RESEARCH NOTE/NOTA DE INVESTIGACIÓN

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *BOLEODORUS VOLUTUS* IN THE UNITED STATES FROM THE RHIZOSPHERE OF MEDICINAL HEMP

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ABSTRACT

Skantar, A. M., M. R. Kantor, L. K. Carta, M. N. Hult, and Z. A. Handoo. 2024. Morphological and molecular characterization of *Boleodorus volutus* in the United States from the rhizosphere of medicinal hemp. Nematropica 54:87-95.

Plant-parasitic nematodes are microscopic worms that cause an estimated $10 billion of crop losses each year in the United States and over $100 billion globally. The current study offers both morphological and molecular insights into a *Boleodorus volutus* population recovered from the rhizosphere soil of medicinal hemp cultivated in a field located in Baltimore County, MD in 2019. Nematodes were extracted from 100 cm^3 soil through sieving and decanting followed by sucrose centrifugal flotation. *Boleodorus volutus* was identified by anatomical features and measurements of females and males. Molecular sequencing and phylogenetic analysis of 18S and 28S rDNA markers were used to characterize *B. volutus*, which represents a new record of this species from the United States.

Key words: Boleodorus volutus, Cannabis sativa, medicinal hemp, plant-parasitic nematodes

RESUMEN

Skantar, A. M., M. R. Kantor, L. K. Carta, M. N. Hult, and Z. A. Handoo. 2024. Caracterización morfológica y molecular de *Boleodorus volutus* en Estados Unidos a partir de la rizosfera del cáñamo medicinal. Nematropica 54:87-95.

Los nematodos fitoparásitos son gusanos microscópicos que causan pérdidas de cosechas estimadas en diez mil millones de dólares cada año en Estados Unidos y más de 100 mil millones de dólares en todo el mundo. El estudio actual ofrece información tanto morfológica como molecular sobre la población de *Boleodorus volutus* recuperada del suelo de la rizosfera de cáñamo medicinal cultivado en un campo ubicado en el condado de Baltimore, MD, en 2019. Los nematodos se extrajeron de 100 cm^3 de suelo mediante tamizado y decantación, seguido de centrifugación en solución azucarada. *Boleodorus volutus.* Fueron identificados por características anatómicas y medidas de hembras y machos. Se utilizó secuenciación molecular y análisis filogenético de marcadores de ADNr 18S y 28S para caracterizar *Boleodorus volutus*, que representa un nuevo registro de esta especie en Estados Unidos.

Plant-parasitic nematodes (PPNs) are microscopic worms that cause an estimated $10 billion in crop loss each year in the United States and up to $118 billion globally (Kantor *et al*., 2022). Nematodes are one of the least-known pests attacking cannabis (*Cannabis sativa* L.) and very little is known about the species of plant-parasitic nematodes infecting this very important medicinal cash crop (Bernard *et al*., 2022). Nematodes parasitizing hemp include root-knot nematodes (*Meloidogyne enterolobii, M. incognita, M. hapla* and *M. javanica*), cyst nematodes belonging to the *Heterodera* group, and reniform, lesion and stem nematodes (Bernard *et al*., 2022).

Cultivation of medicinal hemp is a very appealing crop for organic farmers. A survey conducted in 2019 in North Carolina indicated that 85% of organic growers are interested in growing hemp on their farms (Dingha *et al*., 2019). However, it is well known that organic agriculture practices can lead to an abundance of plant-parasitic nematodes as well as other soil organisms (Adam *et al*., 2013; Briar *et al*., 2016; Pascual *et al*., 2017;) so a better understanding of nematodes affecting hemp is necessary for successful production.

In the United States, the area under hemp production increased from zero in 2013 to 59,110 ha in 2019, according to the United States Department of Agriculture (USDA) Economic Research Service report released in February 2020 (Mark *et al*., 2020). However, according to the latest National Hemp Report released by the USDA National Agricultural Statistics Service (NASS), Agricultural Statistics Board on April 19, 2023, the value of industrial hemp production was down 71% from 2021 (USDA NASS, 2023).

The aim of the present study was to isolate and characterize plant-parasitic nematode species in the soil rhizosphere of medicinal hemp from one field in Maryland. One of the nematodes was characterized by morphological and molecular means and identified as *Boleodorus volutus*. It represents the first report of this species in the United States.

Soil samples were collected in 2019 in October and November from a hemp field in Baltimore County, MD. The field was planted with hemp in May and the previous crop in the field was strawberry. Ten soil samples were collected at random across the field. Nematodes were extracted from 100 cm^3 soil through sieving and decanting followed by sucrose centrifugal floatation.

Besides *B. volutus*, other plant-parasitic nematode species were identified in the hemp rhizosphere, including *Pratylenchus penetrans*, *Paratylenchus projectus*, *Helicotylenchus pseudorobustus*, *Basiria* sp., *Anguina* sp., *Ditylenchus* sp., and *Hemicriconemoides* sp. Because of the lower numbers of these species present in the soil samples, we focused on *B. volutus*, which was more abundant than the other species, allowing for a more thorough analysis. In addition, several free-living nematodes belonging to the order Rhabditida and Dorylaimida were recovered.

Boleodorus volutus (N = 10) were fixed in 3% formaldehyde and processed by the formalin glycerin method (Hooper 1970; Golden, 1990). Photomicrographs of the specimens were made with a Nikon Eclipse Ni (Melville, NY) compound microscope using a Nikon DS-Ri2 camera. Measurements were made with an ocular micrometer on a Leica WILD MPS48, Leitz DMRB (Wetzlar, Germany) compound microscope.

For molecular characterization, single *B. volutus* were mechanically disrupted with a micro knife in 20 µl nematode extraction buffer [500 mM KCl, 100 mM Tris-Cl (pH 8.3), 15 mM MgCl$_2$, 10 mM dithiothreitol (DTT), 4.5% Tween 20, and 0.1% gelatin] and stored at –80°C until needed. Frozen nematodes were thawed, 1 µl proteinase K (from 2 mg/ml stock solution) was added, and the tubes were incubated at 60°C for 60 min, followed by 95°C for 15 min to deactivate the proteinase K. Two or 3 µl of extract were used for each PCR reaction. Two individuals were used for molecular analysis. Molecular markers included the small subunit 18S rRNA (SSU) amplified with primers 18S1.2 [5'– GGCGATCAGATACCGCCCTAG TT– 3'] and 18Sr2b [5'– TACAAAGGGCAGGGACGTAAT– 3'] (Kantor *et al.,* 2023). The D2-D3 expansion segments of the 28S rRNA gene were amplified with primers D2A [5'-ACAAGTACCGTGAGGGAAAGTT-3'] and D3B [5'- TCGGAAGGAACCAGCTACTA-3'] (Skantar *et al*., 2012). Newly obtained sequences

from *Boleodorus volutus* were submitted to GenBank under accession numbers MT994500 for 18S and MT994501 for 28S. DNA sequences were analyzed by BlastN to identify similarity to those in GenBank. Evaluations of intraspecific and interspecific variation were conducted using sequence alignment algorithms within Geneious Prime 2020.2.1 (Boston, MA). Phylogenetic analysis was conducted by Bayesian Inference (Huelsenbeck and Ronquist, 2001) via the CIPRES Gateway (Miller *et al.*, 2010) plug-in within Geneious. The best fitting model of nucleotide evolution was determined with jModel Test 2.1.7 (Darriba *et al.*, 2012) to be GTR+I+G, according to Akaike Information Criteria (AIC). Bayesian analysis was run with random starting trees, four chains for 2×10^6 generations, with Markov chains sampled every 500 generations. Two runs were performed for each analysis. Burn-in samples were discarded, and convergence was evaluated, with remaining samples retained for further analysis. Topologies were used to generate a 50% majority rule consensus tree with posterior probabilities (PP) greater than 0.5 shown on appropriate clades.

Measurements

Females (n = 10; Fig. 1): Body length (mean = 480 ± 21 μm, range = 430 - 510 μm,), stylet (9 μm ± 1.0, 8-10 μm), body width (19 μm ± 1.4, 16-20 μm), head end to posterior end of esophageal glands (98 μm ± 8.0, 80-110 μm), anal body width (11 μm ± 0.8, 10-12 μm), tail length (48 μm ± 6.8, 43-65 μm), a (25 ± 1.2, 24-27), b (5 ± 0.4, 4-6), c (10 ± 1.1, 9–12), c' (4 ± 0.5, 4-5) and V (68% ± 1.6, 64–70%). Four lines in the lateral field.

Males (n = 3): Body length (mean = 420 ± 4.1 μm, range = 415 - 425 μm), stylet (7.5 μm ± 0, 7.5-7.5 μm), body width (14 μm ± 0.5, 14-15 μm), head end to posterior end of esophageal glands (80 μm ±14.1, 70-100 μm), tail length (51 μm ± 1.0, 52-50 μm), spicule (14 μm ± 0.5, 14-15 μm), gubernaculum (5 μm ± 0.5, 5-6 μm), a (29 ± 0.9, 28-30), b (5 ± 0.9, 4-6), c (8 ± 0.5, 8–9). Four lines in the lateral field.

The morphometric details of females (Table 1) were recorded and compared to closely related species that were consistent with the original description of *Boleodorus volutus* Lima and Siddiqi, 1963 and with the recently reported populations from South Africa (Shokoohi, 2021)

and Canada (Munawar *et al.*, 2021). Morphologically, this species is very similar to *B. thylactus* Thorne, 1941 from which it can be differentiated by the presence of a depression at the middle of the lip region present in *B. thylactus*; by the tail length, which is much longer in *B. thylactus* (62-88 μm *vs* 35-60 μm) (Geraert, 2008; Shokoohi, 2021).

Molecular analysis

An 18S sequence 590 bp in length was obtained from two individuals with no intraspecific variation observed. Attempts to obtain full-length 18S sequences were unsuccessful, so the shorter fragment sequence was included in further analysis. The sequence was 99.65 to 99.83% similar (1-7 bp differences) to several *B. thylactus* sequences and 99.65% similar (8 bp differences) to the only other *B. volutus* 18S sequence in GenBank, a population from the Netherlands (FJ969117). A 28S sequence 742 bp in length was obtained from two individuals, with no intraspecific variation observed. This sequence was 99.69% similar to a population from Canada (MZ081097; 2 bp differences) and >98.7% similar to other *B. volutus* populations from South Africa (MW704025, MW704026) and Canada (MZ081095, MZ081096). Multi-sequence alignments with selected Tylenchidae were used to generate the 18S tree using Bayesian Inference (Fig. 2). The population from hemp grouped in a clade that included sequences of both *B. volutus* and *B. thylactus,* but with low support (PP = 0.62).

The 28S D2-D3 sequence was also used for alignments and phylogenetic analysis (Fig. 3). The 28S tree better resolved these two species, with the hemp population forming a maximally supported clade (PP = 1) with other sequences of *B. volutus*. The 18S sequence from Maryland was shorter than the Canada *B. volutus* sequence, and they appeared separated from each other in the 18S tree; however, the 28S sequences from U.S. and Canada did group together within the same clade. The tree positions of the U.S. population of *B. volutus* are consistent with previous studies that focused on *Boleodorus* spp. (Shokoohi, 2021; Munawar *et al.*, 2021; Monemi *et al.*, 2022). There are relatively few *Boleodorus* sequences in GenBank, so future analysis of additional markers such as COI may provide improved resolution of the Boleodorinae.
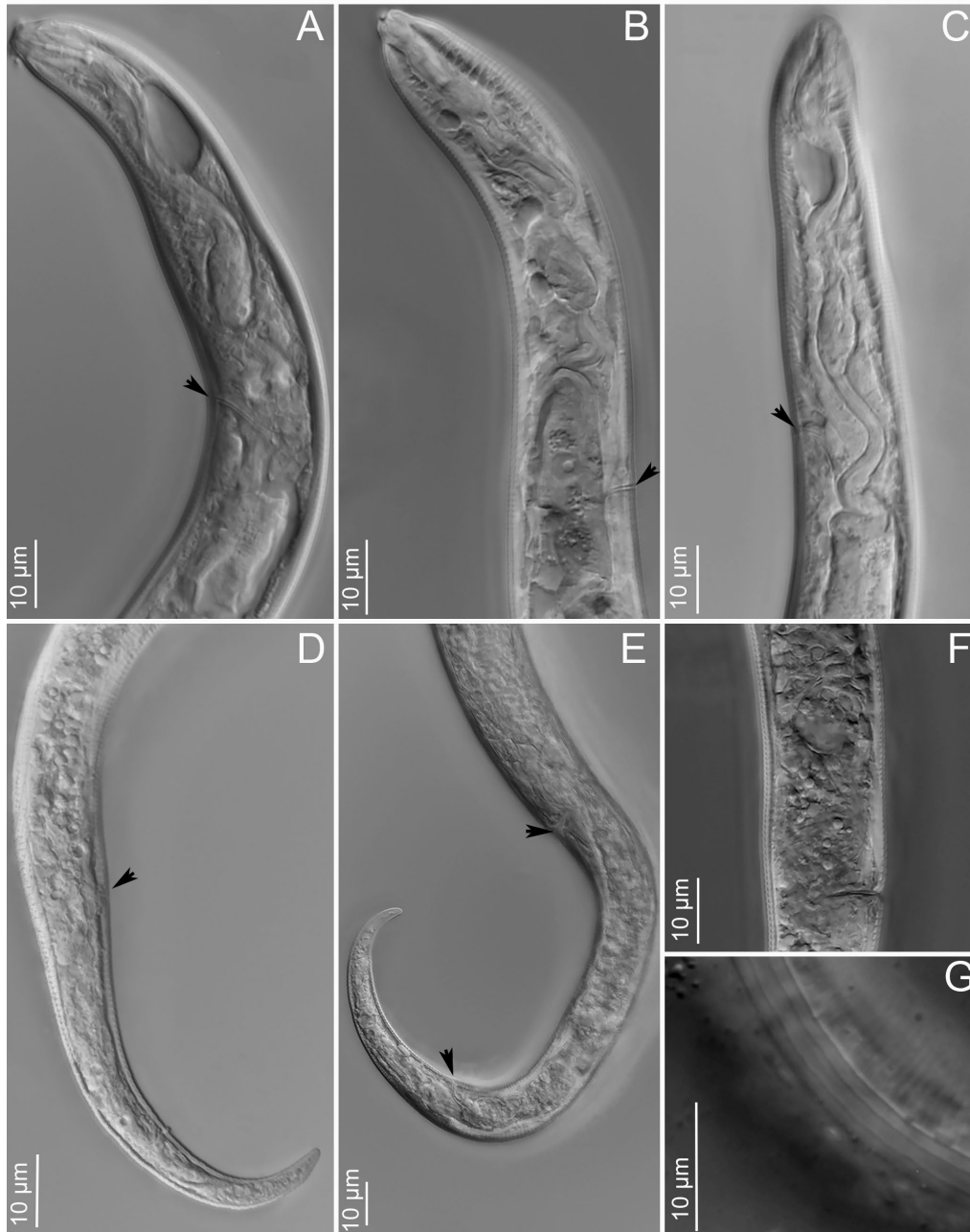
Figure 1. Photomicrographs of *Boleodorus volutus*. A-B) heads with arrows showing the excretory pore; C) Female esophagus with arrow pointing towards the excretory pore; D-E) Female tails with arrow pointing towards the anus area (D) and towards the anus and vulva (E); F) Vulva area; and G) Lateral lines.

Nematodes belonging to the *Boleodorus* genus have short generations, high reproduction rates and are considered herbivores, which feed on plant roots of higher plants (epidermal cells and root hairs) being able to cause mechanical injuries to the roots with their small stylets (Munawar *et al*.,

2021).

While the *B. volutus* specimens were retrieved from the hemp rhizosphere, we cannot conclude that they were parasitizing the hemp root. To our knowledge, this represents the first report of *B. volutus* in the United States.

Table 1. Morphometrics of *Boleodurus volutus* females. Unless indicated, all measurements are in micrometers and presented as mean ± standard deviation (range).

| Character	Maryland	Canada (Munawar *et al.*, 2021)		South Africa (Shokoohi, 2021)	England (Lima and Siddiqi, 1963)
n	10	10	8	7	16
Body length	480 ± 21 (430 - 510)	547.4 ± 31.5 (501.0–581.0)	533.0 ± 30.7 (487.0–577.0)	461.7 ± 59.8 (379-554)	480 (450–510)
a	25 ±1.2 (24-27)	27.4 ± 2.5 (24.5–31.3)	28.9 ± 2.6 (24.9–32.1)	27.2 ± 4.0 (23.7-31.5)	26 (20–28)
b	5 ± 0.4 (4-6)	4.6 ± 0.2 (4.2–4.8)	4.5 ± 0.2 (4.2–4.9)	4.7 ± 0.8 (2.9-5.3)	5.3 (4.9–5.7)
c	10 ± 1.1 (9–12)	9.6 ± 0.8 (8.5–10.6)	10.0 ± 1.0 (8.8–11.5)	9.5 ± 2.0 (7.8-14.7)	10 (8.7–12)
c′	4 ± 0.5 (4-5)	5.4 ± 0.8 (4.3–6.3)	5.2 ± 0.5 (4.6–5.8)	5.1 ± 0.8 (4.2-6.5)	–
V(%)	68 ± 1.6 (64–70%)	68.9 ± 0.7 (68.0–70.0)	69.0 ± 1.2 (68.0–71.0)	68 ± 5.3 (58-76)	69 (67–72)
Lip height	–	2.7 ± 0.2 (2.4–3.0)	2.3 ± 0.3 (2.1–2.8)	2.8 ± 0.5 (2-3)	–
Lip width	–	5.9 ± 0.2 (5.4–6.3)	5.6 ± 0.1 (5.5–5.8)	4.4 ± 0.7 (3.5-5.4)	–
Stylet length	9 ±1.0 (8-10)	8.5 ± 0.4 (8.0–9.3)	9.2 ± 0.6 (8.2–9.8)	9.6 ± 1.1 (8.5-11)	8.7 (8.3–9.2)
Anterior end to excretory pore	–	90.7 ± 4.2 (86.0–97.0)	88.4 ± 3.8 (85.0–95.0)	82.8 ± 8.5 (71-90)	78

18S
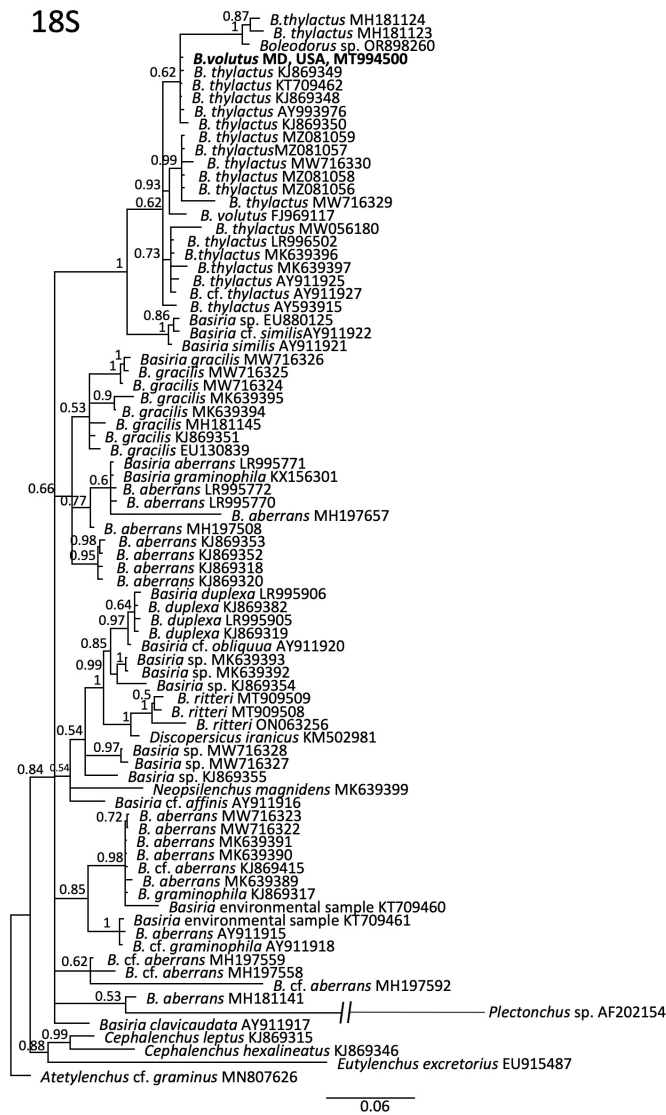
Figure 2. Bayesian 50% Majority rule consensus tree inferred from 18S rDNA alignment under GTR+I+ model. Posterior probability values exceeding 50% are given on appropriate clades. New sequence of *Beleodorus volutus* in bold font.
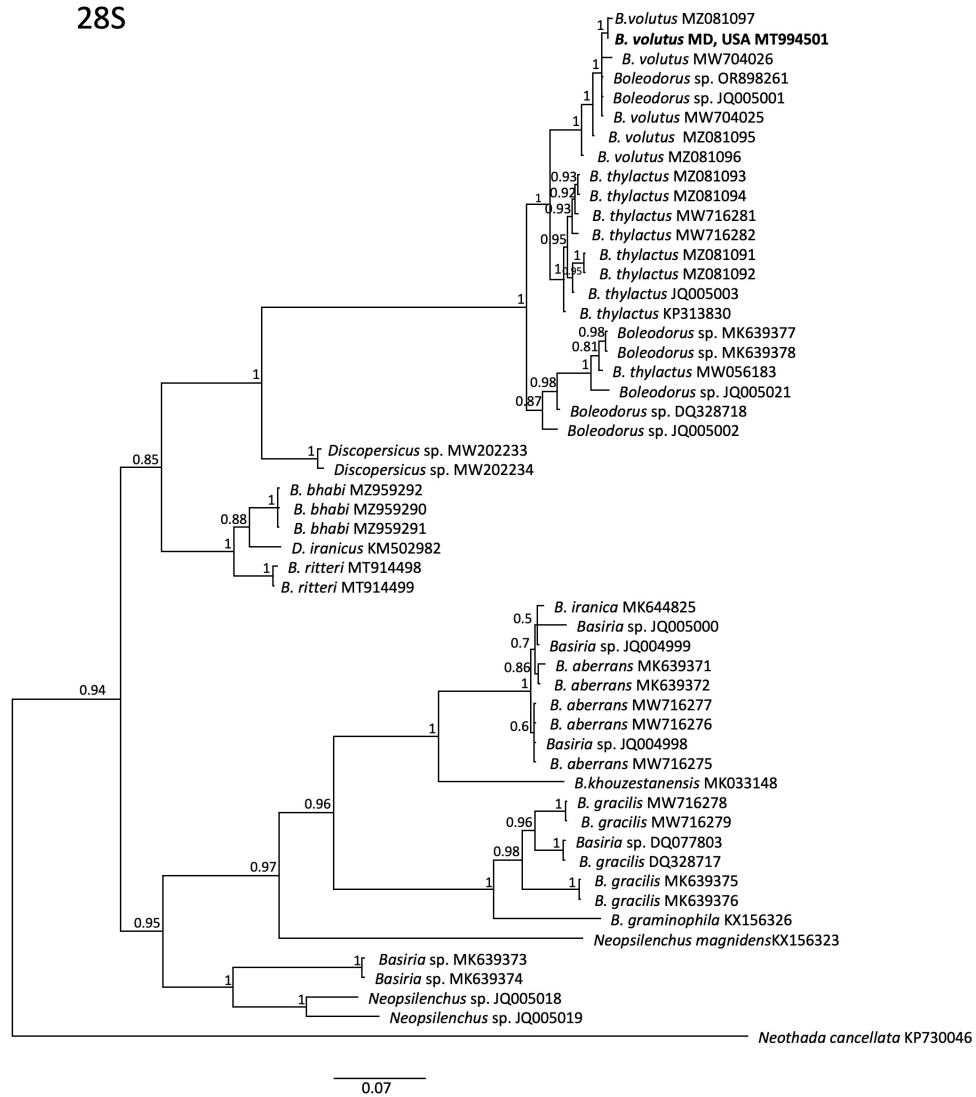
28S

Figure 3. Bayesian 50% Majority rule consensus tree inferred from 28S rDNA alignment under GTR+I+ model. Posterior probability values exceeding 50% are given on appropriate clades. New sequence of *Beleodorus volutus* in bold font.

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