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A New Species of *Branchellion* (Hirudinea: Piscicolidae) Parasitizing the Gills of Short-tail Stingrays (Batoidea: Dasyatidae) From the West Pacific

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A new fish leech, *Branchellion brevicaudatae* sp. n., is described based on specimens parasitizing the gills of the short-tail stingray, *Bathytoshia brevicaudata* (Hutton, 1875), collected from Japanese waters. The new species can be distinguished from other congeners by having: i) pulsating vesicles emerging from posterior base of branchiae, one pair per somite; ii) dorsal white spots, not arranged in longitudinal row; and iii) blackish body. A phylogenetic tree based on partial sequences of the mitochondrial cytochrome c oxidase subunit I gene from the new species and other piscicolid worms showed that the new species is sister to *Branchellion torpedinis* Savigny, 1822. This is the first record of *Branchellion* Savigny, 1822 from Japanese waters.

Key words: Annelida, COI, cox1, Hirudinida, Oceanobdelliformes, taxonomy

INTRODUCTION

Some leeches can reduce the value of marine products by parasitizing the body surface of fish (Cruz-Lacierda et al., 2000). The extent of the damage caused by leeches to the fishing industry is not well known due to the paucity and inaccuracy of records (Govedich et al., 2005). Outbreaks of marine leeches can stress fish and sometimes cause death (Cruz-Lacierda et al., 2000). Certain leeches are known to be intermediate hosts for fish-disease-causing unicellular organisms such as *Trypanosoma* (Sawyer, 1986; Negm-Eldin, 1997). Studies on marine leeches are thus important for the management of fish health.

The short-tail stingray *Bathytoshia brevicaudata* (Hutton, 1875) is a common cartilaginous fish in Japanese waters (Nakabo, 2018). It lives on the sandy bottom of subtidal areas from Hokkaido to southern Honshu and is frequently but unintentionally caught by fishermen. For the fishing industry, the species is almost valueless. Its value, if any, would be as an aquarium exhibit. Our internet search suggests that *Ba. brevicaudata* is currently harbored by more than 10 aquaria in Japan (e.g., Kaiyukan, 2022). The knowledge of the parasite diversity of this stingray is thus important for better keeping of it in aquaria. In a South African aquarium, the monogenean flatworm *Heterocotyle tokoloshei* Vaughan and Chisholm, 2020 is known to have heavily infested *Ba*. *brevicaudata* (Vaughan and Chisholm, 2020). To our knowledge, however, no leech infestations on *Ba*. *brevicaudata* have ever been published, although information on leeches that parasitize the stingray can be found in blog posts.

The genus *Branchellion* Savigny, 1822 consists of eight species of marine leeches that parasitize chondrichthyans, mainly batoids (Ruiz-Escobar and Oceguera-Figueroa, 2019). In a few cases, members parasitizing teleosts were reported (Savigny, 1822; van Beneden and Hesse, 1864; Blanchard, 1894). They are found on the surface of the body, particularly near the gills (Caira et al., 2012). Species in this genus have been reported worldwide (Sawyer, 1986). *Branchellion parkeri* Richardson, 1949 is the sole species that has been reported from the Western Pacific Ocean

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(Richardson, 1949; Ruiz-Escobar and Oceguera-Figueroa, 2019). Some DNA sequences of *Branchellion* spp. that were collected from Australia and Malaysia are registered in GenBank (Williams and Burreson, 2006). One of them was identified as *Branchellion lineare* Baird, 1869 (Burreson, 2020). In Japan, there is no record of the genus (see Nagasawa et al., 2008).

In the present study, we report on the discovery of a new leech species parasitizing the gills of short-tail stingrays in the Pacific Ocean. The leech is described here as a new species in the genus *Branchellion* based on an integrative approach combining morphological data and DNA barcodes (*COI* region).

MATERIALS AND METHODS

Short-tail stingrays were collected by a set net at Sagami Bay, Japan. The caught rays were brought to Nagai fishing port (35°12′22″N, 139°36′32″E). Leeches were removed from gills of the stingrays with forceps. After being photographed, the specimens were fixed in 70% ethanol and observed under stereomicroscopes (Nikon SMZ800N and Nikon Ni). Additionally, examination, dissection, and drawing of one of the specimens were conducted using a Leica M125 stereoscopic microscope with a drawing tube. The type specimens were deposited in the Zoological Collection of Kyoto University (KUZ).

Total DNA of the specimens was extracted using a DNeasy Tissue Kit (Qiagen) from a branchia of the holotype. The reaction mixture (0.2 µl TaKaRa Ex Taq [Takara, Japan], 2 µl of 10× Ex Taq Buffer [Takara, Japan], 1.8 µl dNTP mixture [Takara, Japan], 1 µl of each primer pair [10 µM], 1 µl of extracted DNA, and 14 µl of distilled water) was used for amplification. PCR amplification was performed with the primer pairs polyLCO (5′-GAYTATWTTCAACAAATCATAAAGATATTGG-3′) and polyHCO (5′-TAMACTTCWGGGTGACCAAARAATCA-3′) for part of the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*) (Carr et al., 2011), using an Applied Systems 2720 thermal cycler following by the protocol: preheating at 94°C for 2 min; 35 cycles at 94°C for 40 s, 50°C for 60 s, and 72°C for 60 s; and a final extension at 72°C for 7 min. Nucleotide sequencing was performed using the same primer pair with an ABI BigDye Terminator ver. 3.1 Cycle Sequencing Kit and an ABI 3100 Avant Genetic Analyser (Applied Biosystems). The newly obtained sequences of the *COI* gene (694 bp) were deposited in GenBank. A total of 14 sequences (nine species) were used for molecular analyses. Twelve of them were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank), and the remaining sequences were from this study. All sequences were aligned using MAFFT ver. 7.205 according to the E-INS-i strategy (Katoh and Standley, 2013). Alignment-ambiguous positions were removed using trimAL with the gappyout method (Capella-Gutiérrez et al., 2009). *Piscicola geometra* (Linnæus, 1761) and *Piscicola milneri* (Verrill, 1871) were used as outgroup. The newly obtained sequences of *COI* were deposited in GenBank (Accession numbers ON920889, ON920890). All sequences were used for maximum-likelihood phylogenetic tree construction in MEGAX (Stecher et al., 2020). Additionally, a total of nine sequences (for six species) were used for calculating K2P genetic distances based on 642 bp of *COI* using MEGAX.

RESULTS

Systematics

Branchellion brevicaudatae Jimi and Nakano, sp. n.
[Japanese name: Hoshiei-era-biru]
(Figs. 1, 2)

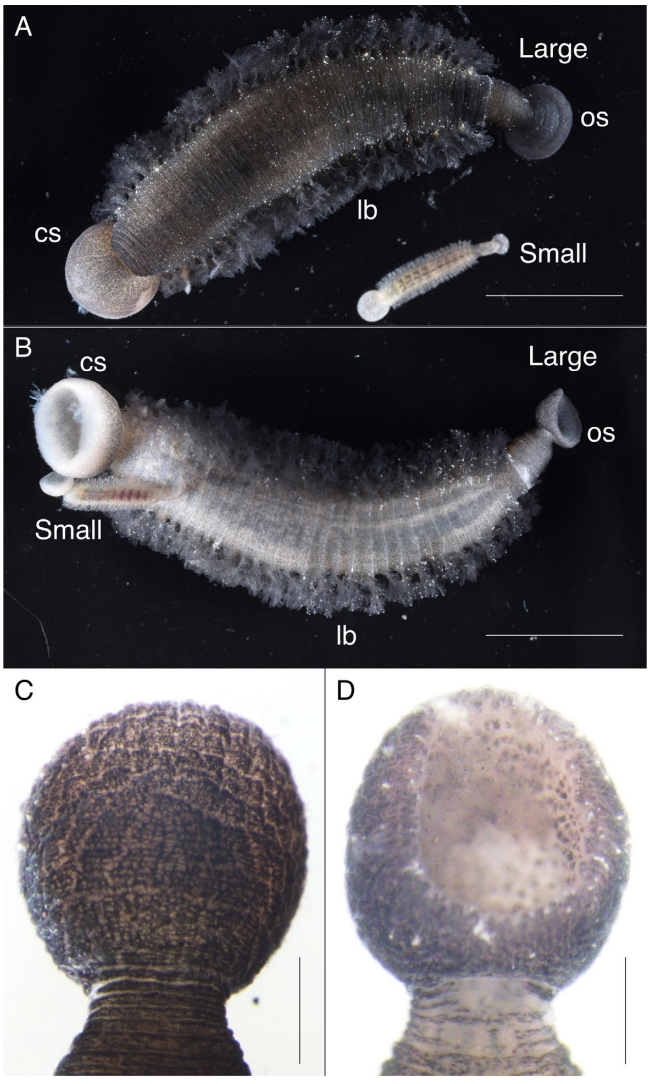

Fig. 1. *Branchellion brevicaudatae* sp. n. Large, holotype (KUZ Z4203). Small, paratype (KUZ Z4206). **(A)** Whole body, dorsal view. **(B)** Whole body, ventral view. **(C)** Oral sucker, dorsal view. **(D)** Oral sucker, ventral view. Scale bars: **(A, B)** 10 mm, **(C, D)** 2.5 mm. Abbreviations: os, oral sucker; lb, lateral branchiae; cs, caudal sucker.

Branchellion sp. Heron Island: Williams and Burreson, 2006, p. 629.

Branchellion sp. Borneo: Williams and Burreson, 2006, p. 629.

Type host. Short-tail stingray, *Bathytoshia brevicaudata* (Hutton, 1875).

Site on host. Gills.

Type locality. Japan: Sagami Bay.

Material examined. Holotype (KUZ Z4203) and five paratypes (KUZ Z4204–Z4206), leg. J. Shinji, on 19 June 2020.

Etymology. The specific name is a substantive noun in the genitive case derived from the specific name of the host species, *Ba. brevicaudata*.

Diagnosis. Body blackish with dorsal white spots. White spots not arranged in longitudinal rows. Lateral branchiae

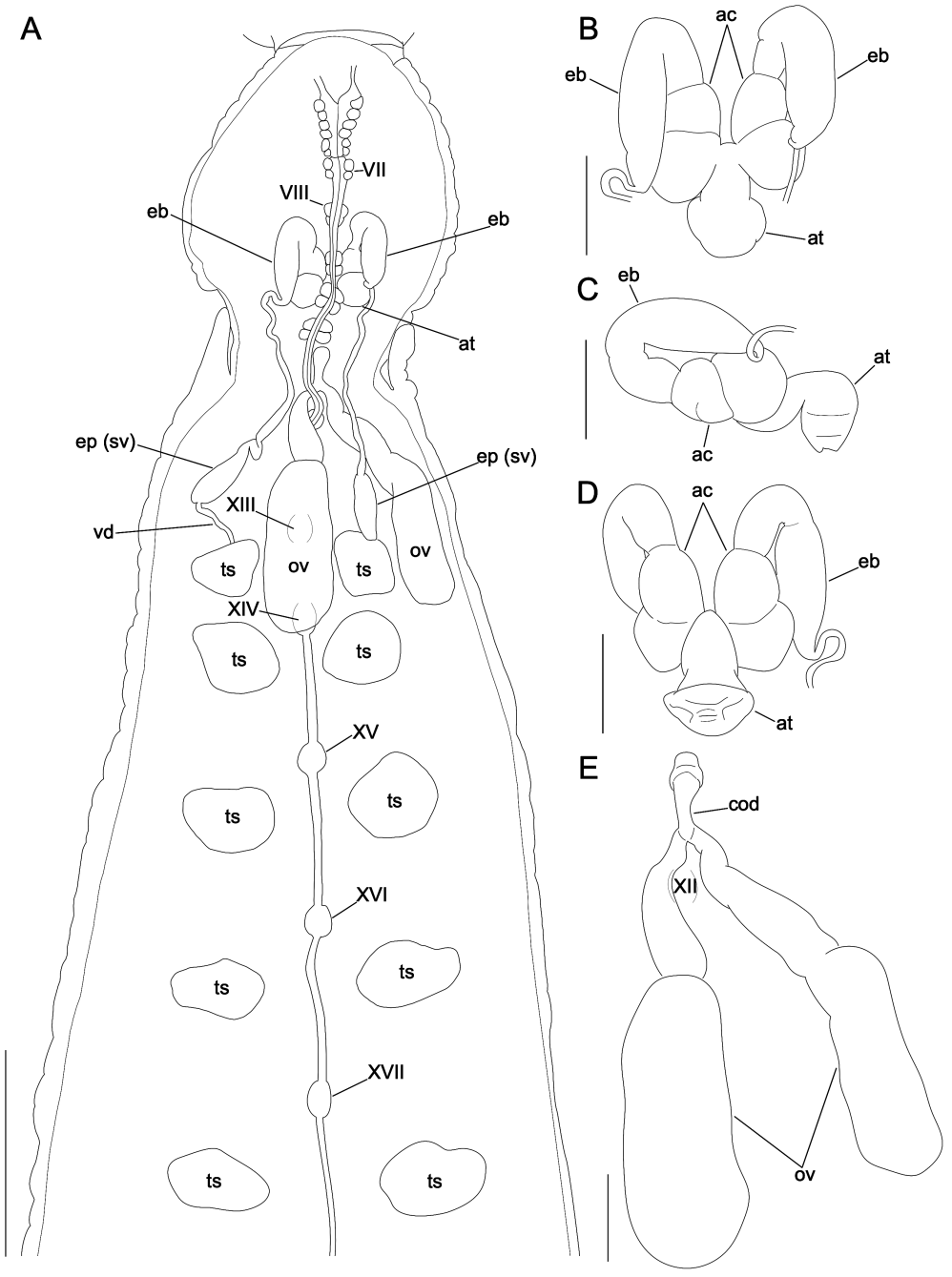

Fig. 2. *Branchellion brevicaudatae* sp. n., paratype (KUZ Z4204). **(A)** Reproductive system, including ventral nervous system, dorsal view. **(B)** Male median reproductive system, dorsal view; **(C)** same, left lateral view; **(D)** same, ventral view. **(E)** female reproductive system, including position of ganglion XII, dorsal view. Scale bars: **(A)** 2 mm, **(B–E)** 0.5 mm. Abbreviations: ac, atrial cornu; at, atrium; cod, common oviduct; eb, ejaculatory bulb; ep (sv), epididymis (seminal vesicle); ov, ovary; ts, testisacs; vd, vas deferens.

33 pairs. Pulsating vesicles emerged from posterior base of branchiae, one pair per somite, 10 pairs.

Description (based on six specimens). Total length 7–30 mm; maximum width (excluding branchiae) 2–8 mm. Body blackish, with numerous small white spots scattered along the body, including suckers and branchiae, not arranged in longitudinal rows. Body distinctly divided into trachelosome and urosome (Fig. 1), ventral and dorsal surfaces without tubercles. Ventral surface with transverse flanges corresponding to furrows, anteriorly projected. Oral sucker circular. Eyespots cannot be identified due to blackish body color. Mouth pore subcentral. Trachelosome slender, elliptical in cross-section, distinctly divided into head, including anterior sucker formed by fused somites I–III, somites IV–VIII uniannulate. Urosome wide, dorsally convex and ventrally flattened, formed by triannulate somites XIII–XXIII. Male gonopore in XIII; female gonopore in XIV fully covered by a collar. Lateral branchiae foliaceous, in 33 pairs, with dorsal and ventral lobes. Pulsating vesicles emerged from posterior base of branchiae, one pair per somite, 10

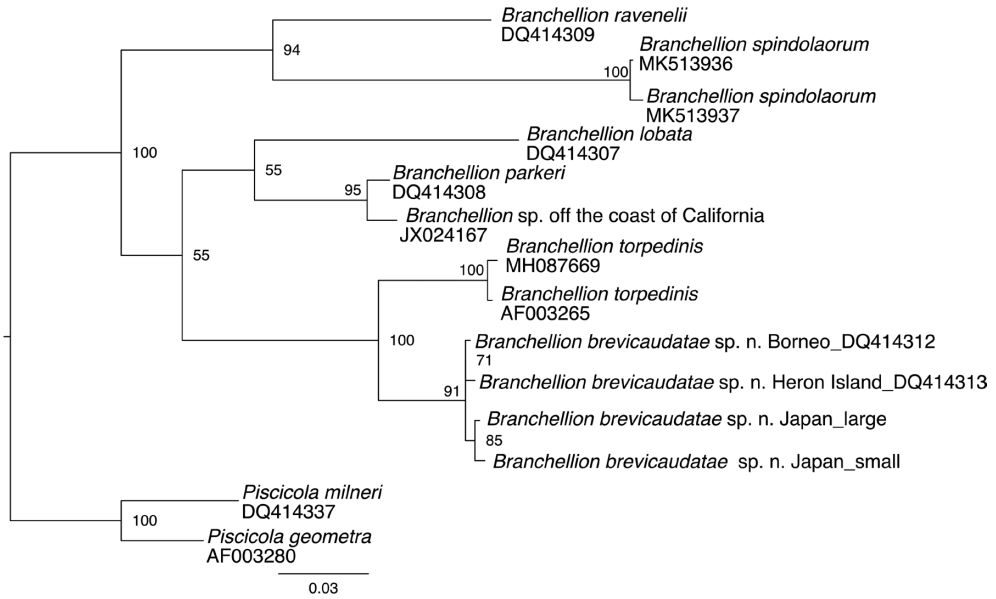

Fig. 3. Maximum-likelihood phylogenetic tree of *Branchellion* species based on partial *COI* sequences in the barcoding region. Nodal bootstrap support (BS) values are indicated for each branch.

Table 1. K2P genetic distances of *Branchellion* spp. in this study.

Species	1	2	3	4	5	6	7	8	9
1. *B. brevicaudatae* sp. n. Japan_Large									
2. *B. brevicaudatae* sp. n. Japan_Small	0.00462								
3. *Branchellion* sp. Borneo (Williams & Burreson 2006) → *B. brevicaudatae* sp. n.	0.00772	0.00618							
4. *Branchellion* sp. Heiron Island (Williams & Burreson 2006) → *B. brevicaudatae* sp. n.	0.00928	0.00773	0.00462						
5. *B. torpedinis*	0.05943	0.05954	0.05954	0.06119					
6. *B. parkeri*	0.12517	0.1234	0.12157	0.12154	0.11611				
7. *B. lobata*	0.1269	0.12692	0.12327	0.12508	0.12509	0.0966			
8. *B. ravenelii*	0.14532	0.14907	0.14532	0.14718	0.15865	0.14164	0.15096		
9. *B. spindolaorum*	0.17393	0.17589	0.17393	0.17586	0.17638	0.16257	0.17411	0.13695	

pairs. Caudal sucker circular, eccentrically attached, flattened, roughly 5× larger than oral sucker, bearing ventral secondary suckers; secondary suckers circular, pedunculated and flattened on their surface.

Digestive system (based on KUZ Z4204, Z4205): proboscis long; esophagus short; bacteriomes undetectable; crop with six pairs of crop-caeca; intestine with eight pairs of caeca.

Reproductive system (based on KUZ Z4204; Fig. 2): male reproductive system: testisacs, on each side, five pairs, 1st pair between somites XIII/XIV, 5th pair between somites XVII/XVIII. Paired vasa deferentia short, in somite XIII. Paired epididymides in somites XII–XIII. Paired ejaculatory ducts reaching ganglion X, then each duct connecting to ejaculatory bulb. Paired ejaculatory bulbs, fusiform in somites VIII–X, folded at position of ganglion VIII, then descending to each atrial cornu (= *cul-de-sac* resembling horn sensu Sawyer, 1986). Atrium short. Female reproductive system: paired ovisacs barrel-shaped, in somites XII–XIV, reaching position of ganglion XIV. Oviducts thick, left oviduct crossing ventrally beneath nerve cord; both oviducts converging into common oviduct in somites XI/XII. Vagina small, globular.

Phylogenetic position and genetic distance

In the resulting phylogenetic tree, *Branchellion brevicaudatae* sp. n. is grouped within the *Branchellion* clade with a 100% bootstrap support [BS]. The Japanese *B. brevicaudatae* sp. n. clade is sister to Borneo and Heron Island clade with high support. The new species forms a clade with *B. torpedinis* with high support. K2P genetic distances for the COI sequences (Table 1) showed distances among the six *Branchellion* species ranging from 5.9% to 17.6%. The lowest interspecific genetic distance is 5.9% between *B. brevicaudatae* sp. n. and *B. torpedinis*. The intraspecific genetic distances among the Japanese specimens of *B. brevicaudatae* and *Branchellion* spp. from Borneo and Heron (Williams and Burreson, 2006) range from 0.4% to 0.7%.

DISCUSSION

The new species resembles *Branchellion angeli* Sigalas, 1921, *B. plicobranchus* Raj, 1954, and *B. torpendinis* Savigny, 1822 in having 33 pairs of branchiae (Savigny, 1822; Harding, 1910; Sigalas, 1921; Raj, 1954). The new species can be discriminated from other congeners with 33 pairs branchiae by having i) pulsating vesicles emerged from posterior base of branchiae, one pair per somite; ii) dorsal white spots, not arranged in longitudinal rows; and iii) blackish body. The body is white in *Branchellion angeli* (Sigalas, 1921), while it changes in the new species according to body length. Small specimens had a whitish body (Fig. 1A), large ones blackish. Sigalas (1921) described *B. angeli* as "Tous, de taille et d'âge différents, avaient un caractère commun : ils étaient entièrement blancs." (= All of them, of different size and shape, had one common character: they were entirely white). This shows that *B. angeli* has a white body color throughout its life, which clearly distinguishes it from the new species. *Branchellion plicobranchus* has a black body without white dorsal spots and pulsating vesicles emerged from anterior/posterior base of branchiae, while the new species has spots and pulsating vesicles emerged from the posterior base of branchiae. Additionally, the new species differs from *B. plicobranchus* in having 10 pairs of pulsating vesicles (vs. 11 pairs in *B. plicobranchus*; Raj, 1954, 1959). *Branchellion torpendinis* has six longitudinal series of yellowish white spots on the dorsal and four on the ventral surface and pulsating vesicles emerged from the anterior/posterior base of branchiae (Quatrefages, 1852; Harding, 1910), while the new species does not have longitudinal white spots or pulsating vesicles emerged from the posterior base of branchiae. In addition to those external diagnostic characters, *B. brevicaudatae* could be characterized by the internal reproductive features, i.e., short and globular ejaculatory bulbs and atrial cornua (= cul-de-sac resembling horns), and barrel-shaped ovisacs.

Our phylogenetic analysis showed that the new species forms a clade with *B. torpedinis* and *Branchellion* sp. from Japan. Although the new species and *B. torpedinis* shared certain common characters such as having 33 pairs of branchiae, these were not considered to be unifying characteristics for the node. A similar observation was suggested in previous studies where the *B. spindolaorum*–*B. ravenelii* clade was composed of species with different numbers of gills. No relationship between the number of gills and phylogenetic relationships was considered to exist.

The Japanese specimens of the new species also formed a clade with specimens collected from Malaysia and Australia based on DNA sequences. These specimens are also considered to be *B. brevicaudatae* sp. n. as they are within the range of intraspecific variation based on genetic distance. In Burreson (2020), an immature specimen referred to as *Branchellion* sp. from Heron Island (Williams and Burreson, 2006) was identified as *B. linear*, which has 31 pairs of simple branchiae. The specimen of *Branchellion* sp. from Heron Island was not examined in the present study, but nonetheless, it is confirmed that immature specimens of *Branchellion* have the same branchial pairs as adult specimens and thus the new species is truly not *B. lineare* (Eugene M. Burreson, personal communication). The new species is suggested to be widely distributed in the western Pacific.

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COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

NJ and TN wrote the main manuscript text, and NH and MO prepared Fig. 1. JS collected the samples. SPW modified the manuscript text and investigated the distribution of the species in Malaysia. All authors reviewed the manuscript.

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