 93/Ipoaga
	BEN 09/Ipodapieh
	BEN 49/Ipohaga
	BEN 21/Ipodapieh
	BEN 35/Ipohaga
	TKK 69/Iporlapiah
	BEN 37/Ipohaga
	TKK 68/Iporlapiah
	TKD 57/Figm
	TKD 56 Figm
	TKD 79/Kiwo
	TKD 76/Kiwo
	TKD 60/Figm
	BEN 03/Tontonga
	TSO 86/Ounvoenikpa
	BEN 12/Ipohaga
	BEN 45/Ipohaga
	BEN 40/Ipohaga
	TPA 37/Ova
	TPA 28/Ova
	TPO 01/Ova
	TPW 44/Ova
	TPA 19/Ova
	TPW 32/Egniva
	TPA 10/Vitchi
	TPW 29/Vafoo
	TPW 30/Egniva

Group	Landraces
IV-B	BEN 18/Iponi
	TAB 95/Ipoeda
	BEN 13/Kpatinafa
	BEN 16/Ikounga
	BEN 15/Ypoda
	BEN 04/Tentenga
	BEN 08/Tentenga
	BEN 22/Tentepera
	BEN 01/Ikantoni
	BEN 44/Poigui
	BEN 23/Ipoda
	TKD 81/Yôlôm
	TSO88/ounfissa
	TKD 58/Djibiga
	TKD 89/Tchapionga
	TKK 67/Itamali
	TKD 61/Langfigm
	BEN 47/Cafera
	BEN 48/Afiyo
	TAB 92/Naman
	TKD 77/Kayara
	TKK 83/Ayôrô
	TKD 62/Tchabigô
	TKB 04/Ipibim
	TKK 66/Kopordagou
	TKD 59/Namba
	TPA 26/Trikpa
	TPW 41/Dikaba
	TPW 42/Ougniva
	TPA 25/Ova
	TPA 13/Ova
	TPA 27/Ezio
	TPW 50/Gnimimbi

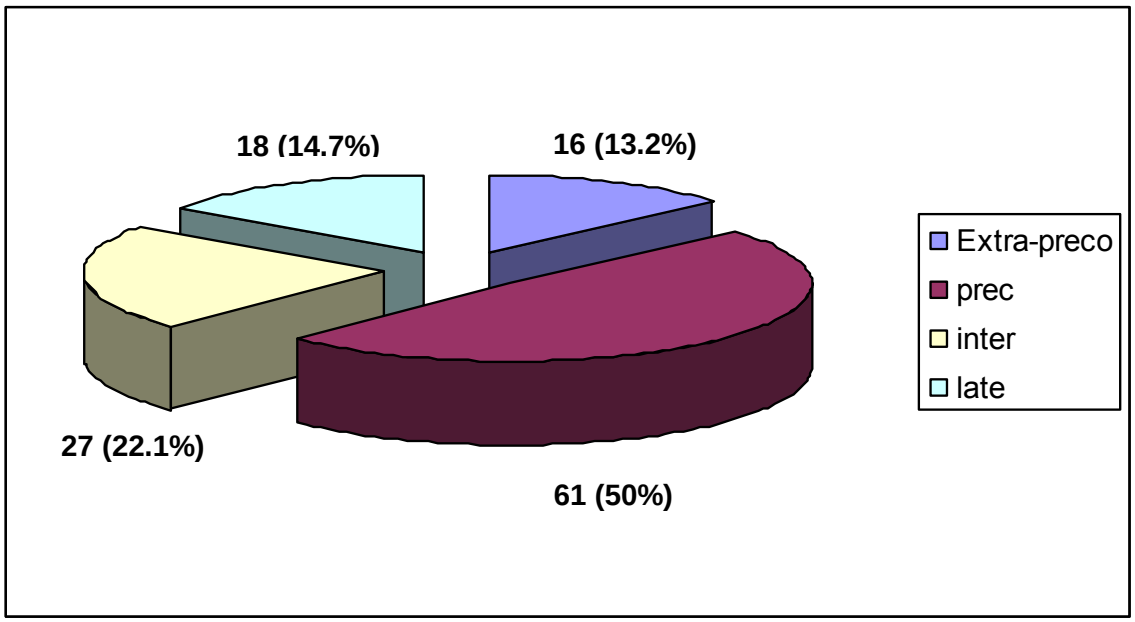
NB: *D. iburua* (**I**)**;** *D. exilis* (**II, III** and **IV-A**&**B**)

Appendix 4. Description of agro-morphological traits used during the phenotypic evaluation

Code	Trait	Description *
DPE	Days to heading	Number of days from seedling until 50% of panicles heading
DPM	Days to maturity	Number of days from seedling until physiological maturity of the majority of the panicles
HPT	Plant height (cm)	From soil level to the highest point of the longest tiller
NIN	Number of internodes	Counted on the longest tiller
LEL	Leaf length (cm)	Performed on the leaf under the flag leaf
LFS	Flag leaf sheath (cm)	Distance from the last node to the beginning of the flag limb
NTP	Number of tillers	Productive tillers counted at maximum heading time
PEX	Panicle exertion (cm)	Distance between the insertion of flag leaf and the beginning of the racems on the panicle
PAL	Panicle length (cm)	Measured from the base of the panicle to the tip of the longest raceme
FBM	Fresh biomass (g/plant)	Weight of fresh plant above soil level taken just after harvesting the panicles
DBM	Dry biomass (g/plant)	Measured after complete deshydratation (one week hard sunshine or in the oven) of the fresh biomass
PAY	Panicles yield (kg/ha)	Harvested panicles weight in a plot, converted to kg/ha
GRY	Grains yield (kg/ha)	Grains weight for all harvested panicles per plot, converted to kg/ha
HID	Harvest index	Calculated as HID = (GRY/DBM) x 100
MNC	Mean number of grains per cm of raceme	Ratio between number of grains on the longest raceme and panicle length (PAL)
GRM	1000-grains mass (mg)	Measurement using three samples taken from bulk-harvested grains

** All measurements were averaged across 3 plants records per replicate*

Appendix 5. Maturity cycle in the fonio collection



Legend. *Extra-preco: extra-precocious landraces (60-70 days); Prec: precocious landraces (80-90 days); Inter : intermediate landraces (100-110 days); Late: late maturing landraces (120-125 days).*

Appendix 6. Eigenvalues and proportion of the variance for the five first Principal Components (PCs) defined by the sixteen agro-morphologic traits analyzed for the 122 genotypes.

	Principal Components				
	1	**2**	3	4	5
DPE	-0.33	0.13	-0.04	-0.21	0.28
DPM	-0.35	0.09	0.024	-0.18	0.28
HPT	-0.22	0.29	0.143	-0.09	-0.48
NIN	-0.19	0.24	0.25	0.07	-0.55
NTP	-0.07	-0.16	-0.49	-0.27	-0.15
LEL	0.04	0.28	-0.17	-0.41	0.18
LFS	0.08	0.49	-0.31	0.08	-0.00
FBM	-0.16	0.05	-0.43	0.49	0.02
DBM	-0.32	-0.12	-0.25	0.25	0.03
PEX	0.03	-0.23	-0.27	-0.27	-0.32
PAL	0.13	0.43	-0.27	-0.04	-0.03
PAY	-0.36	-0.08	-0.12	0.19	0.045
GRY	-0.38	0.00	-0.05	-0.03	0.061
HID	-0.28	0.14	0.165	-0.36	0.12
MNC	-0.30	0.12	0.19	0.20	-0.03
GRM	-0.13	-0.20	-0.26	-0.22	-0.33
Eigenvalue	***6.1***	***2.1***	***1.9***	***1.4***	***1.1***
% of Variance	***36.1***	***12.4***	***11.3***	***8.3***	***6.4***
Cum. % of Variance	***36.1***	***48.5***	***59.8***	***68.1***	***74.5***

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Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

DECLARATION

I hereby declare that the thesis entitled "***Genetic characterization of traditional fonio millets (Digitaria exilis, D. iburua STAPF) landraces from West-Africa: implications for conservation and breeding***" is my original work, except otherwise acknowledged in the text. I have not submitted this thesis or part of it for credit towards a degree to any other institution.

Giessen, July 2010

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(Hubert Adoukonou A. Sagbadja)