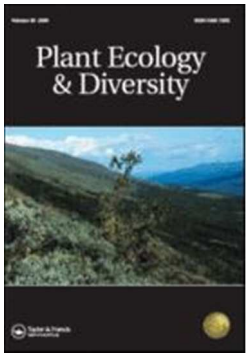

Genetic profiling of the critically endangered palm tree species *Medemia argun* using newly developed chloroplast DNA markers

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Genetic profiling of the critically endangered palm tree species *Medemia argun* using newly developed chloroplast DNA markers

Background: *Medemia argun* is a rare wild palm tree species. Its global existence is assumed to include the main population of about one thousand trees in the Nubian Desert of Sudan and some individually scattered trees in southern Egypt. The species was previously proposed to be extinct, but then reported being alive about twenty years ago.

Aims: seven chloroplast DNA markers were developed for use in the genetic characterization of this threatened species. An additional set of 42 markers were designed and their characteristics are now provided.

Methods: genome sequence mining approach was applied to identify microsatellites in the chloroplast genome of *Bismarckia nobilis*, a near relative of *M. argun*. Seven markers were validated in *M. argun* originating from Sudan and in its wild relatives.

Results: five markers were found polymorphic in *M. argun*, which enabled the genetic diversity assessment. Significant genetic differentiation was observed among generations ($P<0.001$) and collection sites ($P<0.05$). The seven developed markers were polymorphic among the wild relatives *Hyphaene thebaica* and *Borassus aethiopum*.

Conclusions: this is the first study that reports molecular markers for *M. argun*. We consider this work as a starting point towards revealing genetic variation and structure in the critically endangered *M. argun*.

Keywords: Nubian Desert of Sudan; rare wild palms; endangered species; microsatellite markers; population genetics; conservation

Introduction

The palm *Medemia argun* belongs to the subtribe Hyphaeninae of the tribe Borasseae (Arecaceae) and it is the only species in the genus *Medemia* (Govaerts and Dransfield 2005). It is native to Sudan (Broun and Massey 1929; Andrews 1956) and adapted to a very dry and hot environment. *M. argun* mainly occurs in the Nubian Desert of Sudan, but it was also reported in the White Nile region as *M. abiadensis*

(Wendl.; Broun and Massey 1929; Andrews 1956), which was later considered being the same *M. argun* species (Govaerts and Dransfield 2005).

M. argun was first reported in Sudan in 1837 and in Egypt 1963 (reviewed by Ibrahim and Baker 2009), later on proposed extinct, until rediscovered in northern Sudan in 1995 (Gibbons and Spanner 1996). The tree is not domesticated and it is in the Red List of Threatened Species of the International Union for Conservation of Nature (IUCN 2015). The most recent reports documented the presence of hundreds of trees in northern Sudan (Gibbons and Spanner 1999) and 31 scattered individuals in southern Egypt (Ibrahim and Baker 2009), which represent the marginal range of the distribution of *M. argun*. Yet, the detailed knowledge of the distribution of its natural populations in Sudan remains unrevealed. The natural habitats of the species in the heart of the Nubian Desert are exceptionally remote and isolated by mountains, and, thus, very difficult to reach. Increased aridity and other environmental changes are assumed to cause further serious threats to the existence of *M. argun* (Blach-Overgaard et al. 2015).

Better knowledge of the distribution, adaptation, population dynamics, genetic traits and speciation of *M. argun* would allow appropriate conservation measures and would also contribute to the current research on the evolution of palms. Concurrently, the utilization potential of *M. argun* palms can be investigated. Although *M. argun* is mainly known for its very strong fibrous leaves, its relatives in Sudan are wild fruit tree species, widely utilized in rural areas and thought as potential components for the food security and sustainability of smallholder agricultural systems (Gebauer 2005; Eltahir et al. 2010; Salih et al. 2014).

As a result of a field expedition, in silico DNA marker development and laboratory analyses, this study provides the first scientific report on the status of *M. argun* palms in the Nubian Desert in Sudan and it introduces novel choloroplast markers for genetic profiling and diversity analyses in *M. argun* and its close relatives.

Materials and methods

Materials and site features

In November 2014, we reached populations of *M. argun* in Sudan near the location previously described by Gibbons and Spanner (1996). Within a distance of around seven kilometers, at least three generations were observable among tens of *M. argun* trees and seedlings scattered along the valley. It was the only palm tree species we found in this valley, surrounded by ranges of mountains. We saw few scattered shrubs of other plant species, gazelles and traces of other animals; all typical for the nature of the Nubian Desert. However, in some locations the creeping sand covered other plant species and only *M. argun* palms seemed to survive there (Fig. 1). The main features of the sampled populations and habitat are shown in (Fig. 2). Leaf samples of 51 *M. argun* individuals representing two sites located about 7 km from each other and three age classes were collected for DNA analyses.

In Silico primers design and development

We utilized chloroplast genome sequence data of *Bismarckia nobilis* (subtribe Hyphaeninae) as a source for the development of *cpSSR* markers *in silico*. *B. nobilis* is the closest species to *M. argun* with available genome sequence data. The complete

sequence of the *Bismarckia nobilis* chloroplast genome (158210 bp) was downloaded from GenBank (JX088664.1) and repeat motifs were identified (mainly mononucleotide repeats and T/A nucleotides in different combinations). The Primer3 software (Rozen and Skaletsky 2000) was employed to design primer pairs with the following specifications: amplicon size range of 80 –350 bp, primer length of 18–28 bp, GC content of 40–60% and annealing temperature of 55–65°C. A total of 49 primer pairs were designed (Table S1). Seven primer pairs were selected for laboratory analyses (Table 1).

DNA extraction and genotyping

Total genomic DNA was extracted from dry leaves of collected samples using the E.Z.N.A.™SP Plant Mini Kit (Omega Bio-Tek, Norcross, Georgia, USA). PCR conditions were optimized and amplification was first confirmed for one DNA sample of *B. nobilis* obtained from the Royal Botanic Gardens, Kew, DNA Bank (RBGDBD 2015), and then tested in *M. argun*, the wild relative *Hyphaene thebaica* as well as in the more distant wild relative *Borassus aethiopum* (20 samples each obtained from Sudan). The PCR amplification reactions were performed in a final volume of 20 µL containing 2 µL DNA (6–10 ng), 0.4 µl of 10 mM dNTP mixture, 0.6 µL of DyNAzyme II DNA polymerase (2U/µL), 2 µL optimized DyNAzyme buffer (Finnzymes) and 3 µM of each primer (forward primers fluorescently labelled). PCR amplifications were conducted using a thermocycler (PTC-200; MJ Research, Watertown, USA) with the following setting: denaturation for 2 min at 94°C followed by 28 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 52°C, elongation for 1 min at 72°C, and a final elongation step of 8 min at 72°C. The presence of amplification products was confirmed using electrophoresis with 1.4% agarose gels (SeaKem LE Agarose; Lonza, Rockland,

Maine, USA) stained with ethidium bromide, and fragment sizes were compared with the expected range of allele sizes. Genotyping was carried out using a ABI 3130xl DNA Sequencer, employing the GeneScan 500 ROX Size Standard at the DNA Sequencing and Genomics Laboratory; Institute of Biotechnology, University of Helsinki. The DNA fragment analysis was performed with Peak Scanner version 1 software (Applied Biosystems).

Data analysis

Genetic variation parameters, including allelic polymorphism, unbiased haploid genetic diversity as well as analysis of molecular variance (AMOVA; Excoffier et al. 1992), were calculated with GenAlEx version 6.503 (Peakall and Smouse 2006, 2012) for haploid microsatellites. Genetic differentiation of *M. argun* was examined among two collection sites and three age classes of trees.

Results

The results showed that five out of seven markers were polymorphic in *M. argun*, two loci were polymorphic in *Hyphaene thebaica* but none in *Borassus aethiopum* (Table 1). The only genotyped sample of *B. nobilis* showed different amplicon lengths at six loci when compared to the available chloroplast genome of *B. nobilis* (GenBank: JX088664.1;Table 1). However, all seven markers showed different allele sizes among the four tested species (Table 1).

The numbers of allelic variants at the polymorphic loci of *M. argun* ranged from two to three (Table 2). The unbiased haploid genetic diversity per locus varied from 0.000 on

site 2 to 0.660 on site 1; and from 0.000 among the medium and small trees to 0.600 among the old trees (Table 2). In general, the unbiased haploid genetic diversity was reduced in descendant generations from old to young trees (Table 2). The AMOVA results indicated significant genetic differentiations among age classes ($p<0.001$) and among sites ($p<0.05$); 40% of variation was present among age classes and 12% of variation was present among trees from different sites (Table 3).

Discussion

The applicability of chloroplast DNA markers for investigations on genetic diversity and structure, and phylogeography is well documented (e.g. Ennos et al. 1999; Provan et al. 2001; Bai et al. 2014). The extinction risk of *M. argun* is based on the documented size of the existing population and the harsh nature of its habitat. The genetic diversity and structure revealed in this study may reflect the presence of very small fragmented populations scattered in the habitat. The cpSSR markers developed and tested in this study are valuable for examining genetic variation in *M. argun* palms and for producing additional biological information that can be used to assess its conservation status and to support the conservation of this critically endangered plant.

Although new information on the occurrence and biology of *M. argun* was revealed as a result of our expedition and analyses, further field research and experiments are required to properly facilitate its conservation and utilization. The polymorphism of the developed markers among the four related species confirms the transferability of the developed cpSSR markers among palm species and validates their wider use in genetic analyses on palms.

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Note: this webpage been used to order the sample of *Bismarckia nobilis* DNA.

Figure legends

Figure 1. Tens of *M. argun* palms scattered along a valley.

Figure 2. Examples of *M. argun* palms: a, b, c. trees of different ages; d, e. seedlings; f. fruiting tree.

Table 1. Characteristics of seven cpSSR loci examined in 51 samples of *Medemia argun*, 20 samples of *Hyphaene thebaica,* 20 samples of *Borassus aethiobium* and in one *Bismarckia nobilis* sample.

Locus name	Primer sequence (5′ – 3′)	Repeat motif	Amplicon position	Amplicon length (bp)	Alleles BN[a]	Alleles MA[b]	Alleles HT[c]	Alleles BA[d]
Mede1	F: AGCCTTCCAAGCTAACGATG R: ACAATGGACGCTTTTCGTTC	$[TA]_2T[TA]_3[TA]_7T$	trnG-GCC/trnR-UCU	234	236	259/243	267	245
Mede2	F: GTGAACCGCAATCAAATCAA R: TGGGTGGTTTACGTTATGGAA	$[TA]_3ATA[AT]_7TA$	atpH/atpI	157	135	288/228	208	137
Mede3	F: GGGGAAGAAGTGGACTCTAGG R: AGTCCCGAGATCCATCGAA	$[A]_{16}$	petN/psbM	211	211	233/236	222/220	209
Mede4	F: GTTGTGTTGGTAGCCGGAAT R: TCGATTCTTCCTTCAACTTCG	$[AT]_9$	trnL-UAA	202	194	200	205	186
Mede5	F: GCAGGATGTCTCATCCGTAGA R: GTCTCCGTCGTTCCTCCATA	$[AT]_8$	petA/psbJ	127	137	149	129	122
Mede6	F: AGCTCCTCGCGAATGAAAC R: CACGTCCAGCTGTATATTTTG	$[T/A]_{94}$	rpl16	280	274	481/505/307	307	331
Mede7	F: TGTTTCCGATTCACCAATTC R: TCGTTGGATGTGAAAGACAT	$[A/T]_{23}G[A/T]_{100}$	ndhF/trnL-UAG	331	447	562/266	341/355	565

[a] *Bismarckia nobilis*: (subtribe: Hyphaeninae)
[b] *Medemia argun* (subtribe: Hyphaeninae)
[c] *Hyphaene thebaica*: (subtribe: Hyphaeninae)
[d] *Borassus aethiopum* (Subtribe: Lataniinae)

Table 2. Numbers of alleles and unbiased haploid genetic diversities at seven chloroplast microsatellite loci developed for *Medemia argun*. Trees of *M. argun* grouped according to the site of collection and age classes.

	Trees grouped by collection site				Trees grouped by age class					
	Site 1 (n=19)		Site 2 (n=32)		Small trees (n=19)		Medium Trees (n=26)		Old trees (n=6)	
Locus	A	uh	A	uh	A	uh	A	uh	A	uh
Mede1	2	0.105	1	0.000	1	0.000	1	0.000	2	0.333
Mede2	2	0.491	2	0.129	1	0.000	2	0.391	2	0.600
Mede3	2	0.526	2	0.490	2	0.199	2	0.453	2	0.600
Mede4	1	0.000	1	0.000	1	0.000	1	0.000	1	0.000
Mede5	1	0.000	1	0.000	1	0.000	1	0.000	1	0.000
Mede6	3	0.660	2	0.083	1	0.000	3	0.667	2	0.600
Mede7	2	0.167	2	0.468	2	0.515	1	0.000	2	0.500
Mean		0.390		0.234		0.143		0.302		0.527

A= number of alleles; uh = unbiased haploid genetics diversity; n= number of individuals

Table 3. Analyses of molecular variance (AMOVA) for 51 samples of *M. argun* based on five polymorphic chloroplast microsatellite loci, including three age classes and two collection sites.

Source of variation	Degree of freedom	Sum of Squares	Mean Squares	Estimate of variance	Amount of variation	Stat	Value	Probability
Among age classes	2	340317.368	170158.684	10318.201	40%			
Within age classes	48	748223.181	15587.983	15587.983	60%	PhiPT	0.398	0.001
Total	50	1088540.549		25906.184	100%			
Among collection sites	1	81530.220	81530.220	2589.163	12%			
Within collection sites	49	970025.858	19796.446	19796.446	88%	PhiPT	0.116	0.028
Total	50	1051556.078		22385.609	100%			

Probability values based on 999 permutations

Tens of *M. argun* palms scattered along a valley.

205x153mm (300 x 300 DPI)

Examples of *M. argun palms*: a, b, c. trees of different ages; d, e. seedlings; f. fruiting tree.

209x147mm (300 x 300 DPI)

Table S1. Features of 49 cpSSR markers designed as potential markers for *Medemia argun.* Markers designed by utilizing the complete chloroplast genome sequence of *Bismarckia nobilis* (GenBank: JX088664.1).

Locus name	Primer sequence (5′ – 3)	Amplicon location	Amplicon position	Type of amplicon region	Repeat motif	Amplicon length (bp)
Mede1	F: AGCCTTCCAAGCTAACGATG R: ACAATGGACGCTTTTCGTTC	10009-10035	trnG-GCC/trnR-UCU	intergenic partly intergenic	$[TA]_2T[TA]_3[TA]_7T$	234
Mede2	F: GTGAACCGCAATCAAATCAA R: TGGGTGGTTTACGTTATGGAA	14613-14637	atpH/atpI	intergenic intergenic	$[TA]_3ATA[AT]_7TA$	157
Mede3	F: GGGGAAGAAGTGGACTCTAGG R: AGTCCCGAGATCCATCGAA	29868-29883	petN/psbM	gene intergenic	$[A]_{16}$	211
Mede4	F: GTTGTGTTGGTAGCCGGAAT R: TCGATTCTTCCTTCAACTTCG	48366-48383	trnL-UAA	intron intron	$[AT]_9$	202
Mede5	F: GCAGGATGTCTCATCCGTAGA R: GTCTCCGTCGTTCCTCCATA	65570-65585	petA/psbJ	intergenic intergenic	$[AT]_8$	127
Mede6	F: AGCTCCTCGCGAATGAAAC R: CACGTCCAGCTGTATATTTTG	84102-84195	rpl16	intron intron	$[T/A]_{94}$	280
Mede7	F: TGTTTCCGATTCACCAATTC R: TCGTTGGATGTGAAAGACAT	115918-116041	ndhF/trnL-UAG	intergenic intergenic	$[A/T]_{23}G[A/T]_{100}$	331
Mede08	F:TCTCCAATCCATAATAAAGA R:AGATAGATTAGTGCCTGATG	3753-3765	trnK-UUU	intron intron	$[A]_{13}$	115
Mede09	F:GCTCGTTGCTTTTTATTTCACC R:TCCTTACCGGATCGTTACAAA	3955-3964	trnK-UUU/	intron intron	$[T]_{10}$	131
Mede10	F:CGCAACGAACAAAACAAGAA R:TCCTTGAAAAAGGTGCTCAA	5029 – 5041	trnK-UUU/ rps16	intergenic gene	$[C]_{13}$	238
Mede 11	F:GCATATCCTGAATGTGATCCTG R:GTGTCAAATGTGGGTGGAAA	7131-7141	Rps/ trnQ-UUG	Intergenic intergenic	$[A]_{11}$	98
Mede 12	F:GTGGTAAAAGTGTGATTCGTTC R:GCCTTTGGATACAAATCGTGA	9315-9324	trnG-GCC	intron intron	$[T]_{10}$	170
Mede 13	F:TTCCCGAACCAAACATGAAT R:CAGAAAAGAGAGGGTAGGCTCA	12519-12529	atpF	intron intron	$[T]_{11}$	122
Mede 14	F:AGGACTCTTCCGACCAGACA R:ATGCCGAATCGACGACCTAC	12784-12794	atpF	intron intron	$[T]_{11}$	212
Mede 15	F:GGACCGAACCGAAACAAAAT R:CAGCGGCATTTTGATACCAC	18852-18861	rpoC2	gene gene	$[T]_{10}$	127
Mede 16	F:AACCCTACCCTACAAAGAGC	23085-23096	rpoC1	intron	$[T]_{12}$	163

	R:CATCATTTGAGGGGAAGTAG			intron		
Mede 17	F:TAGGCCTGTCGATACTTGATTT	28690-28697	trnC-GCA/ petN	Intergenic	$[A]_8$	84
	R:CCAGAACCCAGATTTTGAGAA			intergenic		
Mede 18	F:AGCAGAGGCAAACGACTAGG	28705-28713	trnC-GCA/ petN	intergenic	$[T]_9$	127
	R:CCTATTGGTACCGGTTTGGA			intergenic		
Mede 19	F:CAAATCGATTCATCGTCGAG	30799 -30808	psbM/ trnD-GUC	Intergenic	$[T]_4$ A $[T]_4$ A	80
	R:TCCCATTTCGGATTTAGTATGG			intergenic		
Mede 20	F:TCTCACTCAAAAAGAGTGTT	49326-49340	trnF-GAA/ ndhJ	intergenic	$[A]_{15}$	105
	R:AAAGTATAGAGGGGTTGAAG			intergenic		
Mede 21	F:TGTGGGGCATGTCTTGTTTA	51710-51719	ndhC/ trnV-UAC	intergenic	$[T]_{10}$	138
	R:AGAAACCGAGACAAAGCGATA			intergenic		
Mede 22	F:ATGGGGTAAAATATCTAGGG	52254-52264	ndhC/ trnV-UAC	Intergenic	$[T]_{11}$	148
	R:ATAATCGTTCGTTCGGTGCT			intergenic		
Mede 23	F:CTTTCATACGGCGGGAGTC	54375-54384	trnM-CAU/ atpE	gene	$[T]_{10}$	153
	R:CTGCCAATTGAACACAATCA			intergenic		
Mede 24	F:GGAAACCACAGGACTAGAAGGA	56420-56427	atpB/ rbcL	gene	$[T]_8$	183
	R:TGGGTGGTACCAACTAAATCG			intergenic		
Mede 25	F:GTGGAAACCACAGGACTAGA	56493-56500	atpB/ rbcL	gene	$[A]_8$	185
	R:TGGGTGGTACCAACTAAATC			intergenic		
Mede 26	F:CATGACATGAGAGAAACCTGTC	59227-59239	rbcL/ accD	intergenic	$[A]_{13}$	236
	R:TTCTTGCCCCCTATTTGATG			intergenic		
Mede 27	F:ATTGAAGTGATACTTTGGAC	59227-59239	rbcL/ accD	Intergenic	$[A]_{13}$	126
	R:TTAAGCATATGAATCCAATC			intergenic		
Mede 28	F:CATGACATGAGAGAAACCTG	59213-59226	rbcL/ accD	Intergenic	$[C]_{14}$	267
	R:AGTCTTCTTGCCCCCTATT			intergenic		
Mede 29	F:TCTCGGATCTAGAAGGAAAGGA	62941-62951	ycf4/ cemA	intergenic	$[A]_{11}$	188
	R:TCCCGGTATTCCACCAATTA			gene		
Mede 30	F:TTATGGCCAATTAACCAACC	67513-67522	psbE/ petL	intergenic	$[A]_{10}$	84
	R:CGAACGACCTGATATTACCTTT			intergenic		
Mede 31	F:TCAGGAAGAAGGGGTCATCT	69051-69064	trnP-UGG/ psaJ	Intergenic	$[A]_{14}$	189
	R:TCGCATTGAAAATCCTCCTT			partly gene		
Mede 32	F:TAGGAATTCCGCGTAAAGT	69050 -69063	trnP-UGG/ psaJ	intergenic	$[A]_{14}$	99
	R:AAATCCTCCTTCTTTATTGTA			intergenic		
Mede 33	F:GAAGGGGTCATCTTTTTCTT	69050 -69063	trnP-UGG/ psaJ	intergenic	$[A]_{14}$	187
	R:GCATTGAAAATCCTCCTTCT			partly gene		
Mede 34	F:TAGGAATTCCGCGTAAAGT	69050 -69063	trnP-UGG/ psaJ	intergenic	$[A]_{14}$	171
	R:TGCTAAAGACCCAAACCATA			gene		
Mede 35	F:CAAGGAACAGGAAGAGGAAGAA	70164-70177	rpl33/ rps18	intergenic	$[TA]_7$	196
	R:GCTTTGATTCGCATCGTTTA			intergenic		
Mede 36	F:GCAATACCAAAGTTCCTTTCTG	72799-72809	rps12/ clpP	intron	$[T]_{11}$	156

	R:GTGTCGGGGGTACATTTCAG			intron		
Mede 37	F:GAACTCGAAGTGCCATGCTA	73133-73144	clpP	intron	$[T]_{11}$	153
	R:TTTCATTCTGGTCGGAGGAG			gene		
Mede 38	F:TGAAGGGGGTTTTCTTCTTCTA	73600-73609	clpP	intron	$[A]_{10}$	167
	R:GGCCCATTCAGGAACAATAA			intron		
Mede 39	F:CAACCCAAACTGCATCTTCC	74293-74302	clpP/ psbB	gene	$[A]_{10}$	129
	R:AACCCATTGTTACGTTTCCA			intergenic		
Mede 40	F:CTACGGATCAGGCGACATTT	82094-82104	rpl36/ infA	gene	$[T]_{11}$	162
	R:ACATTCCTTTCATGGGGACA			intergenic		
Mede 41	F:CCGAATCTACTCTTTTGAAG	82094-82104	rpl36/ infA	intergenic	$[T]_{11}$	153
	R:GATTCAACCAAAGGACGTAT			gene		
Mede 42	F:CGATCCACCCATATAGTGAC	85113-85128	rpl16	intron	$[T]_{16}$	172
	R:TGCTTAGTGTGTGACTCGTT			intron		
Mede 43	F:GAACATGCTGTACGAAATGA	113498-113507	ycf1	gene	$[A]_{10}$	164
	R:TACAGGTCTCCATGGGATAA			gene		
Mede 44	F:TGACCTTACTAAATGGTCCAG	121897-121910	ndhG/ ndhI	intergenic	$[T]_{14}$	152
	R:CAATCCCCATTACTCTTTTC			intergenic		
Mede 45	F:TTGTTCCTGTTCTTCTGTCTC	123760-123769	ndhA	intron	$[A]_{10}$	159
	R:GGGCTTTAAGTTGGTAGAAA			intron		
Mede 46	F:CTTGTCCCTGCTGTTCGTTT	127832-127841	ycf1	gene	$[T]_{10}$	190
	R:TTTGTGCCACTTTTTGATGC			gene		
Mede 47	F:TCAATTCCGGTTCTCTCTCG	130175-130184	ycf1	gene	$[A]_{10}$	172
	R:TCCATGCGTACTCAAAGACG			gene		
Mede 48	F:TCTCCATGGGATAATTTCGTG	131222-131231	ycf1	gene	$[T]_{10}$	164
	R:CGGTTTGAACATGCTGTACG			gene		
Mede 49	F:TCGAGTCAATCTCCTCAGTT	84735–84752	rpl16	intron	$[T]_6$	141
	R:AAGGCAGTGTGATAAAGCAT			intron		