

Presence and fragmentomic characteristics of plant cell-free DNA

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Dear Editor,

The presence of cell-free DNA (cfDNA), also known as extracellular DNA (exDNA), in bodily fluids is first discovered more than 70 years ago. Over the past decades, numerous studies have investigated the biological characteristics and translational significances of cfDNA in human plasma. Human cfDNA hold substantial diagnostic value, as exemplified by noninvasive prenatal testing and cancer liquid biopsies. Moreover, the biology of human cfDNA has also been characterized. In particular, the fragmentation procedure during cfDNA generation is non-random and significantly correlates with nucleosomal structure, endonuclease activities, and cleavage preferences.¹ Recent studies also suggest the presence of cfDNA in plants. For example, in rhizosphere, plant roots release cfDNA for soil-microbe communications, defense mechanisms, and stress responses.² Despite the extensive studies in humans and mammals, the biological characteristics of plant cfDNA remain poorly profiled, partly because cfDNA in roots or leaves is present in low volumes, posing challenges for extraction and comprehensive investigations. Here we report the presence of cell-free DNA (cfDNA) in coconut water (*Cocos nucifera*), exhibiting characteristic fragment size distributions and end motif patterns that differ markedly from human plasma cfDNA. Furthermore, nuclear cfDNA exhibits high DNA methylation level, while organellar cfDNA is barely methylated. Remarkably, this plant-derived cfDNA is readily detectable in murine circulation after ingestion, suggesting that dietary cfDNA may exert regulatory effects on mammalian physiology beyond mere nutrition.

We first examined coconut water from three fresh coconuts under a microscope and confirmed the absence of intact cells (Figure 1A & <https://github.com/hellosunking/plant-cfDNA/blob/main/SF.pdf>). We then extracted DNA from coconut water and analyzed the products (see Methods at <https://github.com/hellosunking/plant-cfDNA/blob/main/SI.pdf>). As a result, we obtained DNA with an average concentration of 89.3 ng per milliliter (range: 68.7–100.0 ng/ml) of coconut water, which is approximately 10-fold higher than that in human plasma. The DNA molecules were short with a prominent peak at ~150 bp (Figure 1B & <https://github.com/hellosunking/plant-cfDNA/blob/main/SF.pdf>), indicating that they were cfDNA rather than genomic DNA. To examine the genomic composition and fragmentomic features, we constructed sequencing libraries from the coconut cfDNA samples (N=3) and sequenced them to ~5x haploid coconut genome coverage (<https://github.com/hellosunking/plant-cfDNA/blob/main/ST.xlsx>). After quality control, ~84% of reads properly mapped to the coconut genome, confirming that these cfDNA molecules originate from coconut rather than contaminants. Among the aligned reads, ~87% were mapped to nuclear chromosomes, while ~4% and ~9% were mapped to chloroplasts and mitochondria, respectively (Figure 1C). However, when normalized to the genome sizes, chloroplast and mitochondrial cfDNA exhibited ~650- and ~350-fold higher sequencing depths, respectively, compared to nuclear cfDNA (Figure 1D). Given that coconut flesh typically lacks chloroplasts, the results suggested that the sources of coconut cfDNA included photosynthesis-related cells (such as leaves). We further analyzed the genomic representation of cfDNA originated from the nucleus. Compared to sonicated genomic DNA extracted from leaves, ~8% and ~14% genomic regions showed higher and lower representations, respectively, in cfDNA (<https://github.com/hellosunking/plant-cfDNA/blob/main/SF.pdf>), suggesting selective release or under-/over-fragmentation of coconut cfDNA might occur in certain genomic regions.

We next profiled the size distributions of cfDNA molecules, grouped by cellular origins. As shown in Figure 1E and <https://github.com/hellosunking/plant-cfDNA/blob/main/ST.xlsx>, in all samples, nuclear cfDNA displayed three notable features: 1) a major peak at 158 bp, likely reflecting DNA wrapped around a nucleosome while distinct from the 166-bp peak in human plasma cfDNA;^{3,4} 2) a 10-bp periodicity below 176 bp, which is similar to human plasma cfDNA but with slightly different offset peaks; and 3) a second peak at ~330 bp, likely representing di-nucleosomal cfDNA. In contrast, chloroplast and mitochondrial cfDNA were shorter than nuclear cfDNA but longer than human mitochondrial cfDNA in plasma, and they lacked 10-bp periodicity (Figure 1E). We then explored the plant nuclease footprints by analyzing 5'-end motif patterns in coconut nuclear cfDNA, focusing on 1- and 2-mers. Of note, most mammalian nucleases lack known orthologs in plants, such as DNASE1L3, which is recognized as the primary nuclease for cfDNA fragmentation and exhibits a strong preference for 5'-C ends.^{3,5} After normalization against genomic background, these cfDNA molecules showed strong preferences for 5'-C and 5'-G ends (especially 5'-GT) and depletion of 5'-T ends, exhibiting patterns distinct from those in human plasma cfDNA (Figures 1F–G). Moreover, for both 1- and 2-mer end motifs, the motif diversity scores (indicating the degree of randomness) were lower in coconut cfDNA compared to human plasma cfDNA (<https://github.com/hellosunking/plant-cfDNA/blob/main/ST.xlsx>).⁶ Together with size distributions, these findings indicated that coconut nuclear cfDNA fragmentation was non-random, akin to human plasma cfDNA, but likely governed by different nucleases.

DNA methylation is a key epigenetic modification, while currently high-resolution genomewide DNA methylation profiling in coconut is lacking. Hence, beyond fragmentomics, we performed whole genome bisulfite sequencing to assess cytosine methylation in coconut cfDNA.⁷ The results were summarized in Figures 1H–I. In nuclear cfDNA, the overall DNA methylation levels were 84.6% and 14.8% in CpG and CpH dinucleotides (where “H” denotes non-G bases), respectively. In contrast, cfDNA of chloroplast and mitochondrial origin showed negligible methylation levels. We further profiled DNA methylation densities in 1-Mb windows for nuclear cfDNA, which revealed that cytosine methylation in both CpG and CpH contexts predominantly occurs in chromosome bodies, with much lower levels in telomeres. These patterns aligned with prior studies on cellular DNA in other plants.^{8,9}

Recent studies have revealed that exogenous DNA can mediate immunoregulatory activities in hosts (e.g., protecting lupus-prone mice from disease progression).¹⁰ We wonder whether plant cfDNA can enter the host circulation upon ingestion. To do this, we administered coconut water or sterile saline (as control) to mice and collected their blood 5–30 minutes afterwards (Figure 1J). We extracted cfDNA from murine plasma and performed PCR experiments using coconut-specific primers. To our surprise, coconut-derived cfDNA was readily detectable in 9 out of 14 (64.3%; 95% confidence interval: 35.1–87.2%) murine plasma samples treated with coconut water, while not in any mice treated with saline (N=3; Figure 1K). Sanger sequencing of the PCR product confirmed a perfect match to the pre-designed sequence in coconut genome (<https://github.com/hellosunking/plant-cfDNA/blob/main/SF.pdf>). We further sequenced murine cfDNA samples with strong coconut signals (N=3) and controls (N=3), which confirmed the presence of coconut-derived cfDNA (Figure 1L). Interestingly, the size of coconut-derived cfDNA in murine plasma turned shorter compared to that isolated from coconut water, along with stronger 10-bp periodicity (Figure 1M), suggesting additional frag-

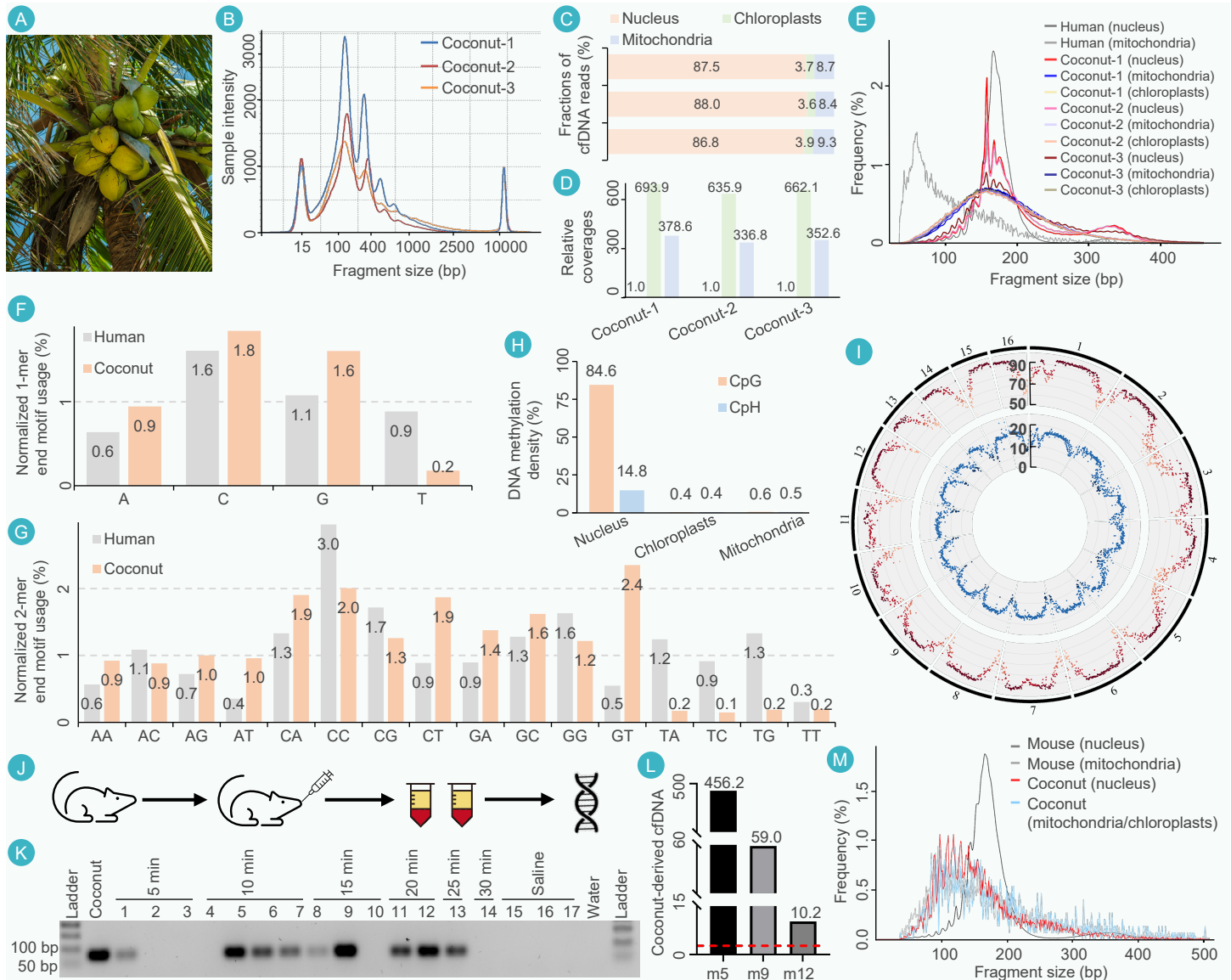


Figure 1. Presence and characteristics of cell-free DNA (cfDNA) in coconut water (A) A photo of coconut. (B) Sample intensity and size distribution of coconut cfDNA examined on an Agilent 4200 TapeStation system. Signals at 15 and 10000 bp represented lower and upper markers, respectively. (C) Fractions and (D) genome coverages of chloroplasts and mitochondria in relative to nucleus in 3 coconut cfDNA samples. (E) Size distribution of coconut versus human cfDNA. (F) 1-mer and (G) 2-mer end motif patterns in coconut cfDNA. The end motif usages were normalized by the frequencies in corresponding genomes. (H) Overall DNA methylation levels (in percentages) in CpG and CpH (where "H" denotes non-G alleles) dinucleotides in coconut cfDNA grouped by subcellular origins. (I) DNA methylation levels (in percentages) per 1 Mb windows in CpG (outer circle) and CpH (inner circle) dinucleotides in coconut cfDNA originated from nucleus. (J) Experimental design to investigate the presence of coconut cfDNA in murine blood upon ingestion. Mice were fasted and water-deprived for 3 hours and then fed with fresh coconut water (m1 to m14; N=14) or saline (m15 to m17; N=3) and sacrificed 5-30 minutes afterwards, and blood was harvested to extract cfDNA. (K) Detection of coconut-derived cfDNA in murine plasma using coconut-specific primers. Coconut cfDNA and water was used as positive and negative controls, respectively. (L) Relative abundance of coconut-derived cfDNA in 3 coconut-treated murine samples (m5, 10 minutes after ingestion; m9, 15 minutes after ingestion; m12, 20 minutes after ingestion), shown as RPM values (coconut-derived cfDNA reads per 1 million total reads). The red line indicated the limit of detection (LOD) determined using control murine samples, which was 2.75 in this study; samples with coconut-derived cfDNA RPMs higher than LOD were considered to contain coconut-derived cfDNA. (M) Size distribution of coconut- and mouse-derived cfDNA in pooled murine plasma data.

mentation occurred in the host upon ingestion. Given that coconuts have served as traditional foods and Chinese herbal medicine for thousands of years, it would be of interest to investigate the potential effect of coconut cfDNA upon entering circulation.

In conclusion, we reveal the presence of cfDNA in coconut water, characterized by size distributions and end-motif patterns distinct from those in human plasma cfDNA. Coconut could serve as an accessible model for studying the fragmentomics of plant-derived cfDNA, while cfDNA in other plant species might also exist with different characteristics, which deserves further investigations.

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AUTHOR CONTRIBUTIONS

Conception, design, and supervision: K.S.; Development of methodology: M.Y., Y.A.,

K.S.; Performed experiments: M.Y., Y.A., H.L., K.S.; Analysis and interpretation of data: all authors; Writing the manuscript: M.Y., K.S.; Review and edit of the manuscript: M.Y., Y.A., K.S. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

Animal study was conducted according to protocols approved by SZBL Animal Center (AESK202302).

DATA AND CODE AVAILABILITY

Raw sequencing data has been deposited to Sequence Read Archive (SRA) in NCBI with BioProject ID PRJNA1348917. Methods, supplementary figures, and tables had been deposited at github (<https://github.com/hellosunking/plant-cfDNA>).

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