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Mitochondrial genome sequencing and phylogenetic analysis of *Cynodon dactylon* × *Cynodon transvaalensis*

Shilian HUANG[1], Yancai SHI[2], Miao CHEN[1,3,*]

[1]College of Life Sciences, South China Agricultural University, Guangzhou, China
[2]Guangxi Key Laboratory of Plant Conservation and Restoration Ecology in Karst Terrain, Guangxi Institute of Botany, Guangxi Zhuang Autonomous Region and Chinese Academy of Sciences, Guilin, China
[3]Faculty of Agricultural Science, Guangdong Ocean University, Zhanjiang, China

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Abstract: *Cynodon dactylon* × *Cynodon transvaalensis* is one of the most important turfgrasses. Sequencing the *C. dactylon* × *C. transvaalensis* mitochondrial genome can help us learn more about its genomic composition and allow further study of the population genetics, taxonomy, and evolutionary biology of Poaceae plants and other related species. Here the *C. dactylon* × *C. transvaalensis* mitochondrial genome was sequenced using Illumina HiSeq combined with PacBio sequencing technology, and the map of the mitochondrial genome was constructed after de novo assembly and annotation. The *C. dactylon* × *C. transvaalensis* mitochondrial genome has 366,612 bp and contains 53 genes, including 31 protein-coding genes, 3 rRNA genes, and 16 tRNA genes. The result of relative synonymous codon usage (RSCU) showed an A or U preference at the third position of the codons. Thirty-three chloroplast DNA fragments were found in *C. dactylon* × *C. transvaalensis* mitochondrial DNA, ranging from 72 to 3003 bp. Phylogenetic trees built based on the chloroplast genome were congruent with the plant taxonomy and NCBI taxonomy common tree, while the phylogenetic trees built based on 9 mitochondrial genes showed some differences from the common tree.

Key words: *Cynodon dactylon* × *Cynodon transvaalensis*, mitochondrial genome, phylogenetics, repeat sequence, gene transfer

1. Introduction

Mitochondria are genetically semiautonomous organelles that can encode some of the genes associated with their own functions. The mitochondrial genomes of plants have the characteristics of polymorphism, heterogeneity, complexity, and variability. Angiosperms have the largest and most complex mitochondrial genomes. Although the size of mitochondrial genomes ranges from approximately 220 kb (*Brassica napus*) (Handa, 2003) to 11.3 Mb (*Silene conica*) (Sloan et al., 2012), the number of basic functional genes has little variation, and the complexity of the genome is relatively conserved (Kubo and Newton, 2008). With the exception of maize S-type cytoplasmic sterile line (Allen et al., 2007) and japonica rice (Notsu et al., 2002), which have linear molecular structures, the mitochondrial genomes of other plants that have been sequenced all have annular structures.

The size differences of plant mitochondrial genomes are mainly caused by repetitive sequences. However, there is no positive correlation between the enlargement of mitochondrial genomes and increased gene number (Palmer et al., 2000). Species with larger mitochondrial genomes do not necessarily contain more genes (Palmer et al., 2000). Plant mitochondrial genomes mainly encode respiratory metabolism and oxidative phosphorylation-related genes, such as cytochrome complex I–V subunit genes, ribosomal protein genes, cytochrome C synthesis-related genes, rRNAs, tRNAs, and a large number of unknown open reading frames (Millar et al., 2011). Plant mitochondrial genomes contain a large number of repetitive sequences in different sizes and are highly diverse in configuration. In the same species, size difference in the mitochondrial genome is mainly caused by repeat sequences, especially the noncoding sequences between the gene intervals (Adams et al., 2000).

Horizontal gene transfer (HGT) is a process by which receptor cells acquire genetic materials from donors; HGT is the driving force of eukaryotic genome evolution (Bermthorsson et al., 2003; Xiong et al., 2008). In the mitochondrial genomes of higher plants, gene fragments derived from chloroplasts are ubiquitous and occupy a high proportion of the mitochondrial genomes. For

* Correspondence: czchenmiao@126.com

example, there are 17 chloroplast-derived DNA fragments in the rice mitochondrial genome, accounting for 6.3% of the mitochondrial genome (Notsu et al., 2002). However, there are no chloroplast-derived sequences in the mitochondrial genome of *Marchantia polymorpha* L. This suggests that the transfer of chloroplast gene sequences to mitochondria is likely to be endemic to flowering plants (Oda et al., 1992).

Cynodon dactylon × *Cynodon transvaalensis* is a hybrid whose paternal parent is *Cynodon dactylon* and maternal parent is *Cynodon transvaalensis*. Due to its strong vegetative growth and its abilities to tolerate trampling, heat, and drought stresses, as well as its good texture and fast vegetative establishment, it is widely used in sports fields, lawns, parks, and golf courses (Huang et al., 2018). Therefore, it has the potential for adversity gene mining. However, little genetic information is known for triploid bermudagrass. We have de novo assembled and annotated the complete mitochondrial genome of *Cynodon dactylon* × *Cynodon transvaalensis*. We have discussed the content, structure, and organization of the mitochondrial genome. We have also analyzed the chloroplast-derived genes/fragments in the mitochondrial genome. We then explored phylogenetic relationships among various plant mitochondrial genomes. Sequencing of the mitochondrial genome of *Cynodon dactylon* × *C. transvaalensis* will allow further study of the population genetics, taxonomy, and evolution biology of Poaceae plants and related species.

2. Materials and methods

2.1. DNA extraction, mitochondrial genome sequence, and assembly

The method of mitochondrial DNA extraction is the same as mentioned by Richardson et al. (2013). Sequencing of the mitochondrial DNA was carried out by using Illumina HiSeq (Illumina, Inc, San Diego, CA, USA) combined with PacBio sequencing technology (Pacific Biosciences, Menlo Park CA, USA). The Illumina sequencing data were preliminarily assembled by SOAPdenovo (v2.04) (Luo et al., 2012); the scaffolds were then compared to the sequencing data of PacBio to correct the single molecule sequencing data. Canu v1.5 (Koren et al., 2017) software was used for subsequent assembly. For the detailed methods, we referred to Koren et al. (2012).

2.2. Mitochondrial genome annotation and analysis

The protein-coding genes (PCGs) were identified by using ORF Finder from the NCBI website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The tRNA and rRNA genes were identified by the online server tRNAscan-SE 2.0 (http://lowelab.ucsc.edu/tRNAscan-SE/) and RNAmmer 1.2 Server (http://www.cbs.dtu.dk/services/RNAmmer/), respectively (Lowe and Chan, 2016; Lagesen et al., 2007). The SSR sequences were identified by MISA (http://pgrc.ipk-gatersleben.de/misa/) using the default parameters. The analysis of codon numbers and relative synonymous codon usage (RSCU) of protein-coding genes and predicted ORFs was computed with GCUA (General Codon Usage Analysis) (McInernev, 1998).

2.3. Phylogenetic analysis

The blast alignment of the mitochondria with the chloroplast genome was performed using Circoletto (http://tools.bat.infspire.org/circoletto/) (Nikos, 2010) with e values ranging from –10 to –20. The alignment of 26 mitochondrial genomes and 24 chloroplast genomes was performed using HomBlocks (Bi et al., 2017), and the sequence was trimmed using the Gblocks method. ModelFinder (Kalyaanamoorthy et al., 2017) was used for model selection. Bayesian analysis (BI) was performed on MrBayes 3.2.5 (Ronquist et al., 2012) with 2 cold chains and 2 hot chains. The Markov Chain Monte Carlo (MCMC) chain was set to 10,000,000 generations, sampling once every 1000 steps, with a relative burn-in of 25%. The convergence of independent runs was evaluated by the average standard deviation of the splitting frequency (<0.01). The maximum likelihood (ML) analysis was performed on IQ-TREE version 1.6.6 (Nguyen et al., 2015) under the substitution model GTR + F + R3. The node support values were estimated with 1000 replicates of ultrafast likelihood bootstrap and SH-aLRT.

3. Results and discussion

3.1. Mitochondrial genome organization and nucleotide composition

The mitochondrial genome of *Cynodon dactylon* × *C. transvaalensis* is a closed circular molecule with 366,612 bp (Figure 1) and a GC content of 43.6% (Table 1). Compared to other Poaceae plants, the mitochondrial genome of *Cynodon dactylon* × *C. transvaalensis* has a similar GC content but a smaller genome size (Table 2). The base content of A, C, G, and T is 28.2%, 21.8%, 21.8%, and 28.2%, respectively. Three long repeats (>500 bp), 216 short repeats (>20 bp, <500 bp), and 28 SSRs account for 9.12% (33,430 bp), 2.30% (8422 bp), and 0.075% (274 bp) of the total mitochondrial genome size, respectively (Tables 1 and 3).

Fifty-three genes were identified in the mitochondrial genome of *Cynodon dactylon* × *C. transvaalensis*, including 31 out of all 41 protein-coding genes in the mitochondrial genome of ancestral flowering plants (Mower et al., 2012), 3 rRNA genes, 6 complete native mitochondrial tRNA genes, and 10 chloroplast-derived tRNA genes (Table 1). To our knowledge, among all sequenced angiosperm mitochondrial genomes, only *Amborella trichopoda* Baill. (Rice et al., 2013) and *Liriodendron tulipifera* L. (Richardson et al., 2013) possess 41 complete protein-coding genes. Loss and metastasis of a large number of mitochondrial

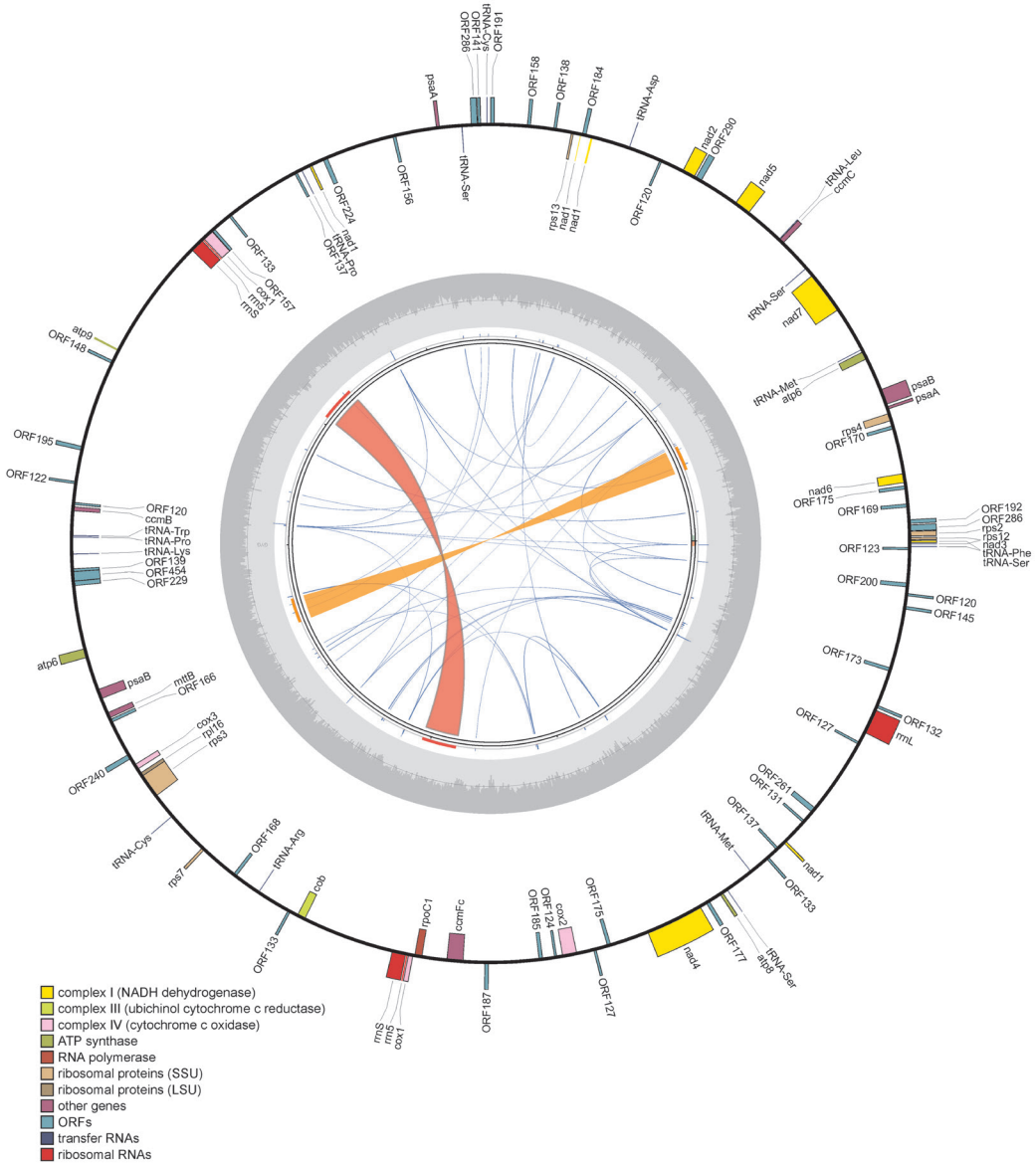

Figure 1. Map of the *Cynodon dactylon* × *C. transvaalensis* mitochondrial genome. Intra-ring genes represent a clockwise direction of transcription, while the outer-ring genes are the opposite. Different functional genes are identified in different colors. The built-in gray histogram shows the genomic GC content, with the middle gray line being a 50% threshold line.

genes in other mitochondrial genomes, especially the loss and transfer of ribosomal protein-coding genes and succinate dehydrogenase (*sdh*) genes, lead to changes in gene contents in angiosperms (Adams et al., 2002). All of the *sdh* genes were lost in the mitochondrial genome of *Cynodon dactylon* × *C. transvaalensis*.

Repetitive genes are prevalent in vascular plants (Goremykin et al., 2009); e.g., *Nelumbo nucifera* Gaertn. and maize (CMS-C) contain 6 and 10 duplicated protein genes, respectively (Allen et al., 2007; Gui et al., 2016). In the mitochondrial genome of *Cynodon dactylon* × *C. transvaalensis*, *psa*B (a chloroplast encoding gene) and 2 rRNA genes (*rrn18* and *rrn5*) have 2 copies. Large repeats are very active and frequently recombined, which may lead to recombination between genes, causing mutations in gene sequences and transcripts (Carlson and Kemble, 1985). In addition, 46 unknown functional open reading frames (ORFs) were also predicted in this study, accounting for 6.65% (24, 369 bp) of the total length (Table 1). Numerous studies have shown that these ORF are not completely dysfunctional (Siqueira et al., 2001). Some conserved ORFs between species may be similar to functional genes lost in some mitochondrial genomes (such as *atp4*, *atp8*, *sdh3*, or *sdh4*) and exert important

Table 1. Features of *Cynodon dactylon* × *C. transvaalensis* mitochondrial genome.

Category	Feature	Number	bp (%)
Genome (366,612 bp)	G + C		159,843 (43.60)
Genes (total)			
	protein coding[1]	32	29,815 (8.13)
	rRNA[2]	5	7681 (2.10)
	mt-derived tRNA	6	453 (0.12)
	cp-derived tRNA	10	784 (0.21)
	ORF	46	24,369 (6.65)
Introns			
	cis-spliced	11	17,781 (4.85)
	trans-spliced	3	n.a
Repeats			
	Repeats (>500)	3	33,430 (9.12)
	Short Repeats (>20,<500)	216	8422 (2.30)
	SSRs	28	274 (0.075)

Table 2. Comparison of GC contents and length in 16 *Gramineae* plant mitochondrial genomes.

Species	GC (%)	Length (bp)	Accession
Aegilops speltoides	44.43	476,091	NC_022666.1
Bambusa oldhamii	43.88	509,941	EU365401
Cynodon dactylon × *C. transvaalensis*	43.60	366,612	MK175054
Hordeum vulgare subsp. *vulgare*	44.23	525,599	AP017301
Oryza rufipogon	44.04	559,045	NC_013816.1
Oryza sativa Japonica group	43.85	490,520	NC_011033.1
Oryza sativa Indica group	43.84	491,515	NC_007886.1
Phoenix dactylifera	45.14	715,001	NC_016740
Sorghum bicolor	43.73	468,628	NC_008360.1
Triticum aestivum	44.35	452,528	NC_007579.1
Tripsacum dactyloides	43.93	704,100	NC_008362.1
Triticum timopheevii	44.35	443,419	NC_022714.1
Zea luxurians	43.93	539,368	NC_008333.1
Zea mays subsp. *mays*	43.93	569,630	NC_007982.1
Zea mays subsp. *parviglumis*	43.88	680,603	NC_008332.1
Zea perennis	43.88	570,354	NC_008331.1

functions during respiratory metabolism (Heazlewood et al., 2003). For example, the presence of a conserved ORF, as identified in the mitochondrial genome of some angiosperms, is functionally similar to the *rpl10* gene (Kubo and Arimura, 2010).

The preference of codon usage is a common phenomenon existing in nature and is mainly determined by the dynamic equilibrium related to gene mutation and natural selection (Bulmer, 1991; Wong et al., 2002). Natural selection often makes organisms prefer to use optimal codons, and mutation can lead to the existence of some nonoptimal codons. Different genes of different species or the same species have different codon preference through long-term evolution (Murray et al., 1989). The result of

Table 3. SSR locus of the *Cynodon dactylon* × *C. transvaalensis* mitochondrial genome.

SSR number	SSR type	SSR	Size	Start	End
1	p1	(A)10	10	29,833	29,842
2	p1	(A)12	12	59,820	59,831
3	p1	(T)10	10	74,461	74,470
4	p1	(A)10	10	83,800	83,809
5	p1	(A)10	10	95,078	95,087
6	p1	(A)12	12	98,637	98,648
7	p2	(TA)6	12	119,497	119,508
8	p1	(G)11	11	125,100	125,110
9	p1	(A)10	10	132,870	132,879
10	p1	(T)10	10	155,779	155,788
11	p1	(A)10	10	175,442	175,451
12	p1	(A)10	10	181,774	181,783
13	p1	(T)10	10	208,990	208,999
14	p1	(T)14	14	214,085	214,098
15	p1	(A)11	11	220,370	220,380
16	p1	(A)10	10	224,504	224,513
17	p1	(A)11	11	224,795	224,805
18	p1	(A)11	11	267,174	267,184
19	p1	(C)10	10	269,881	269,890
20	p3	(AGT)5	15	273,280	273,294
21	p1	(T)10	10	277,210	277,219
22	p1	(T)10	10	284,452	284,461
23	p1	(T)10	10	289,308	289,317
24	p1	(T)10	10	306,533	306,542
25	p1	(A)11	11	322,144	322,154
26	p1	(T)12	12	325,004	325,015
27	p1	(A)10	10	328,461	328,470
28	p1	(C)10	10	361,658	361,667

Table 4. Codon usage in protein-coding genes of the *Cynodon dactylon* × *C. transvaalensis* mitochondrial genome.

AA	Codon	N	RSCU	AA	Codon	N	RSCU
Phe	UUU	835	1.17	Ser	UCU	476	1.33
	UUC	589	0.83		UCC	339	0.95
Leu	UUA	490	1.14		UCA	377	1.05
	UUG	511	1.19		UCG	276	0.77
Tyr	UAU	489	1.31	Cys	UGU	253	1.03
	UAC	259	0.69		UGC	236	0.97
ter	UAA	134	0	ter	UGA	149	0
ter	UAG	128	0	Trp	UGG	430	1
Leu	CUU	577	1.34	Pro	CCU	437	1.26
	CUC	354	0.82		CCC	323	0.93
	CUA	359	0.83		CCA	351	1.01
	CUG	294	0.68		CCG	276	0.8
His	CAU	471	1.29	Arg	CGU	262	0.89
	CAC	258	0.71		CGC	190	0.65
Gln	CAA	455	1.2		CGA	301	1.02
	CAG	301	0.8		CGG	256	0.87
Ile	AUU	635	1.26	Thr	ACU	372	1.25
	AUC	431	0.86		ACC	308	1.03
	AUA	445	0.88		ACA	286	0.96
Met	AUG	473	1		ACG	227	0.76
Asn	AAU	529	1.27	Ser	AGU	357	1
	AAC	307	0.73		AGC	326	0.91
Lys	AAA	560	1.09	Arg	AGA	424	1.44
	AAG	469	0.91		AGG	330	1.12
Val	GUU	400	1.25	Ala	GCU	501	1.45
	GUC	240	0.75		GCC	311	0.9
	GUA	328	1.03		GCA	349	1.01
	GUG	310	0.97		GCG	223	0.64
Asp	GAU	524	1.32	Gly	GGU	426	1.04
	GAC	269	0.68		GGC	266	0.65
Glu	GAA	616	1.18		GGA	516	1.26
	GAG	430	0.82		GGG	435	1.06

relative synonymous codon usage (RSCU) showed that the frequency of A or U at the third position of *Cynodon dactylon* × *C. transvaalensis* mitochondrial genome codons is higher than that of G or C and reflects a high A/T content in the third position of each codon (Table 4). Furthermore, the codons AGA, CUU, GAU, GCU, UAU, and UCU showed a higher usage frequency, as the numerical values of RSCU are greater than 1.3.

3.2. Chloroplast DNA insertions in *Cynodon dactylon* × *C. transvaalensis* mitochondrial DNA

Plant mitochondrial genomes typically contain DNA from plasmid and nuclear genomes and, in some cases, from other species including bacteria, viruses, and plants (Timmis et al., 2004; Goremykin et al., 2009; Alverson et al., 2010; Rodríguez-Moreno et al., 2011; Rice et al., 2013). The plastid-like sequences were first identified by Nakazono and Hirai (1993). The plastid-derived sequences are variable in the mitochondrial genomes of seed plants, accounting for 1%–12% (Mower et al., 2012). In this study, 33 chloroplast DNA fragments were identified in the *Cynodon dactylon* × *C. transvaalensis* mitochondrial genome, ranging from 72 to 3003 bp. The total length of

chloroplast DNA fragments is 18,091 bp, which is about 4.93% of the mitochondrial genome and 13.47% of the chloroplast genome (Figure 2, Table 5). Five intact plastid genes (*ndh*A, *psa*B [×2], *rpo*A, *rrn23*, *ycf3*), 8 tRNAs (trnA [UGC], trnC [GCA] [×2], trnM [CAU], trnP [UGG], trnR [ACG], trnS [GGA], trnW [CCA]), 2 ribosome RNAs (rrn4.5, rrn5), and numerous partial genes and intergenic spacer regions were identified. All plasmid fragments are located in intergenic regions. The protein-coding genes transferred from the plastid seem to be nonfunctional. However, tRNAs of plastid origin are likely to be functional (Gui et al., 2016; Nakazono and Hirai, 1993).

3.3. Phylogenetic analyses

Because organelles are maternally inherited and have unique characteristics in evolution, they are important for reconstructing phylogenetic relationships between organisms (Jansen et al., 2005). Nine common genes in 26 mitochondrial genomes, including *atp6*, *atp9*, *ccm*C, *cob*, *cox1*, *cox3*, *nad3*, *nad6*, and *rps3*, were selected for phylogenetic analysis. The trees were completed with both maximum likelihood (ML; Figure 3A) and neighbor joining (NJ; Figure 3B) methods. The 24 complete chloroplast genome sequences were also used for constructing trees with the methods of ML (Figure 4A) and NJ (Figure 4B). In the phylogenetic trees, *Cycas taitungensis* was a sister of angiosperms; the monocots and the eudicots were monophyletic; *Butomus umbellatus*, *Spirodela polyrhiza*, and *Phoenix dactylifera* were sisters of Poaceae plants. In the branch of Poaceae, Bambuseae (containing *Bambusa oldhamii*, *Ferrocalamus rimosivaginus*), Triticeae (containing *Hordeum vulgare*, *Triticum aestivum*) and Oryzeae (containing *Oryza rufipogon*, *Oryza sativa*) have closer genetic relationships. *Cynodon dactylon* × *C. transvaalensis*, a species belonged to Cynodonteae, showed a closer genetic relationship with Andropogoneae (containing *Sorghum bicolor*, *Tripsacum dactyloides*, *Zea luxurans*, *Zea perennis*, and *Zea mays*). All of the trees showed that the topology was consistent with the representatives of the Angiosperm Phylogeny Group (APG) IV (Byng et al., 2016). The phylogenetic trees built on the chloroplast genome or 9 mitochondrial genes by using different methods showed the same result. The phylogenetic trees built on the chloroplast genome were congruent with the plant taxonomy and NCBI taxonomy common tree (Liu et al., 2013). However, the phylogenetic trees built on 9 mitochondrial genes showed some differences from the common tree. This may be due to different evolutionary rates of mitochondrial genes in different plants (Ma et al., 2012). In contrast to the chloroplast genome, the mitochondrial genome evolves at a slower rate (less than

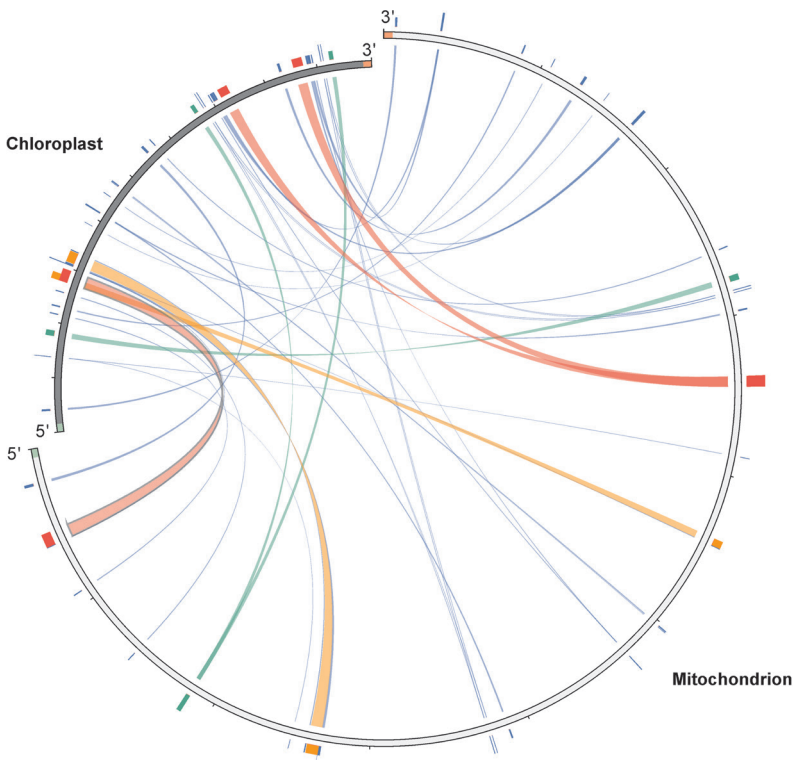

Figure 2. Schematic representation of chloroplast DNA transferred into the *C. dactylon* × *C. transvaalensis* mitochondrial genome. The connected regions represent highly similar regions on both genomes. The color of the wiring area, according to the length of the match, is standard and appears in red, yellow, green, and blue.

Table 5. Chloroplast insertions in the *Cynodon dactylon* × *C. transvaalensis* mitochondrial genome.

No.	Length	Identity	Position	Genes contained
1	3003	99%	18982–21984	*psa*B–*psa*A (partial)
2	2400	99%	240419–242818	*rrn*23 (partial)–*rrn*4.5–*rrn*5–*trn*R (ACG)–*trn*N (GUU) (partial)
3	2686	94%	96002–98645	*ycf*3
4	1577	99%	204012–205588	*psa*B (partial)
5	1273	99%	264107–23527	*rpo*C1 (partial)
6	947	99%	66782–67728	*rps*12 (partial)
7	574	99%	305568–306141	*trn*A (UGC)–*rrn*23 (partial)
8	559	98%	7671–8226	*rpo*A
9	527	99%	98637–99163	*psa*A (partial)
10	557	97%	319989–320540	*ndh*A–*ndh*H (partial)
11	377	100%	353602–353978	*rrn*23 (partial)
12	316	93%	257339–257654	*rbc*L (partial)
13	316	93%	141494–141809	*rbc*L (partial)
14	287	92%	363784–364068	*rps*16 (partial)
15	286	92%	334993–335273	*atp*I (partial)
16	212	91%	182359–182564	*trn*P (UGG)
17	191	92%	52055–52241	*atp*A (partial)
18	145	96%	21986–22130	*psa*A(partial)
19	145	96%	203866–204010	*psa*A (partial)
20	189	91%	95621–95809	*trn*S (GGA)
21	214	85%	271605–271810	*rpl*14 (partial)
22	195	86%	33785–33979	*rpo*C2 (partial)
23	182	86%	173025–173194	*trn*A (UGC) intron
24	119	91%	136961–137079	*rrn*16 (partial)
25	119	91%	262069–262187	*rrn*16 (partial)
26	97	93%	224583–224676	*trn*C (GCA)
27	82	96%	182152–182233	*trn*W (CCA)
28	76	97%	92083–92158	*trn*C (GCA)
29	121	88%	327947–328067	*pet*A (partial)
30	75	94%	363652–363726	n.a.
31	86	91%	137546–137631	*rrn*16 (partial)
32	86	91%	261517–261602	*rrn*16 (partial)
33	72	93%	314068–314139	*trn*M (CAU)

1/3 the rate of the chloroplast genome), with sequence variations smaller than chloroplasts and nuclear DNA, providing limited evolutionary information (Norman and Gray, 2001; Perrotta et al., 2002).

Contribution of authors

Shilian HUANG and Yancai SHI contributed equally to this work.

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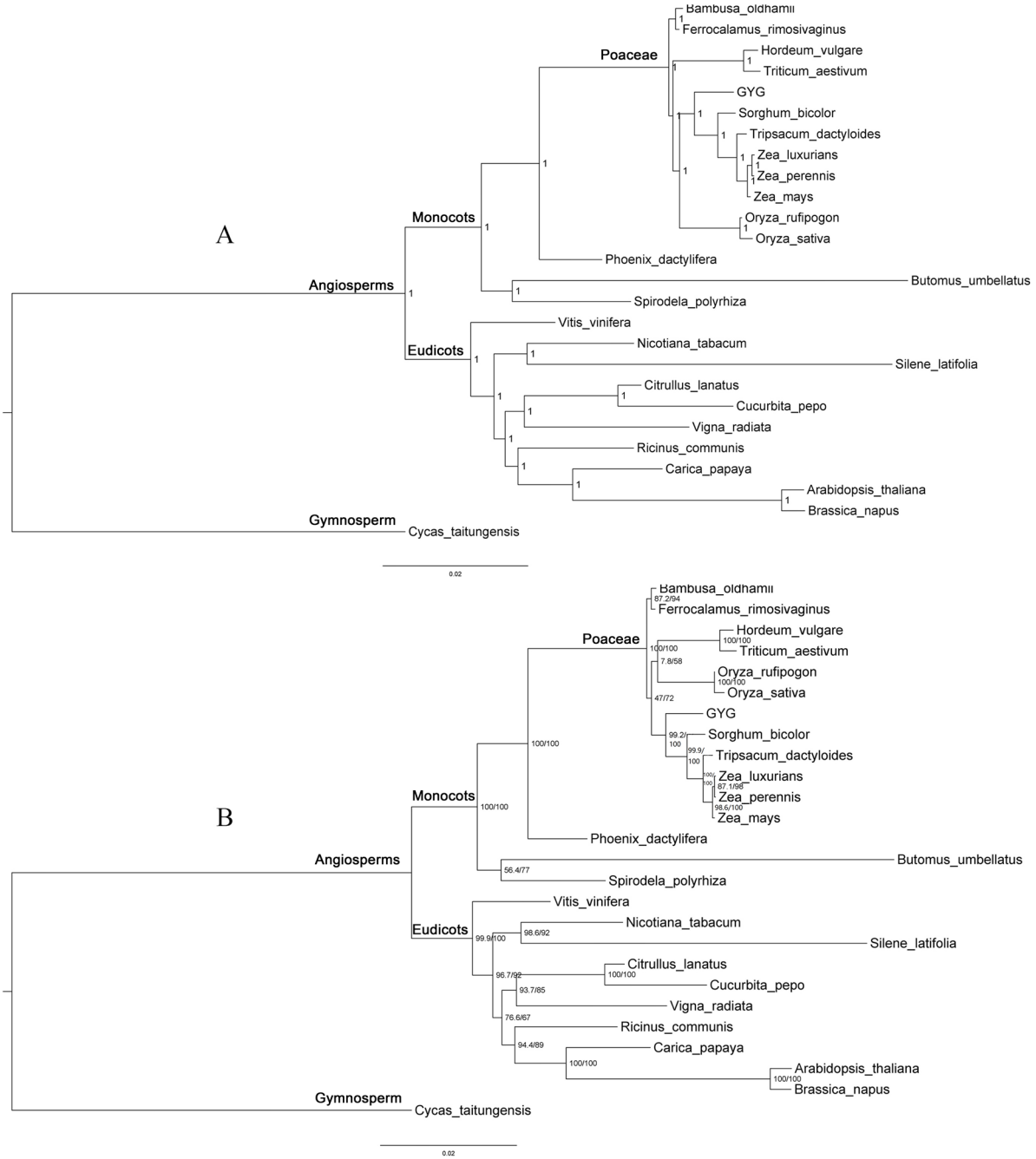

Figure 3. Phylogenetic relationships inferred from 9 common genes in the 26 mitochondrial genomes are shown in the BI tree (A) and the ML tree (B). GYG represents *C. dactylon* × *C. transvaalensis*.

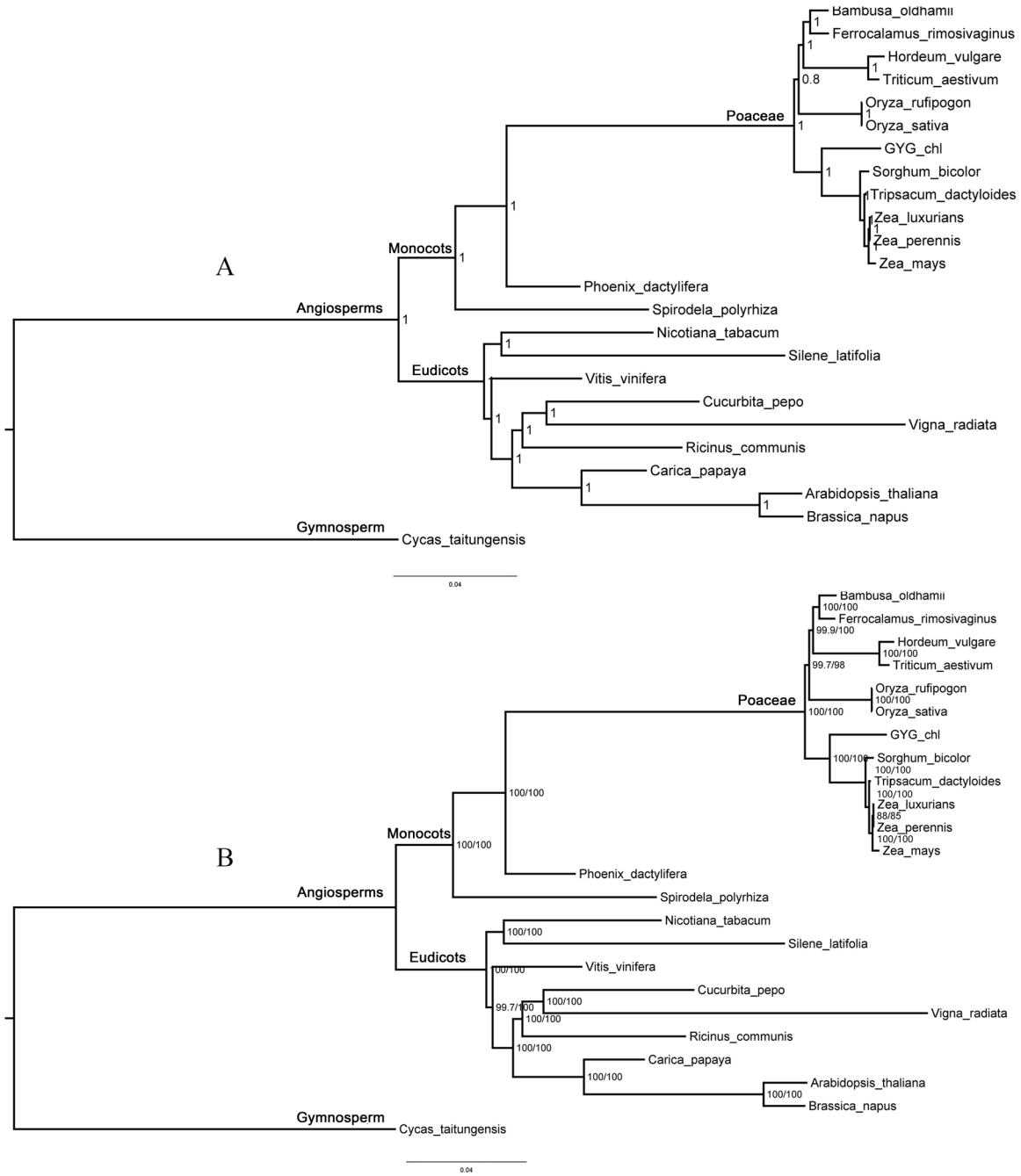

Figure 4. Phylogenetic relationships inferred from 24 complete chloroplast genome sequences are shown in the BI tree (A) and the ML tree (B). GYG_chl represents *C. dactylon* × *C. transvaalensis*.

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