REGULAR ARTICLE

Comparative analysis of genetic diversity of 8 millet genera revealed by ISSR markers

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ABSTRACT

The genetic diversity among different millet genera is not very well known; therefore, an investigation was undertaken with Inter Simple Sequence Repeats (ISSR) markers to ascertain the possibility of using these DNA markers to reveal the genetic relationships among different millet genera/species. Twelve out of 30 screened ISSR primers amplified successfully, and a total of 258 bands were scored for all 69 millet accessions, with a high level of polymorphism being detected. Nei's genetic distance among all accessions varied from 0.0241 to 0.3786, and from 0.0163 to 0.1476 at the genera level, respectively. The Shannon's index was estimated as 0.9689. The Neighbour joining (NJ) tree created, using the UnWeighted Neighbor-Joining method and Dice's dissimilarity coefficient, grouped all 69 millet accessions into eight clusters, indicating that the majority of accessions of a given genus tend to group together. The ISSR markers revealed the close relatedness between the *Eragrostis* and *Panicum* genera, as well as between *Eragrostis* and *Pennisetum*; while the greatest distance was found between the *Coix* and *Setaria* genera. Such a determination of relatedness is useful for a better understanding of the difficult relationships among the different millet genera, which are generally considered to be a complex group.

Keywords: Genetic distance; Genetic diversity; ISSR; Millet species; Polymorphism

INTRODUCTION

Millets represent a diverse group of cereal crops, comprising about a dozen crop species. They belong to different genera, which originated in Africa and Asia, were then subsequently domesticated, and are still cultivated there (McKevith, 2004; Baltensperger and Cai, 2004; FAO, 1995). Millets are small-grain cereals from the grass family (*Poaceae*) (Baltensperger and Cai, 2004). The millet group is split into two tribes. The tribe *Paniceae* comprises a number of different species such as *Pennisetum glaucum* (L.) R. Br., *Setaria italica* (L.) P. Beauv., *Panicum miliaceum* L., *Coix lacryma-jobi* L., *Eragrostis tef* (Zuccagni) Trotter, *Echinochloa crus-galli* (L.) P. Beauv., *Digitaria exilis* (Kippist) Stapf (Belton and Taylor, 2003). Finger millet (*Eleusine coracana* Gaertn.) is the only species of millet belonging to the second tribe, *Chlorideae* (Desai, 2004). A distinctive attribute of the millets are their adaptability to adverse agroecological conditions, minimal input requirements,

and good nutritional properties. Millets represent a unique biodiversity component in agriculture, and play a significant role in food security for the developing countries in Asia and Africa. They also play a growing role in the processing, and new alternative products for the developed world (Obilana and Manyasa, 2002). From the nutritional point of view, millets are equivalent (or even superior to) other cereals (Lasztity, 1996; Obilana and Manyasa, 2002); moreover, they do not contain gluten-forming proteins, making them important in a celiac diet (Amadou et al., 2013; Taylor et al., 2006). Compared to other cereals, millets are mainly suited to less fertile soils and poorer growing conditions, such as intense heat and low rainfall, where other cereal crops may likely fail (National Research Council, 1996; Winch, 2006). Beyond these indisputable qualities, many millet species have an important cultural significance, and play an irreplaceable role in social events and celebrations of the local people. Millets represent crucial plant genetic resources for the

agricultural and food security of poor farmers inhabiting arid, infertile, and marginal lands (Garí, 2002).

Traditionally, the genetic resources of the millet species were evaluated by: descriptions of their morphological characteristics (Andrews and Kumar, 2006; Brink and Belay, 2006; Kaume, 2006; Jansen and Ong, 1996; Jansen, 2006), by health-impact traits (Kalinová and Moudrý, 2006), and by molecular data. Current studies, based on DNA fingerprinting in millet species, have mainly been carried out for the identification of unknown accessions or genotypes within a single millet species (Supriya et al., 2011; Le Thierry d'Ennequin et al., 2000; Hu et al., 2009; Arya et al., 2013; Qin et al., 2005; Zeid et al., 2012, Nozawa et al., 2006; Adoukonou-Sagbadja et al., 2010).

Knowledge about the genetic diversity, and revelations of the genetic relationships among millet species is essential for the suitable conservation and increased use of millet genetic resources, and it also plays an important role in millet breeding. The application of methods using DNA analysis is pivotal for the description of genetic variability within different millet species. One of the alternatives is the use of Inter Simple Sequence Repeats (ISSR) markers, which is known to be a highly variable, reproducible, and cost effective method (Wolfe and Liston, 1998; Yang et al., 1996). Comparing ISSR markers with Random Amplified Polymorphic DNA (RAPD) analysis, there are many advantages on the side of ISSR, which can reveal a greater level of genetic variability. Using longer primers and higher annealing temperatures, they provide results that are more reliable and reproducible (Wolfe and Liston, 1998).

ISSR has been widely used in studies of the genetic structure of plants (Li and Jin, 2008), genetic diversity (Sheeja et al., 2009), genetic relationships (Li et al., 2009), phylogeny and evolution (Zamani et al., 2011). They have also been successfully applied to a number of monocotyledonous plants (Ben El Maati et al., 2004; Mondini et al., 2014; Bahieldin et al., 2012; Virk et al., 2000). Findings of genetic similarities in millet species have been performed using ISSR markers in *Pennisetum, Setaria, Eleusine* and *Eragrostis* genera (Pedraza-Garcia et al., 2010; Lin et al., 2012; Salimath et al., 1995; Assefa et al., 2003). On the other hand, ISSR markers have never been applied to studies focusing on the genetic diversity of the various other species of millet genera (such as *Panicum, Echinochloa, Coix,* and *Digitaria*).

There is a deficit of information about the levels of genetic variability among different millet species, and there is also no information available about the genetic relationships among the different genera. The motivation for this study was to uncover the linkages within the group of millet species by the use of ISSR markers.

MATERIALS AND METHODS

Plant materials
A set of 69 accessions, belonging to 8 millet genera, was used (Table 1). Selected millet samples were obtained from the Czech Gene Bank of the Crop Research Institute (CRI), Prague, Czech Republic; from the Botanical Garden of Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague, Czech Republic; as well as from the United States Department of Agriculture (USDA), Iowa State University.

DNA extraction, ISSR amplification, and scoring
Young leaves were obtained from plants grown in the greenhouses at the Botanical Garden of Faculty of Tropical AgriSciences CULS Prague, Czech Republic. The fresh leaves were frozen using liquid nitrogen to be ground into a fine powder. Total genomic DNA was isolated using an Invisorb® Spin Plant Mini Kit (Stratec Molecular, Berlin, Germany). The DNA concentration was determined using a Micro-spectrophotometer, UVS-99 (ACT Gene, Piscataway, NJ, USA). A portion of the DNA was diluted to 50 ng/µl, for use in the ISSR analysis, and both the stock and diluted portions were stored at -20°C.

A set of 30 ISSR primers (University of British Columbia, Vancouver, Canada) were tested. A set of testing samples, which consisted of every millet species, was used to screen for suitable primers. Twelve reproducible ISSR primers were selected for the final analysis (Table 2).

Every 20 µl of PCR reaction mixture was composed of: 10 µl of 2x PPP Master Mix [150 mM Tris-HCl, pH 8.8 (25°C), 40 mM $(NH_4)_2SO_4$, 0.02% Tween, 20.5 mM $MgCl_2$, 400 µM dATP, 400 µM dCTP, 400 µM dGTP, 400 µM dTTP, 100 U/ml Taq-Purple DNA polymerase, monoclonal antibody anti-Taq (38 nM), stabilizers, and additives (Tob-Bio, Czech Republic)], 10 µM of respective ISSR primer (Integrated DNA Technologies, Belgium), 2 µl of DNA (50 ng/µl), 0.2 µl of BSA (Thermo Scientific, USA), and 7.3 µl PCR H_2O (Top-Bio, Czech Republic). The ISSR analysis was carried out using a QB96 Server Gradient Thermal Cycler (Quanta Biotech, UK).

The PCR was carried out with modifications of the annealing temperature to optimize the reaction for individual primers. The cycling conditions were as follows: initial denaturation step at 95°C for 4 min, followed by 45 cycles of denaturation at 94°C for 30 s, primer annealing at 45 - 58°C for 45 s (Table 2), and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. Amplified products were mixed with loading dye (Thermo Scientific, USA) and loaded onto the gel. Electrophoretic separation was performed on 2% agarose

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Table 1: Millet accessions

	Name	Donor	Code of donor	Origin
1	*Pennisetum ciliare* (L.) Link	USDA	PI 161631	South Africa
2	*Pennisetum ciliare* (L.) Link	USDA	PI 229662 02	Madagascar
3	*Pennisetum glaucum* (L.) R. Br.	USDA	PI 288801	India
4	*Pennisetum glaucum* (L.) R. Br.	USDA	PI 337492	Brazil
5	*Pennisetum glaucum* (L.) R. Br.	USDA	PI 331353 01	Uganda
6	*Pennisetum glaucum* (L.) R. Br.	USDA	PI 343842 01	Senegal
7	*Pennisetum glaucum* (L.) R. Br.	USDA	PI 527413 01	Algeria
8	*Pennisetum glaucum* (L.) R. Br.	USDA	PI 532176 01	Oman
9	*Pennisetum purpureum* Schumach.	USDA	PI 316421 02	Mexico
10	*Pennisetum sieberianum* (Schltdl.) Stapf & C. E. Hubb.	USDA	PI 532675	Mali
11	*Setaria italica* L. subs. *italica*	USDA	PI 464525	India
12	*Setaria italica* L. subs. *italica*	USDA	PI 212626	Afghanistan
13	*Setaria italica* L. subs. *italica*	USDA	PI 433391	Taiwan
14	*Setaria incrassata* (Hochst.) Hack.	USDA	PI 209210	South Africa
15	*Setaria pumila* (Poir.) Roem. & Schult.	USDA	PI 206460	Turkey
16	*Panicum bergii* Arechav.	USDA	PI 310031 01	Brazil
17	*Panicum coloratum* L.	USDA	PI 224991 01	South Africa
18	*Panicum coloratum* L.	USDA	PI 225580 01	South Africa
19	*Panicum coloratum* L.	USDA	PI 225582 01	Zambia
20	*Panicum coloratum* L. var. *coloratum*	USDA	PI 226085 01	Kenya
21	*Panicum deustum* Thunb.	USDA	PI 364955 01	South Africa
22	*Panicum dregeanum* Nees	USDA	PI 364956 01	South Africa
23	*Panicum lanipes* Mez	USDA	PI 238346 01	Zaire
24	*Panicum miliaceum* L.	CULS		not known
25	*Panicum miliaceum* L.	CULS		not known
26	*Panicum schinzii* Hack.	USDA	PI 284153 01	Cyprus
27	*Panicum sumatrense* Roth	USDA	Ames 14464	India
28	*Panicum virgatum* L.	USDA	PI 421901 01	Florida, USA
29	*Eleusine coracana* (L.) Gaertn.	USDA	PI 214059	India
30	*Eleusine floccifolia* (Forssk.) Spreng.	USDA	PI 196853	Ethiopia
31	*Eleusine indica* (L.) GAERTN.	CRI	14Z2500001	Belgium
32	*Eleusine indica* (L.) *Gaertner.*	USDA	PI 226270 01	Zimbabwe
33	*Eleusine multiflora* Hochst. ex A. Rich.	USDA	PI 226067 01	Kenya
34	*Eleusine tristachya* KUNTH.	CRI	14Z2500002	Sweden
35	*Coix lacryma-jobi* L.	USDA	PI 324509	Japan
36	*Coix lacryma-jobi* L.	USDA	PI 320865	India
37	*Eragrostis bahiensis* Schrad. ex Schult.	USDA	PI 203648	Brazil
38	*Eragrostis capensis* (Thunb.) Trin.	USDA	PI 364803	Brazil
39	*Eragrostis cilianensis* subs. *starosselskyi* (Grossh.) Tzvelev	USDA	PI 212297	Afghanistan
40	*Eragrostis curvula* (Schrad.) Nees	USDA	PI 156818	South Africa
41	*Eragrostis cylindriflora* Hochst.	USDA	PI 364817	South Africa
42	*Eragrostis lappula* Nees	USDA	PI 364260	Brazil
43	*Eragrostis lugens* Nees	USDA	PI 203862	Brazil
44	*Eragrostis obtusa* Munro ex Ficalho & Hiern	USDA	PI 344546	South Africa
45	*Eragrostis pilosa* (L.) P. Beauv.	USDA	PI 223259	Afghanistan
46	*Eragrostis plana* Nees	USDA	PI 364340	South Africa
47	*Eragrostis racemosa* (Thunb.) Steud.	USDA	PI 192959	Kenya
48	*Eragrostis rigidior* Pilg.	USDA	PI 364824	Afghanistan
49	*Eragrostis rotifer* Rendle	USDA	PI 208131	South Africa
50	*Eragrostis rotifer* Rendle	USDA	PI 364825	Afghanistan
51	*Eragrostis secundiflora* subsp. oxylepis (Torr.) S. D. Koch	USDA	PI 295692	South Africa
52	*Eragrostis superba* Peyr.	USDA	PI 442111	Japan
53	*Eragrostis tef* (Zuccagni) Trotter	USDA	PI 494388	Ethiopia
54	*Eragrostis tef* (Zuccagni) Trotter	USDA	PI 442115	Japan
55	*Echinochloa crus-galli* (L.) BEAUV.	CRI	01Z3010001	Czech Republic
56	*Echinochloa crus-galli* (L.) BEAUV.	CRI	01Z3010002	Czech Republic

(Contd)

Table 1: (Continued...)

	Name	Donor	Code of donor	Origin
57	*Echinochloa esculenta* (A. Braun) H. Scholz	USDA	PI 315699	Florida, USA
58	*Echinochloa frumentacea* LINK	CRI	01Z3000001	not known
59	*Echinochloa frumentacea* LINK	USDA	PI 463803	India
60	*Echinochloa pyramidalis* (Lam.) Hitchc. & Chase	USDA	PI 207924	South Africa
61	*Digitaria brownii* (Roem. & Schult.) Hughes	USDA	PI 238286 01	South Africa
62	*Digitaria debilis* (Desf.) Willd.	USDA	PI 299596 02	Lesotho
63	*Digitaria eriantha* Steud.	USDA	PI 299794 01	South Africa
64	*Digitaria exilis* (Kippist) Stapf	USDA	PI 349688	Nigeria
65	*Digitaria gazensis* Rendle	USDA	PI 258431 01	South Africa
66	*Digitaria leptorrhachis* (Pilg.) Stapf	USDA	PI 364517 01	South Africa
67	*Digitaria milanjiana* (Rendle) Stapf	USDA	PI 409804 01	South Africa
68	*Digitaria sanguinalis* (L.) SCOP.	CRI	01Z2900002	Czech Republic
69	*Digitaria swazilandensis* Stent	USDA	PI 238290 01	Zimbabwe

Table 2: Characterization of ISSR markers (Zietkiewicz et al., 1994) used in this study and their polymorphism

No.	Primers code (UBC)	Sequence 5' - 3'	Annealing temperature (°C)	Total number of bands amplified	Number of scorable bands per primer	No. and frequency of polymorphic bands per primer	Range of amplification (bp)
1	UBC810	$(GA)_8T$	55	262	16	16 (100%)	250-2500
2	UBC812	$(GA)_8A$	52	175	16	16 (100%)	350-2500
3	UBC824	$(TC)_8G$	55	329	25	25 (100%)	350-2500
4	UBC834	$(AG)_8YT$	48	392	22	22 (100%)	350-2000
5	UBC840	$(GA)_8YT$	52	437	24	24 (100%)	250-3000
6	UBC841	$(GA)_8YC$	48	532	24	23 (95.83%)	250-2500
7	UBC846	$(CA)_8RT$	48	361	21	21 (100%)	400-2500
8	UBC848	$(CA)_8RG$	48	442	22	22 (100%)	350-2500
9	UBC854	$(TC)_8RG$	52	425	22	22 (100%)	300-2500
10	UBC855	$(AC)_8YT$	52	434	24	24 (100%)	300-2500
11	UBC859	$(TG)_8RC$	50	326	21	21 (100%)	400-2000
12	UBC873	$(GACA)_4$	52	314	21	21 (100%)	350-2500
		Total	-	4429	258	257 (99.61%)	-
		Mean	-	-	21.5	21.42	-

gel in 1x TBE buffer. Gels were run for about 2.5 - 3 h at 4 V.cm⁻¹. Gels were stained with SYBR® Safe DNA Gel Stain (Life technologies, USA), and visualized with a UV transilluminator. The banding pattern was recorded using a CSL-MICRODOC System (CLEAVER, United Kingdom). PCR amplification of the samples with each primer was carried out in duplicate to ensure the consistency and reproducibility of the results.

Data analysis
ISSR fragments were scored for the presence (1) or absence (0) of bands in the gel profile. Only strong and clear bands were used to construct a binary matrix. The binary matrix was used to calculate a dissimilarity matrix using Dice's coefficient (Dice, 1945). Data were analyzed using DARwin5 software (Perrier and Jacquemoud-Collet, 2006), and then a final Neighbour joining (NJ) dendrogram (Saitou and Nei, 1987) was constructed by means of the UnWeighted Neighbor-Joining method. Shannon's information index (*I*, LogBase = e) was estimated by fingerprinting analysis with missing data (FAMD) software,

version 1.31 (Schlüter and Harris, 2006) for all accessions according to Hutchenson (1970) and normalised according to Ramezani (2012). The percentage of polymorphic bands and Nei's genetic distance (Nei, 1972; Nei and Takezaki, 1983) were calculated by using FAMD. The Principal Coordinates Analysis (PCoA) was performed by software DARwin 5.0 using the data obtained from the calculation of the Dice's coefficient.

RESULTS

ISSR profile and analysis
In this study, 12 ISSR (Table 2) were then used to analyze the genetic diversity of 69 millet accessions. These 12 primers A total of 258 fragments, ranging from 250 to 2500 bp, were amplified with a mean of 21.5 bands per primer, of which 257 (99.61%) were polymorphic. Absolute polymorphism (100.0%) was observed with primers UBC810, UBC812, UBC824, UBC834, UBC840, UBC846, UBC848 UBC854, UBC855, UBC859, and UBC873; while the lowest level of polymorphism (95.83%) was observed

with primer UBC841 (Table 2). The highest number of polymorphic bands was produced by UBC824 (25 polymorphic bands); while the lowest number was obtained from UBC810 and UBC812 (16 polymorphic bands).

The number of bands generated by the ISSR primers within a single genus varied from 31 to 230. The level of polymorphism within a single genus varied from 12.06% in the *Coix* genus to 89.88% in the *Eragrostis* genus, with a mean of 65.81%.

Genetic diversity within and among genera

Shannon's index among all 69 samples was estimated at 0.9689. The Nei's genetic distance matrix among all millet accessions was found to be in the range of 0.0241 to 0.3786 (mean of 0.1922). The maximum genetic distance was between *Eragrostis tef* (accession No. 53) and *Panicum sumatrense* (accession No. 27), while the lowest genetic distance was between *Eragrostis cylindriflora* (accession No. 41) and *Eragrostis racemosa* (accession No. 47).

At the genus level, the values of Nei's genetic distance indicated a high level of variation (Table 3). The greatest distance was found between the *Coix* and *Setaria* genera (0.1476), while the lowest genetic distance was between the *Eragrostis* and *Panicum* genera (0.0163).

Cluster analyses based on the ISSR genotyping profile

A dendrogram based on Neighbour joining analysis of the ISSR data was constructed in order to be able to infer the phylogenetic relationships among 69 millet accessions belonging to eight genera. The dendrogram showed that, in most cases, accessions of the same millet genus clustered together (Fig. 1).

According to the dendrogram, all 69 accessions were separated into eight clusters. Cluster I consists of 11 millet accessions belonging to four millet genera, viz. *Eragrostis, Eleusine, Panicum*, and *Digitaria*. The *Eragrostis* accessions formed a distinct branch consisting of four accessions; whereas, *Eragrostis cylindriflora* -No. 41 (South Africa origin) and *Eragrostis racemosa* -No. 47 (Kenyan origin) showed the highest similarity to all millet accessions. Three *Digitaria* accessions (Nos. 62, 67, and 68) also comprised

a distinct branch. Similar to Cluster I, Cluster II was also mostly formed by *Eragrostis* accessions. Also present were accessions of other millet genera, viz. *Eleusine*, *Pennisetum*, *Panicum*, and *Digitaria*.

Cluster III primarily contained *Pennisetum* accessions, followed by *Coix*, *Digitaria*, and *Eragrostis* accessions. *Pennisetum* accessions of this cluster grouped together; another five *Pennisetum* accessions did not fall within the same group, but fell into a different cluster (Cluster 7); meaning that the intraspecific genetic diversity among those accessions was large. Although *Coix* accessions (Nos. 36 and 35) clustered with other millet genera, these two accessions formed a distinct branch. Both accessions originated in Asia, specifically in India and Japan.
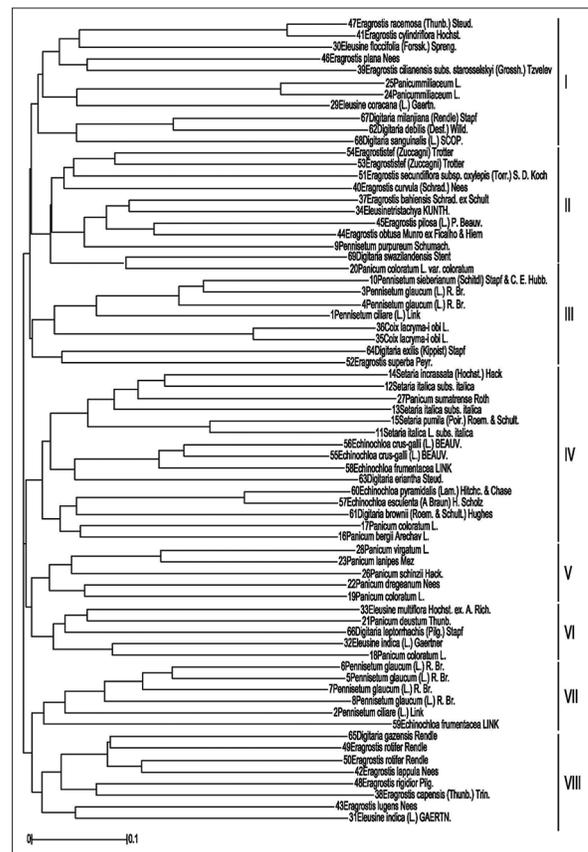

Fig 1. NJ dendrogram showing relationships among and wihtin different millet genera. Dendrogram constructed on the basis of ISSR markers.

Table 3: Genetic distance matrix among 8 millet genera

	Pennisetum	Coix	Eleusine	Eragrostis	Digitaria	Echinochloa	Panicum	Setaria
Pennisetum	0.0000							
Coix	0.1185	0.0000						
Eleusine	0.0369	0.1259	0.0000					
Eragrostis	0.0206	0.1229	0.0271	0.0000				
Digitaria	0.0305	0.1250	0.0266	0.0234	0.0000			
Echinochloa	0.0385	0.1293	0.0451	0.0403	0.0423	0.0000		
Panicum	0.0229	0.1270	0.0295	0.0163	0.0257	0.0443	0.0000	
Setaria	0.0625	0.1476	0.0685	0.0573	0.0586	0.0624	0.0648	0.0000

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In Cluster IV, *Setaria*, *Digitaria*, *Echinochloa*, and *Panicum* accessions clustered together. The *Setaria* accessions formed a distinct branch consisting of five *Setaria* accessions (Nos.11, 12, 13, 14, and 15), with one accession of *Panicum sumatrense* - No. 27. The other two accessions of *Panicum*, viz. *Panicum coloratum* - No. 17 and *Panicum bergii* - No. 16 showed quite high dissimilarities.

Cluster V was composed entirely of *Panicum* accessions, viz. *Panicum virgatum* - No. 28, *Panicum lanipes* - No. 23, *Panicum schinzii* - No. 26, *Panicum dregeanum* - No. 22, and *Panicum coloratum* - No. 19. Other *Panicum* accessions appeared scattered in the dendrogram, possibly due to the large intraspecific genetic diversity among the *Panicum* accessions.

Cluster VI showed a high degree of admixtures of millet genera; the cluster was formed by *Panicum*, *Eleusine*, and *Digitaria* accessions. The accessions *Panicum coloratum* -No. 18 and *Eleusine indica* - No. 32 were quite similar.

Cluster VII was formed almost completely by accessions of one millet genus, specifically by the *Pennisetum* genus. Four of these five *Pennisetum* accessions belong to the same species - *Pennisetum glaucum*, specifically Nos. 5, 6, 7, and 8. The fifth *Pennisetum* accession of that cluster was accession No. 2 – *Pennisetum ciliare*. From these five accessions, two accessions of *Pennisetum glaucum* were the most similar, specifically, Nos. 6 and 5.

Cluster VIII primarily included *Eragrostis* accessions and one accession of *Digitaria* and *Eleusine,* Nos. 65 and 31, respectively. *Digitaria gazensis* - No. 65 showed a high similarity with *Eragrostis rotifer* - No. 49. The most similar were *Eragrostis rotifer* - No. 50 and *Eragrostis lappula* - No. 42.

PCoA was performed to further explore the relationships among millets with ISSR data. Millet accessions were scattered on the graphic area of PCoA diagram with no evident clusters. Only *Setaria* and *Echinochloa* accessions were quite well separated from another millet accessions, which corresponds with the dendrogram.

DISCUSSION

Despite the importance of millets, the available information of both their phylogenetic relationships and genetic diversity, using molecular markers, is still rather limited. In most of the studies, which were focused on genetic diversity and the relationships of millets, only one millet species or genus (Li et al., 2012; Assefa et al., 2003; Yu et al., 2006; Salimath et al., 1995, Arya et al., 2013, Kim et al., 2014) was often involved; whereas, the current study was aimed at revealing the relationship among and within different millet genera, simultaneously.

In the present study, a Neighbour joining dendrogram offers a new perspective towards an understanding about the relationships at the inter-specific/intra-generic levels. The accessions were divided into eight clusters (Fig. 1), where a single genus for the most part grouped together, but did not form separate clusters, contrary to our expectations.

The *Eragrostis* accessions were split into three different clusters, which affirmed the fact that *Eragrostis* is a large and taxonomically complex genus (Tefera et al., 2006). The level of polymorphism among the evaluated *Eragrostis* accessions was 89.88%, which corresponds with the findings of Bai et al. (2000), who found a high level of polymorphism in wild *Eragrostis* species by using RAPD analysis. At the same time, they revealed a relatively low polymorphism in the *Eragrostis tef* accessions. ISSR analysis was also used to uncover the genetic diversity in *Eragrostis tef* (Assefa et al., 2003). They obtained much lower estimates for genetic similarity among the *Eragrostis* accessions (0.26 - 0.86). Bai et al. (2000) reported that *Eragrostis tef* is very close to *Eragrostis pilosa*, supporting the hypothesis of Ebba (1975) that *Eragrostis tef* originated from this species. Ayele and Ngyuen (2000) reported that *E. pilosa* was more closely related to *E. tef* than *E. curvula*. Our data are at variance with those findings, because Nei's genetic distance between *Eragrostis tef* (accession Nos. 53 and 54) and *Eragrostis pilosa* (accession No. 45) were observed at 0.1532 and 0.1725, respectively. Our findings also showed that *E. curvula* was more closely related to *E. tef*.

In order to enhance an understanding of the diversity and relationships in the *Pennisetum* genus, an ISSR analysis incorporated cultivated, wild, and weed *Pennisetum* species - *P. glaucum*, *P. purpureum*, *P. ciliare*, and *P. sieberianum*, respectively. Unfortunately, revealing the relationships among *Pennisetum* species is rather complicated because *Pennisetum* is a highly cross-pollinated crop, with large numbers of wild relatives, including those that can be inter-crossed (Jauhar, 1968, 1981; Jauhar and Hanna, 1998). The results of present study may also support this fact, since *Pennisetum purpureum* was highly differentiated from another *Pennisetum* accession. The *Pennisetum purpureum* accession was even present in another/different cluster, and could be detected as an admixed individual, as was similarly revealed by Oumar et al. (2008). Regarding to the genetic relationships of another *Pennisetum* accession, the clustering showed a close relatedness among the domesticated species *P. sieberianum* and *P. glaucum*. Donadío et al. (2009) obtained similar results; however, they also uncovered a close relatedness of these *Pennisetum* species to *P. purpureum*, which is in disagreement with the data obtained in current study. In all likelihood, these differences might be attributed to variations in the type and number

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of genotypes, as well as to the techniques employed. Additionally, it should be noted that the grouping of *P. glaucum* and *P. ciliare* (syn. *Cenchrus ciliaris*) accessions strongly supports the finding that *Pennisetum* and *Cenchrus* are closely related genera (Clayton and Renvoize, 1986; Crins, 1991). According to Clayton and Renvoize (1986), *Cenchrus ciliaris* is even considered to be on the boundary between *Cenchrus* and *Pennisetum*, and findings of present study support this fact. Although *P. ciliare* accessions were present in the *Pennisetum* clusters, they were rather distant from other *Pennisetum* accessions. Regarding the clustering of all *Pennisetum* accessions, the clustering reflects both the complicated taxonomy of the *Pennisetum* genus and of clustering according to geographical origin, which is evident in one of the *Pennisetum* clusters.

Surprisingly, the *Coix lacryma jobi* accessions occurred in the *Pennisetum* cluster, as well (Cluster III). The reason for the clustering of these two *Coix* accessions with the *Pennisetum* accessions might have been caused by cross-pollination, which is predominant in *Coix* sp. (Jansen, 2006). Similarly, the *Digitaria exilis* accession was present in the same cluster, which supports the view that *Pennisetum* and *Digitaria* are considered being a distantly related (Hacker 1995). Additionally, results of the current study demonstrate the complexity of the *Digitaria* species. These accessions are scattered throughout the entire dendrogram, which reflects considerable variability in the *Digitaria* genus. Hayward and Hacker (1980) attributed this large specific diversity within the *Digitaria* genus to its great antiquity, but also to its significant rate of speciation. Findings of present study showed high genetic divergences between the cultivated *Digitaria exilis* and the other taxonomically distant *Digitaria* species (Table 1), which is in concordance with the results obtained by Adoukonou-Sagbadja et al. (2010). Among the wild species investigated, *D. eriantha* and *D. sanguinalis,* there were observed as the most distant, genetically, from the cultivated *Digitaria exilis*. Nevertheless, it should be noted that different hypotheses exist on the reproductive system of *Digitaria* species, ranging from inbreeding (Watson and Dallwitz, 1992; Sarker et al., 1993) to out-crossing (Hilu et al., 1997).

Although the *Setaria* genus is also a complex genus containing crop, wild, and weedy species, with different breeding systems at the life cycle and ploidy levels (Wanous, 1990), all *Setaria* accessions investigated in the present study grouped together (Fig 1). Thus, our findings do not support the results of the phylogenetic studies performed by Doust et al. (2007) or Kellogg et al. (2009), which indicated that the *Setaria* genus is a collection of unrelated groups. Although *Setaria* accessions formed a distinct branch in the present study, no clear geographic structure within the genus was found, contrary to the findings of Li et al. (2012), in which a clear geographic structure was revealed by using ISSR markers. In general, the geographic center from which *Setaria* originated is still controversial. Single and multiple centers of origin for *Setaria* have been suggested in Eurasia. A center in northern China was first suggested by Vavilov (1926), and confirmed by many archaeologists and archaeobotanists (Smith, 1998; Lu, 1999; Shelach, 2000); now with some genetic studies having confirmed the existence of this center (Hirano et al., 2011; Li et al., 2012). Nevertheless, the multiple domestication theory is widely accepted (Kawase and Sakamoto 1987; Li et al., 1998; Benabdelmouna et al., 2001; Kawase et al., 2005; Fukunaga et al., 2005, 2006). Despite the limited number of accessions used in the present study, the clustering of the *Setaria* accessions, and relatively high level of similarity, might suggest a hypothesis of a single center of domestication. Unfortunately, the origin of *Setaria* still has remained unresolved, and further detailed analysis using large numbers of accessions is required.

The *Echinochloa* genus is also a taxonomically complicated genus, because clear-cut boundaries between species seldom exist, and the species are very variable. Introgression between species is also common (Brink and Belay, 2006). Last but not least, its great diversity is also caused by its easy adaptation to a wide range of aquatic and ruderal habitats, combined with self-pollination (Partohardjono and Jansen, 1996). These genetic and morphological differences in the *Echinochloa* species lead to taxonomic problems. Thus, many studies have attempted to understand the population genetic structures of some *Echinochloa* species, and have revealed their genetic diversity by using molecular markers (Asins et al., 1999; Roy et al., 2000; Rutledge et al., 2000; Tasrif et al., 2004; Altop and Mennan, 2011; Nozawa et al., 2006; Danquah et al., 2002). As with previous studies, the *Echinochloa* species in the present study were differentiated, and might confirm the theory of Yabuno (1966) and Scholz (1992), that the weedy *Echinochloa crus-galli* has its cultivated counterpart *Echinochloa esculenta*. Altop and Mennan (2011) mentioned that the variability among *Echinochloa* accessions from various locations might be due to its adaptability to the geographic locations, as well as differences in weed management practices.

ISSR analysis revealed a high level of polymorphism in the *Panicum* genus (89.49%). In the present study, the scattering of *Panicum* accessions throughout the NJ dendrogram (Fig. 1) and PCoA diagram (Fig. 2) clearly confirmed the fact that the *Panicum* genus is extremely variable (Hacker, 1995). The most variable *Panicum* species in the current study is *Panicum coloratum*, which is a polymorphic species native to tropical Africa. Furthermore, attributes such as predominant cross-pollination or the development of ecotypes adapted to a wide range of soils (Hacker 1995) might be responsible
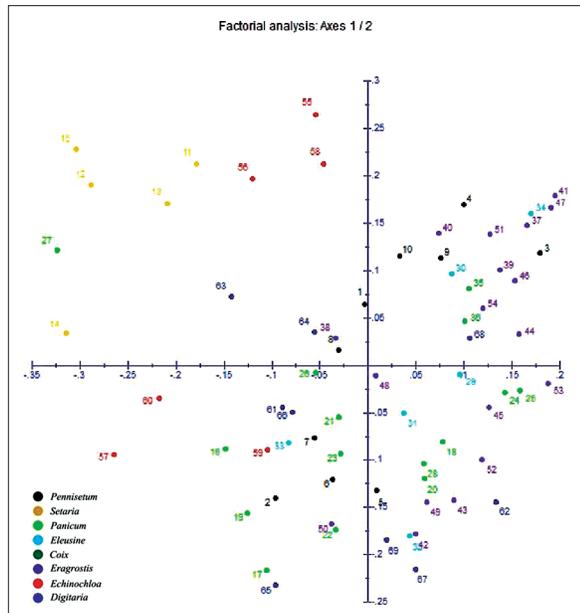
Fig 2. Prinicpal Coordinates Analysis for ISSR data.

for the segregation of *P. coloratum* accessions from other *Panicum* accessions. Also, the *Panicum sumatrense* accession was separated from other *Panicum* accessions, which reflected a high variability (van der Hoek and Jansen, 1996). Further, the clustering of *P. sumatrense* with *Setaria* accessions is quite in agreement with data obtained by Lakshmi et al. (2002), where *P. sumatrense* and *Setaria italica* accessions were present in the same cluster. In addition, Aliscioni et al. (2003) mentioned that the genus shows a wide range of variation, and relationships within the *Panicum* genus are not completely clear, which was also evident in our study. Also, M'Ribu and Hilu (1994) studied the variation among *Panicum* species, and their findings revealed the differentiation of individual millet species, which is in accordance with the data obtained in this study. Although most *Panicum* accessions employed in the present analysis originated in Africa, there were no clear separations of the accessions according to their geographic origins, contrary to the data obtained by Hu et al. (2008) and Hunt et al. (2011).

In contrast to *Panicum*, the *Eleusine* genus belongs to a relatively small genus (Phillips, 1972; Hilu and de Wet, 1976), but the classification of the genus has been notoriously difficult, not only at the intra-generic level, where considerable disagreements on species delimitation and their relationships have persisted (Bisht and Mukai, 2001, 2002; Lye, 1999; Phillips, 1972, 1995). This is also true at the supra-generic level, where its closest allies are disputed (Clayton and Renvoize, 1986). Therefore, some studies have used a comparative analysis of DNA markers in order to get greater knowledge of the interrelatedness of the *Eleusine* species (Gupta et al., 2010; Hiremath and Salimath, 1992; Salimath et al., 1995). Hiremath and Salimath (1992) used

molecular markers to uncover the genetic affinities between *Eleusine coracana* and three diploid species (viz. *Eleusine indica*, *Eleusine floccifolia*, and *Eleusine tristachya*), which are believed to form a close genetic assemblage within the genus. These results are inconsistent with those obtained in the present study. The *Eleusine* species were clearly separated and scattered throughout the dendrogram, which might indicate an implemented sorting of the *Eleusine* species. Hence, these findings could be of value towards a better characterization of the genus. Another interesting finding is that the ISSR technique used in the present study revealed a high level of polymorphism (74%), which is suggestive of the ISSR technique being a most promising tool for uncovering of plant diversity. These findings are in agreement with a comparative study performed by Salimath et al. (1995).

Regarding the level of polymorphism at the genera level, the findings revealed in the present study a broad range of polymorphism. These unique findings confirmed that millets are more or less related. The determination of relatedness might help to resolve the difficult interrelationships among the different millet genera. Despite the fact that *Eragrostis* and *Panicum* are complex and variable genera (Tefera and Belay, 2006; Hacker, 1995), the data demonstrated that these genera are the most similar. Further, a close relatedness was also observed between the *Eragrostis* and *Pennisetum* genera (Table 3). The genus *Pennisetum* is also considered to be distantly related to *Digitaria* (Hacker 1995), which relatively corresponds with our findings. Furthermore, according to our data, the *Digitaria* genus is close to the *Eleusine* genus. On the other hand, the *Eleusine* genus is most distant from the *Setaria* genus. There has been a hypothesis that *Setaria* had evolved from *Panicum* (Brink and Belay, 2006). Nevertheless, the results of this study could not unambiguously confirm this hypothesis due to the relatively high genetic distance. However, the most distant genus from other millet genera implemented in the present study was *Coix*; with knowledge about this species still being limited.

CONCLUSIONS

To the best of our knowledge, this is the first report where different millet genera were compared simultaneously using ISSR markers. These markers confirmed the presence of a high level of genetic variability among and within the different millet genera. Further, the ISSR cluster analysis, revealed that the majority of accessions of a given genera tend to group together. On the other hand, it must be noted that in some cases the genus boundaries were not very rigid, and accessions of a given genera were scattered throughout the dendrogram. This clustering is probably due to the variation in the types and number of

genotypes, species admixtures, different origins of the accessions, different propagation systems, and their ability to adapt to different geographic conditions. Additionally, the results of the present study clearly confirmed the concept that millets are a complex group. Although the present investigation shed some light on a better understanding of the genetic diversity in millets, further studies are required to improve our understanding of the phylogenetic relationships as well as the genetic diversity in millets at the genera/species level.

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Author contributions

Z. D. performed all the experimental procedures and statistical calculations, carried out drafting and writing the manuscript, and prepared the figures. E. S. helped with experimental procedures and data analysis. P. H. Č. made special data analysis, and helped in drafting the manuscript. D. J., I. V., E. F. C. and L. M. conceived the project, provided guidance in the study design. All authors were involved in revising the manuscript in progress, and critically reading and approving the final manuscript.

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