TECHNICAL NOTE

GENERAL

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Whole Plastome Sequences of Two Drug-Type *Cannabis*: Insights Into the Use of Plastid in Forensic Analyses†

ABSTRACT: DNA is one of the fastest growing tools in forensic sciences, increasing reliability in forensic reports and judgments. The use of DNA has increased in different areas of the forensic sciences, such as investigation of plant species, where plastid DNA has been used to elucidate and generate evidence in cases of traceability of genetically modified and controlled plants. Even with several advances and the practice of using DNA in forensic investigations, there are just few studies related to the identification of genetic tools for the characterization of drug and nondrug-types of *Cannabis*. Herein, the whole plastomes of two drug-type *Cannabis* are presented and have their structures compared with other *Cannabis* plastomes deposited in the GenBank, focusing in the forensic use of plastome sequences. The plastomes of *Cannabis sativa* "Brazuka" and of the hybrid *Cannabis* AK Royal Automatic presented general structure that does not differs from the reported for other *C. sativa* cultivars. A phylogenomic analyses grouped *C. sativa* "Brazuka" with the nondrug *C. sativa* cultivars, while the hybrid *Cannabis* AK Royal Automatic placed isolated, basal to this group. This suggests that the analysis of plastomes is useful toward genetic identification of hybrids in relation to *C. sativa*.

KEYWORDS: forensic science, forensic genetics, CPDNA, hemp, marijuana, chloroplast genome, next generation sequencing

DNA is one of the fastest growing tools for forensic analysis, which increases reliability in forensic reports and judgments, overcoming routine biochemical analyses, mainly in investigations involving humans (1). However, the use of DNA has been increasing in other areas of the forensic sciences, such as investigation of plant species, where plastid DNA (cpDNA) has been used to elucidate and generate evidence in cases of traceability of genetically modified and controlled plants (2).

The genus *Cannabis* L. (Cannabaceae) is one of the oldest botanical genera used in human history, with records dating from the prehistoric eras, being used for therapeutic, religious, and recreational purposes (3–5). Currently, the taxonomic classification of the genus *Cannabis* is still under discussion. While some authors consider it as a monospecific genus (i.e., *C. sativa*; [6]), others consider the existence of three *Cannabis* species (i.e., *C. sativa*, *C. ruderalis,* and *C. indica* [7,8]). Aside of this discussion, *Cannabis* is roughly classified into two groups, according to its main use: hemp (nondrug-type), applied for fiber production, and marijuana (drug-type), used for recreational proposes due to it psychotropic action, provided by high concentration of the compound Δ-9-tetrahydrocannabinol (THC). In addition, plants of *Cannabis* with a high concentration of cannabidiol (CBD) have been used as a medicinal compound, and the ratio CBD/THC is the determinant of its therapeutic instead of psychotropic use. Currently, several hybrid cultivars of *Cannabis* have been developed, aiming to improve the production of THC or CBD (6,9).

The plastid genome (plastome) is quite conserved; however, significant differences are observed, which allows the use of cpDNA in analyses of taxonomic, biogeographic, and evolutionary divergence. Due to the small length, simple structure, mainly uniparental inheritance and sequence conservation, plastomes have been considered effective in studies concerning plant

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evolution, phylogeny, and traceability (2). In general, plastomes range from 35,000 to 217,000 base pairs, coding for 63 to 209 genes, the majority of which are responsible for the regulation of the plants photosynthetic mechanisms (10). Some plastid DNA sequences as the genes *rbc*L and *mat*K and the noncoding spacer *trn*H-*psb*A have been suggested as genetic barcodes, toward taxonomic/phylogenetic identification of plants at species level (11,12). However, barcode regions for identification at subspecies, strain or population levels are somewhat difficult to find. A segment of about 561 bp of the *rbc*L region was suggested as a potential tool for forensic geographic discrimination of *Cannabis sativa* (13), while four intergenic plastid regions and five plastid microsatellites were used intending to recover useful forensic information on the biogeography and crop-use characteristics of *Cannabis* populations (14). In spite of the advances generated from these two studies, both highlight the need of further investigations toward the use of plastid information as forensic tools and none of them differentiated cultivars or species of *Cannabis*.

While the drug-type strains of *Cannabis* are regulated in Canada, Uruguay, and some European countries (15), both drug- and nondrug-types are illegal in numerous countries including Brazil. As these two *Cannabis* types cannot be easily discriminated by morphological analysis (mainly after processing), different methods for identifying the drug-type *Cannabis* have been proposed for forensic investigation. Such methods include the analysis of DNA sequence polymorphisms in the THCA synthase gene (16), gas chromatography/mass spectrometry and chemometric tools (17), genomic SNP analysis (18), and loop-mediated isothermal amplification of the THCA synthase gene (19). Until the present study, five varieties of *Cannabis sativa* had their whole plastomes sequenced, assembled, and deposited in the NCBI (National Center for Biotechnology Information) database: *C. sativa* Carmagnola, *C. sativa* Dagestani (20), *C. sativa* Cheungsam, *C. sativa* Yoruba-Nigeria (21), and *C. sativa* Chinese hemp (GenBank ID: KY084475.1). However, even with these advances and the practice of using DNA in forensic investigations, there are just few studies related to the identification of genetic tools for the characterization of *Cannabis* cultivars (e.g., [14,16,22–25]). Intending to contribute to this field by expanding the currently small database of *Cannabis* plastome, the present study had two aims: (i) to sequence and characterize the plastomes of two different drug-type *Cannabis* samples seized by the Brazilian Federal Police, and (ii) to compare their structures with other *Cannabis* plastomes deposited in the GenBank, focusing in the forensic use of whole plastome sequences.

Materials and Methods

Plant Materials and cpDNA Isolation

In this study, we follow the parallel hypothesis for species concepts in the Cannabaceae (*sensu stricto*), recognizing the existence of three binomials for *Cannabis*, as proposed by Boutain (8), based on plastome phylogenies. The two samples employed in this study are drug-type *Cannabis* and were kindly provided by the Brazilian Federal Police (BFP). One sample was identified as a hybrid plant (*C. sativa* × *C. indica* × *C. ruderalis*), *Cannabis* AK Royal Automatic. This sample was obtained from seeds seized and indoor germinated in the laboratory of the BFP. The other sample, identified as *Cannabis sativa* (hereafter called *Cannabis sativa* "Brazuka"), was seized by the BFP in an illegal plantation in Pernambuco State, northeastern Brazil. Fresh green leaves from both samples were separately collected for chloroplast isolation. Whole chloroplasts were isolated from these samples with the methodology described by Vieira et al. (26) slightly modified and using the isolation and wash buffers described by Jansen et al. (27). The complete procedure is described in the Data S1. Total DNA was extracted using the CTAB protocol (28), adding proteinase K (10 mg/mL) in the lysis buffer. All procedures of chloroplast and DNA isolation were performed in the laboratories of the BFP.

After isolation, DNA samples were transferred to the laboratories of the Universidade Federal do Pampa, Campus São Gabriel, for purification and sequencing. DNA quality was determined using NanoVue™ spectrophotometer (GE Healthcare, Chicago, IL) and purified twice with the Agencourt® AMPure® XP Reagent (Beckman Coulter, Brea, CA). The final concentration of the DNA was quantified using a Qubit Fluorometer kit (Invitrogen, Carlsbad, CA) following manufacturers recommendations, and samples were stored at −20°C until use.

Plastomes Sequencing

Aiming to maximize the coverage of the sequencing due to reduced amount of initial sample, the plastome of *Cannabis* AK Royal Automatic was sequenced using two NGS sequencing strategies: the PGM Ion Torrent (short reads sequencing) and the Oxford Nanopore MinION™ (long reads sequencing) platforms. The sequencing of the *C. sativa* "Brazuka" plastome was performed using only the PGM Ion Torrent™ platform.

For short reads sequencing, the library preparation was performed using the Ion OneTouch™ 2 System with the Ion PGM™ Template OT2 400 Kit Template (Thermo Fisher Scientific, Waltham, MA) using the isolated cpDNA. The sequencing was performed using Ion PGM™ Sequencing 400 on Ion PGM™ System using Ion 318™ Chip v2. Long reads sequencing was performed using the Oxford Nanopore MinION™ technology. The library was prepared by using the rapid sequencing kit of genomic DNA (SQK-RA002) for the MinION™ device with the flow cell FLO-MIN 107 R9, following the manufacturer's instructions. The raw reads were acquired through the MinKNOW software in a 48-h run experiment.

Plastomes Assembly and Annotation

Both plastomes were reconstructed by reference-guided assembly with the plastid genome of *Cannabis sativa* var. Yoruba-Nigeria (KR363961.1) as a reference, using the CLC Genomics software (Qiagen, Hilden, Germany). The analysis of plastome coverage and confirmation of circularization were performed in the Tablet software, the preliminary annotation in GeSeq platform (29) and visualized on OGDraw tool (30). The analysis of the RNAs and the comparison with the annotation were performed in the software tRNAscan-SE v.2.0 (31). The physical map of the circular plastome was built using the OGDraw software.

The plastomes of the two *Cannabis* sequenced in this study were deposited at NCBI under the access numbers MK878538 for *Cannabis* AK Royal Automatic and MK878537 for *Cannabis sativa* "Brazuka".

Phylogenomic Analysis and SSR Loci Prospection

The plastomes of the two *Cannabis* specimens sequenced in this study were aligned with all other *C. sativa* cultivars that

TABLE 1—*Data of the species used for the phylogenomic analysis, containing the access code in the NCBI and corresponding references.*

Species/variety	NCBI access	Reference
Cannabis AK Royal Automatic	MK878538	This study
C. sativa "Brazuka"	MK878537	This study
C. sativa var. Yoruba-Nigeria	NC_027223.1	Oh et al. 2015
C. sativa var. Cheungsam	KR184827.1	Oh et al. 2015
C. sativa var. Dagestani	KR779995.1	Vergara et al. 2015
C. sativa var. Carmagnola	KP274871.1	Vergara et al. 2015
Humulus lupulus	NC_028032.1	Vergara et al. 2015

have their plastid genomes deposited in the NCBI database at the time of this study. The plastome of *Humulus lupulus* L. (Canabaceae) was used as outgroup (Table 1).

Alignment of the whole plastomes was performed using the MAFFT platform (32). A dotplot analysis was performed in order to verify the consistence of the alignments. This analysis revealed the presence of a discontinuous region in the plastome of the Chinese cultivar that makes its alignment inconsistent. Therefore, all further analyses were performed using the two plastomes sequenced and assembled in this study and the *C. sativa* cultivars Carmagnola, Dagestani, Cheungsam,

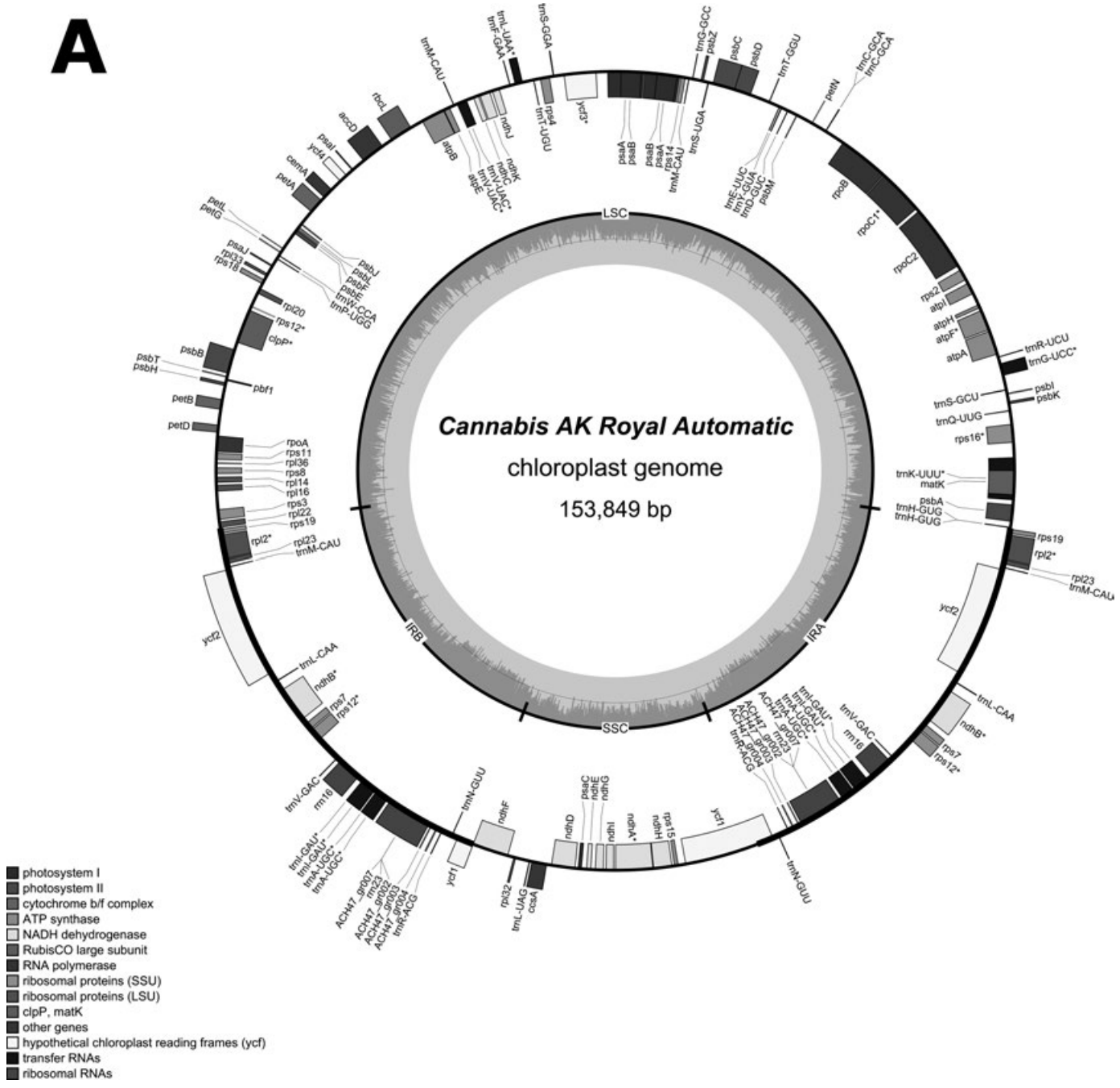

FIG. 1—*Physical maps of the plastomes of the hybrid Cannabis AK Royal Automatic (A) and of Cannabis sativa "Brazuka" (B). Genes containing introns are coded with an asterisk (*). [Color figure can be viewed at wileyonlinelibrary.com]*

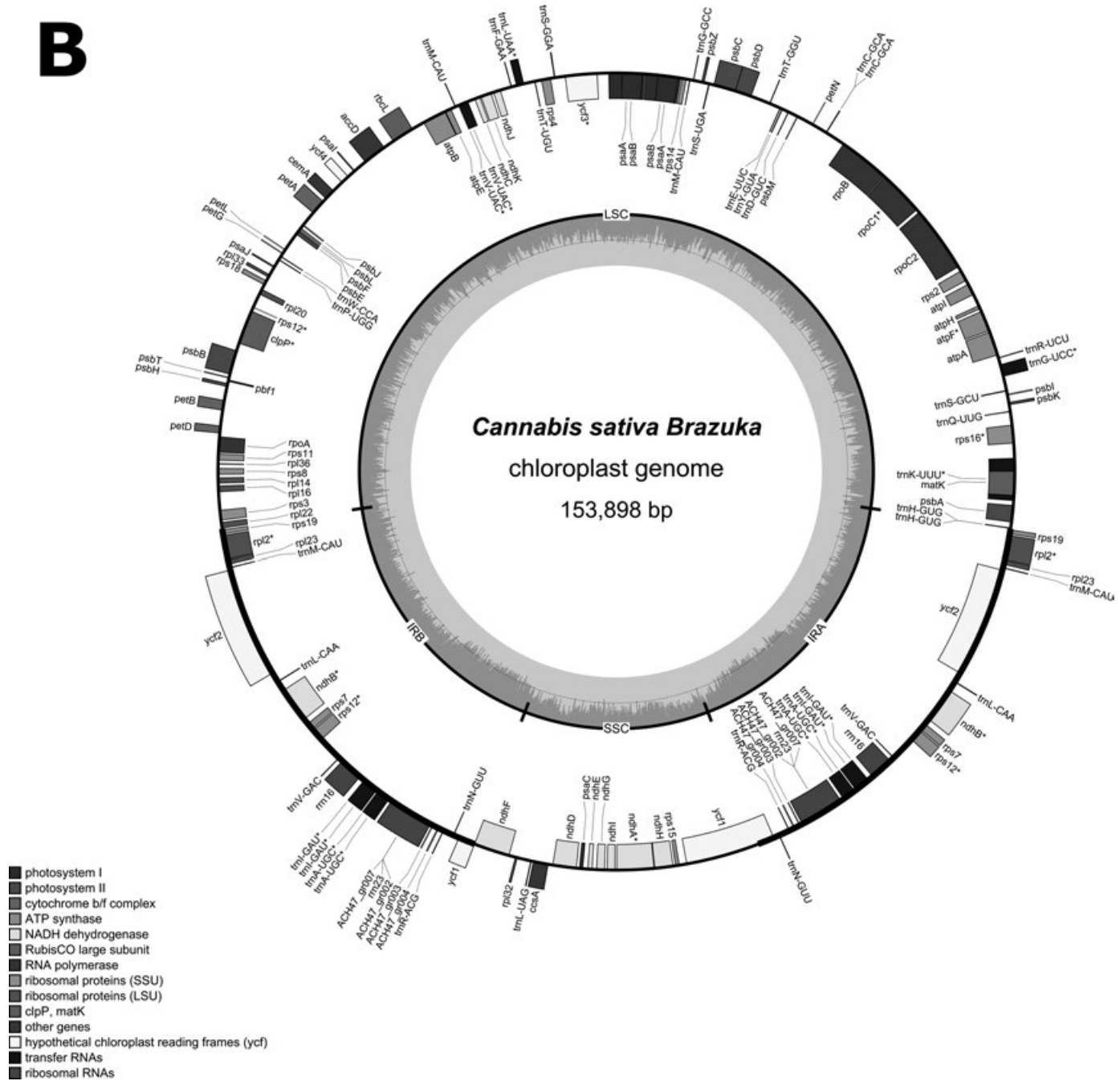

FIG. 1—*Continued. [Color figure can be viewed at wileyonlinelibrary.com]*

and Yoruba-Nigeria. The phylogenomic analysis was performed in the MEGA 6.0 software (33), using the general time reversible evolution model with Gamma distribution, as determined through the model test performed in the same software. Evolutionary analysis was performed using the maximum likelihood approach, with a 1000 replicate bootstraps. The consensus trees were visualized using FigTree software v.1.4.3.

In order to compare the number and distribution of putative cpSSR markers, motifs were prospected for all plastomes using the software SSRLocator (34). The search was performed for monomers, dimers, and trimers motifs, with at least ten, six, and four replicates, respectively.

Results

After assembly, the plastome of the hybrid *Cannabis* AK Royal Automatic resulted in a total of 153,849 bp, with a GC content of 36.6%. The plastome of the *C. sativa* "Brazuka" has 153,898 bp in length with a GC content of 36.7%. These values are consistent with the plastomes already known for other *C. sativa* cultivars (20,21).

Both plastomes were similar to those of the majority of angiosperms, with a quadripartite structure. In the *Cannabis* AK Royal Automatic plastome, the inverted repeat regions (IRs) have 26,009 bp separated by the 17.808 bp small single-copy region (SSC) and the large single-copy region (LSC) with 84,023 bp

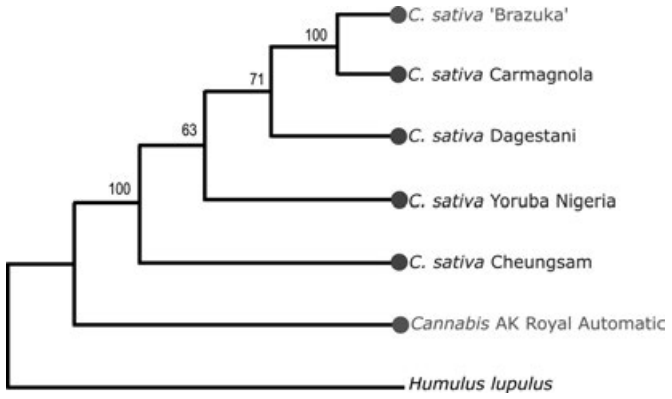

FIG. 2—*Consensus phylogenomic tree obtained using the maximum likelihood algorithm. Numbers at nodes are the percentage of bootstrap support after 1000 replicates. Red dots represent the drug cultivars and blue dots represent the nondrug cultivars. The Cannabaceae species Humulus lupulus, sister taxa to Cannabis, was used as outgroup for the analysis. [Color figure can be viewed at wileyonlinelibrary.com]*

(Fig. 1*A*), with a total of 162 genes identified (including RNAs and repeated genes). The *C. sativa* "Brazuka" plastome presents a pair of IR regions with 26,007 bp separated by the SSC region with 17,822 bp and the LSC region with 84.062 bp (Fig. 1*B*), with 155 genes identified. A total of 38 tRNA regions were identified in both *C. sativa* "Brazuka" (Data S2) and the hybrid *Cannabis* AK Royal Automatic (Data S3) plastomes.

The phylogenomic analyses grouped all *Cannabis sativa* cultivars into a highly supported clade (BP = 100%), while the hybrid *Cannabis* AK Royal Automatic formed a clade basal to the *C. sativa* one (Fig. 2). Within the *Cannabis sativa* clade, no clear partitioning of drug and nondrug cultivars is observed. *Cannabis sativa* "Brazuka", a drug-type cultivar, grouped with high bootstrap support (100%), together with *C. sativa* var. Carmagnola, a nondrug cultivar.

The total number of SSR monomers, dimers, and trimers prospected in the analyzed plastomes was very similar among *C. sativa* "Brazuka", Carmagnola, Cheugsam, Dagestani, and Yourubá-Nigeria, while the hybrid *Cannabis* AK Royal Automatic presented a significative higher number of monomers and less loci with the AAT motif (Table 2).

Discussion

Forensic applications have mostly focused on the differentiation of *Cannabis* plants as drug or nondrug-types (35). Aiming to analyze this differentiation at genomic level, an analysis of commonly cultivated *Cannabis* cultivars (81 drug-types and 43 nondrug-types), Sawler et al. (18) used 14,031 genomic SNPs. This study revealed a trend of genetic structure between drug-types and nondrug-types, showing that genetic differences between these groups are not limited to genes involved in THC production (18). This study also suggests that nondrug-type has a larger fraction of alleles in common with *C. indica* than with *C. sativa*. Nevertheless, the identification of a plant as a drug (*C. sativa*) or nondrug (*C. indica*) based on these SNPs is not correlated with *C. sativa* or *C. indica* for all cultivars. Even the direct analysis of the THC synthase gene based on the relative quantification of this region through real-time PCR assays failed to differ drug- and nondrug-type of *Cannabis* (23). No correlation between THCA synthase gene copy number and the content of THC in *Cannabis* samples was observed. These outcomes ratify the difficulty in identifying drug- and nondrug-types of *Cannabis* through genetic analysis. Our study based on whole plastomes also revealed such difficulty in genetically partitioning drug and nondrug-types of *Cannabis sativa* cultivars, which grouped together in the maximum likelihood tree (Fig. 2), while the hybrid *Cannabis* AK Royal Automatic (*C. sativa* × *C. ruderalis* × *C. indica*) clustered basal to all other samples. We were not able to detect coding plastid regions (single or combined) with potential forensic utility for identification of drug- and nondrug-types or hybrid and nonhybrid cultivars. Thus, the forensic usefulness of plastomes seems to be dependent of the full sequence analysis.

For species of the Cannabaceae family, a variation in the size of the plastomes is usual, mainly in the IR regions, with occurrence of contraction or expansion of <100 bp (36). In our study, the four varieties of *C. sativa* which plastomes were previously characterized presented a closer relationship to *C. sativa* "Brazuka", while *Cannabis* Ak Royal Automatic is somewhat differentiated, although the plastomes length varied just slightly among cultivars. Thus, the main difference related to the clustering of those cultivars is related to the DNA sequence of the plastomes. As the number and position of the genes in all cultivars is virtually identical, the differences are related to noncoding intergenic regions and introns.

Overall, the genetic differentiation between drug and nondrug *Cannabis* based on the putative origin from *C. sativa*, *C. ruderalis*, *C. indica* or its hybrids seems to be a big challenge. In this study, we presented the whole plastome sequences of two drug cultivars: the pure *C. sativa* "Brazuka" and a hybrid originated from the cross of three species, *C. sativa*, *C. ruderalis,* and *C. indica*. The maximum likelihood tree (Fig. 2) and the prospection of SSR loci (Table 2) demonstrated high correspondence among the *C. sativa* "Brazuka" and cultivars Carmagnola, Cheugsan, Dagestani, and Yoruba-Nigeria, while *Cannabis* Ak Royal Automatic revealed significant difference from all other cultivars. This distinctness may be related to the hybrid origin of this cultivar. While the other five cultivars are originated from direct selection of plants with the desired characteristics, *Cannabis* Ak Royal Automatic is a hybrid originated from two interspecific crosses: First *C. sativa* × *C. indica*, resulting in the

TABLE 2—*Data from regions prospected with the SSR Locator software for Cannabis plastomes based on motifs and base pairs (bp) for each motif.*

	SSR Motifs		
	Monomer	Dimer	Trimer
Cannabis AK Royal Automatic	A = 331; T = 506; C = 14	AT/TA = 6	AAT = 8; ATG = 4; ATA = 4; TTC = 4; TTA = 14; TAT = 4
C. sativa "Brazuka"	A = 283; T = 387; C = 11	AT/TA = 6	AAT = 12; ATG = 4; ATA = 4, TTC = 4; TTA = 4
C. sativa var. Carmagnola	A = 268; T = 381; C = 11	AT/TA = 6	AAT = 12; ATG = 4; ATA = 4, TTC = 4; TTA = 4
C. sativa var. Cheungsam	A = 232; T = 352; C = 13	AT/TA = 6	AAT = 12; ATG = 4; ATA = 4; TTC = 4; TTA = 4
C. sativa var. Dagestani	A = 268; T = 359; C = 11	AT/TA = 6	AAT = 12; ATG = 4; ATA = 4; TTC = 4; TTA = 4
C. sativa var. Yorubá-Nigeria	A = 256; T = 363; C = 12	AT/TA = 6	AAT = 12; ATG = 4; ATA = 4; TTC = 4; TTA = 4

cultivar *Cannabis* AK47, followed by the crossing of *Cannabis* AK47 with *C. ruderalis*, resulting in the *Cannabis* AK Royal Automatic. This hybrid was created aiming to improve the environmental resistance and a high content of THC which was present in the above 20% range of compost for the AK 47 hybrid cultivar (19,37). Changes in plastomes are genetic phenomena already observed in other species that undergo speciation or hybridization (38–42) and are the likely explanation for this genetic differentiation.

Our study suggests that the analysis of whole plastomes is a useful tool toward genetic identification of hybrids in relation to *C. sativa*. However, due to the absence of plastome sequences designated as *C. indica* and *C. ruderalis* deposited in the Genbank database, we are not able to determine the parental origin of the *Cannabis* AK Royal Automatic plastome. So, the next step needed in this issue is the sequencing of the whole plastomes of *C. indica* and *C. ruderalis*, allowing the inter-specific comparison of cpDNA sequences and the determination of plastid barcodes for each *Cannabis* species. A sliding window analysis for all six plastomes evaluated in this study revealed very low variation in coding genes (data not shown), suggesting that the differentiation concerning the hybrid *Cannabis* to the *C. sativa* cultivars occurs primarily in noncoding regions. Thus, such barcodes have to be prospected in introns and intergenic regions of the plastome. With these barcodes, the forensic differentiation of *Cannabis* species will be possible, as well as the determination whether a cultivated population is a drug- or a nondrug-type of *Cannabis*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Buffers and Procedures for the chloroplast isolation.

Data S2. Total of tRNA regions were identified in the *Cannabis sativa* "'Brazuka"' plastome.

Data S3. Total of tRNA regions were identified in the *Cannabis* AK Royal Automatic plastome.