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NOTE

PHYLOGEOGRAPHY OF *CUTLERIA CYLINDRICA* (CUTLERIALES, PHAEOPHYCEAE) IN NORTHEASTERN ASIA, AND THE IDENTITY OF AN INTRODUCED POPULATION IN CALIFORNIA[1]

Keita Kogishi

Graduate School of Science and Technology, Kobe University, 1-1 Rokkodai, Kobe 657-8501, Japan

Taiju Kitayama

National Museum of Nature and Science, Amakubo 4-1-1, Tsukuba 305-0005, Japan

Kathy Ann Miller

University Herbarium, University of California, Berkeley, California 94720, USA

Takeaki Hanyuda and Hiroshi Kawai[2]

Kobe University Research Center for Inland Seas, 1-1 Rokkodai, Kobe 657-8501, Japan

***Cutleria cylindrica* Okamura was described from Japan in 1902 and has been reported only from northwestern Asia until its relatively recent discovery in California, USA, and Baja California, Mexico. To clarify the genetic relationships within and among the disjunct populations, we carried out a molecular phylogenetic study, as well as the examination of sex ratio and the life-history patterns, of populations in Japan, Korea, and California. Based on the DNA sequences of mitochondrial genes *cox2*, *cox3*, the open reading frame (ORF) region, and the spacer between *cox*3 and ORF, a total of 23 haplotypes were detected in the 85 individuals from 20 localities in Japan, Korea, and California. All localities in Japan and Korea included multiple haplotypes, but only a single haplotype was found in California. There was a positive relationship between distance and genetic divergence in Japan and Korea. The single haplotype found in California was the same as one occurring in Japan (Aomori Pref. and Fukuoka Pref.) and Korea (Daedaepo, Pusan). Both male and female gametophytes were distributed in most northeastern Asian populations. Only female gametophytes, developing parthenogenetically from female gametes, were found in California and Aomori Pref., Japan. On the basis of these results, we conclude that the disjunct population of *C. cylindrica* in California originated from a relatively recent introduction from Japan and shares its origin with the parthenogenetic population in the Tsugaru Strait.**

***Key index words:* *cox2*; *cox3*; *Cutleria cylindrica*; genetic diversity; introduction; Phaeophyceae**

***Abbreviations:* cox, cytochrome *c* oxidase; ORF, open reading frame; SAMOVA, spatial analysis of the molecular variance**

C. cylindrica (Cutleriales, Phaeophyceae) was described from Japan (Okamura 1902) and recorded from Korea (Cotton 1906). However, in contrast to its frequent occurrence in northeastern Asia (Okamura 1936, Segawa 1956), it has not been reported outside Asia until its relatively recent discovery in California (Hollenberg 1978, Stewart 1991) and Baja California (Aguilar-Rosas 1994). This disjunct biogeographic distribution (i.e., restricted to northeastern Asia and northwestern America) is relatively rare in temperate/warm-temperate brown algae [c.f. *Ishige sinicola* (S. et G.) Chihara; *Petrospongium rugosum* (Okamura) Setch. et N. L. Gardner] (Setchell and Gardner 1924, Dawson 1944, Yoshida 1998).

Disjunct distributions may be explained by one of the following scenarios: (1) a natural circum-Pacific distribution that is a relict of a continuous distribution in the past, (2) a relatively recent introduction by human activity, or (3) that one or more disjunct population represents a cryptic species. Considering the limited number of species exhibiting such a distribution, and the rarity of natural long-distance dispersal in species lacking buoyant structures, the second scenario is more plausible than the first. Many macroalgal species have been unintentionally transported by transoceanic shipping (i.e., attached

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[2]Author for correspondence: e-mail kawai@kobe-u.ac.jp.

to rock ballast or ship hulls, and/or entrained in ballast water), oyster mariculture, or the aquarium trade (Padilla and Williams 2004, Mineur et al. 2007, Williams and Smith 2007).

The closely related species *Cutleria multifida* (Turner) Grev., which was originally described from Europe, is now known to occur in New Zealand, Chile, Australia, and Japan. Although *Cutleria* species exhibit heteromorphic life histories alternating between macroscopic dioecious gametophytes and small prostrate sporophytes, only female gametophytes were found in the New Zealand and Chile populations of *C. multifida*. As these populations were limited to the vicinity of international ports, they were considered the parthenogenetic descendents of individuals transported from their native range by shipping (Adams 1983, Santelices et al. 1989). However, it is difficult to prove that the species was not present in the locality prior to its discovery and to conclude that a particular population originated from recent anthropogenic introductions. Furthermore, Church (1898) also reported the frequent occurrence of populations dominated by female gametophytes in the native range of this species (Normandy and Plymouth).

Similarly, for *C. cylindrica*, populations in which female plants are dominant have been reported from several localities on the Tsugaru Strait between Hokkaido and Honshu, Japan (Sasaki et al. 1987, Kitayama et al. 1992). In a culture study, Kitayama et al. (1992) showed that these female-dominated populations were maintained by the parthenogenesis of female gametes that directly developed into female gametophytes. They also showed that under lower temperature conditions (<15°C), erect thalli (morphological gametophytes) developed only in parthenogenetic populations.

The possibility that populations in the northeastern Pacific constitute a cryptic species (scenario 3) is plausible because many cryptic taxa have been reported by recent molecular studies (McIvor et al. 2001, Zuccarello and West 2003, Sasaki and Kawai 2007). Therefore, to clarify the biogeography of *C. cylindrica*, we carried out a molecular phylogenetic analysis combined with analyses of sex ratios based on herbarium specimens and life-history studies in culture.

For the molecular analyses, the following specimens were newly collected from Japan, Korea, and California and used for DNA extractions (Table 1): Japan (68 individuals/17 localities), Korea (two individuals/one locality), and California, USA (15 individuals/two localities). Part of each specimen was quickly dried in silica gel and used for molecular studies. Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. PCR amplifications and sequencing of the mitochondrial *cox2* (cytochrome oxidase subunit II) gene, *cox3* (cytochrome oxidase subunit III) gene, ORF region (homolog of ORF379 [*Fucus vesiculosus,* AY494079]), and intergenic region between *cox3* gene and ORF region were performed to examine genetic diversity. Primers for PCR amplification and sequencing are listed in Table 2. PCR was carried out with a GeneAmp PCR Cycler 9700 (Applied Biosystems, Foster City, CA, USA) and a TaKaRa PCR Thermal Cycler Dice (Takara Shuzo, Shiga, Japan) using a TaKaRa ExTaq Reaction Kit (Takara Shuzo) or KOD FX (ToYoBo, Osaka, Japan). After polyethylene glycol (PEG) purification (Lis 1980), PCR products were sequenced using a CE DTCS quick start kit (Beckman Coulter, Fullerton, CA, USA) and a CEQ8000 DNA analysis system (Beckman Coulter) according to the manufacturer's instructions.

In the DNA sequences of mitochondrial *cox2* and *cox3* genes and the intergeneric region adjacent to *cox3* and the ORF region, a total of 23 haplotypes

TABLE 1. Populations of *Cutleria cylindrica* examined in the present study.

Population code	Locality and collection date	No. of samples used for examining sex ratio, molecular study, and culture, respectively	Haplotypes (no. of samples)
1	Netanai/Ohma, Aomori Pref., Japan (15 June 2006)	12, 10, 4	E (2), G (1), K (6), L (1)
2	Senjyoujiki, Sado Island, Niigata Pref., Japan (12 June 2005)	10, 7, 0	A (6), J (1)
3	Miura Peninsula, Kanagawa Pref., Japan (27, 28 April 2005)	8, 8, 2	E (4), G (4)
4	Shimoda, Shizuoka Pref., Japan (29 April 2005)	13, 12, 0	A (2), E (7), H (1), I (1), N (1)
5	Maiko, Kobe, Hyogo Pref., Japan (23 March 2006)	3, 4, 0	M (2), N (2)
6	Awaji Island, Hyogo Pref., Japan (1, 12, 22 April 2005)	58, 13, 2	D (1), M (8), N (1), R (1), T (2)
7	Shishikui, Tokushima Pref., Japan (4 June 2005)	4, 4, 3	B (3), C (1)
8	Suouoshima, Yamaguchi Pref., Japan (22 June 2005)	1, 1, 0	S (1)
9	Shikanoshima, Fukuoka Pref., Japan (25 April 2005)	4, 4, 0	K (2), N (1), O (1)
10	Miyazaki, Miyazaki Pref., Japan (6 May 2005)	3, 2, 0	U (1), V (1)
11	Onyu Island, Oita Pref., Japan (15 March 2006)	2, 2, 0	P (1), Q (1)
12	Otsuku Island, Kumamoto Pref., Japan (March 2005)	1, 1, 0	W (1)
13	Dadaepo, Pusan, Korea (11 March 2006)	2, 2, 0	F (1), K (1)
14	Santa Catalina Island, California, USA (16, 21, 23, 24 April 2006)	15, 15, 5	K (15)

TABLE 2. List of primers used for PCR and sequencing.

Code	F/R	Sequence (5′–3′)	Annealing position
trnY-P1	F	TCYATCRTAGGTTCGAATCC	*trnY* (52–71)
cox3-P1	F	GAYCCWAGTCCMTGGCCWTTAG	*cox3* (49–70)
cox3-P5	F	CHCCHGTTTTTAATATTGGAGG	*cox3* (341–362)
cox3-P5.2	F	KCHCCHGTYTTTAATATTGG	*cox3* (340–359)
cox3-P6	R	CDACAATHGCATGATGAGCCC	*cox3* (478–457)
cox3-P6.2	R	CRATTGCATGRTGAGCCCAAG	*cox3* (475–455)
cox3-P2	R	ACAAARTGCCAATACCAAGC	*cox3* (755–736)
ORF379-P1	R	CACAATATTTAACTTTATCG	ORF379 (133–114)
cox2-P5	F	GAKGAGATAAAAGAAATKTTATC	*cox2* (2,359–2,381)
cox2-Cc1	F	TKTTATCTAAGATAGGGAGC	*cox2* (2,375–2,394)
cox2-Cu1	F	GATGACGATTTAGCTATTCC	*cox2* (2,812–2,831)
cox2-Cu2	R	TTMGTAGGMACAARAAGACG	*cox2* (2,909–2,890)
cox2-Cu3	R	GCCCAWGAATGYARAACATC	*cox2* (2,960–2,941)
cox2-P2	R	GAGCATAAYCTTTTWCCAACCC	*cox2* (3,152–3,131)

Annealing positions correspond to the sequences of *Fucus vesiculosus* (AY494079, Oudot-Le Secq et al. 2006).

were recognized in the individuals collected in Japan, Korea, and California (Table 3). Figure 1 shows the distribution of the haplotypes and their ratios in each local population. Each local population in Japan and Korea included multiple haplotypes (two to five haplotypes depending on localities), except for Yamaguchi, where only one individual was examined. Figure 2 shows the haplotype-spanning network tree of all the haplotypes. The genetic distances (number of substitutions) among the haplotypes were relatively small in each local population and tended to be greater between geographically distant populations. This tendency was also confirmed by the statistical analyses: significant, although weak, correlation between geographic and genetic distances was observed among the Pacific and Seto Inland Sea populations in Japan; for example, correlation between the genetic distance based on Phist and geographic distance, $r = 0.5414$, $z = -20.2833$, $P = 0.0138$, $R^2 = 0.293$.

TABLE 3. List of haplotypes revealed from DNA sequences of mitochondrial *cox2* and *cox3* genes, the intergeneric region adjacent to *cox3,* and the ORF region.

Haplotype	Accession no. (*cox2*/*cox3*-ORF)
A	AB499618/AB499641
B	AB499619/AB499642
C	AB499620/AB499643
D	AB499621/AB499644
E	AB499622/AB499645
F	AB499623/AB499646
G	AB499624/AB499647
H	AB499625/AB499648
I	AB499626/AB499649
J	AB499627/AB499650
K	AB499628/AB499651
L	AB499629/AB499652
M	AB499630/AB499653
N	AB499631/AB499654
O	AB499632/AB499655
P	AB499633/AB499656
Q	AB499634/AB499657
R	AB499635/AB499658
S	AB499636/AB499659
T	AB499637/AB499660
U	AB499638/AB499661
V	AB499639/AB499662
W	AB499640/AB499663

In contrast, all of the 15 individuals collected from two localities on Santa Catalina Island had the same haplotype (type-K). This haplotype was also found at Ohma facing the Tsugaru Strait; Shika Island in northern Kyushu (Japan); and at Dadaepo, Pusan (Korea).

To examine genetic relationships among the haplotypes, a statistical parsimony network was created using TCS ver. 1.21 (Clement et al. 2000) based on the DNA sequences of mitochondria haplotypes. The sequence data of each haplotype and the frequency of the haplotypes in each population were used to define groups of populations that were homogeneous geographically and differentiated maximally from each other (spatial analysis of the molecular variance, SAMOVA), using SAMOVA ver. 1.0 (Dupanloup et al. 2002). SAMOVA combines geographic information of each population with AMOVA. A simulated annealing procedure that maximizes the total amount of molecular variance due to differences between groups of geographically homogeneous populations was performed, based on pair-wise differences and for a varying number of groups ($k = 2$–5). For each simulated annealing, 200 permutations were performed. Haplotype diversity (h) and nucleotide diversity (π) were calculated for each SAMOVA group using ARLEQUIN ver. 2.001 (Schneider et al. 2000).

In the SAMOVA analyses, different numbers of groups (two to five groups) of populations were assumed (Table 4). When $k = 3$–5, significant, although weak, genetic differentiation among groups was found ($\phi_{ct} = 0.42859$–0.49999, $P < 0.05$). In these categories ($k = 3$–5), Kumamoto and Miyazaki populations (Kyushu Island, southern Japan) were recognized as genetically differentiated groups.

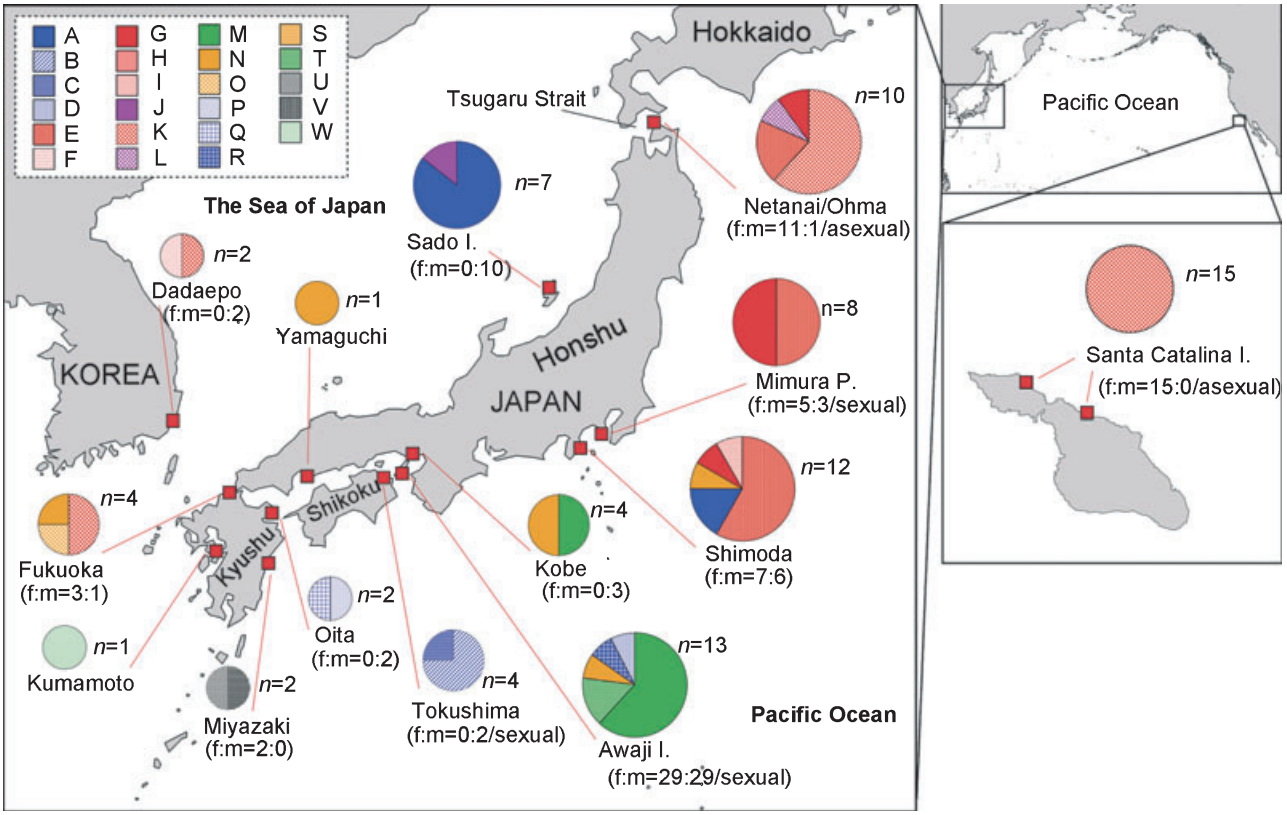

FIG. 1. Distribution and ratio of the haplotypes in the local populations studied based on mitochondrial *cox2* and *cox3* genes, the intergeneric region adjacent to *cox3*, and the open reading frame (ORF) region; sex ratio of female and male gametophytes in local populations; and summary of life-history patterns found in the culture studies.

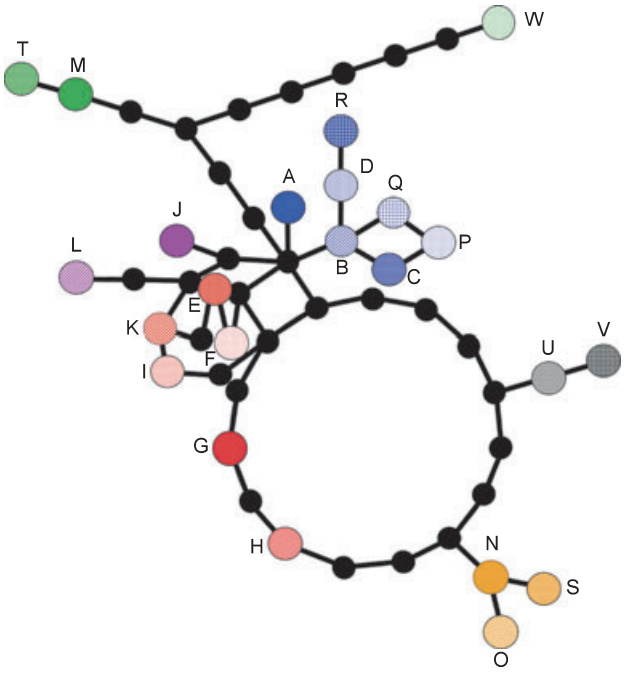

FIG. 2. Haplotype-spanning network tree based on mitochondrial *cox2* and *cox3* genes, the intergeneric region adjacent to *cox3*, and the open reading frame (ORF) region.

TABLE 4. Summary of SAMOVA.

Number of groups (*k*)	Structure tested	ø-statistics
2	Group 1 {10} Group 2 {1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14}	$ø_{ct}$ = 0.50299
3	Group 1 {10} Group 2 {12} Group 3 {1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 13, 14}	$ø_{ct}$ = 0.49999*
4	Group 1 {10} Group 2 {11} Group 3 {12} Group 4 {1, 2, 3, 4, 5, 6, 7, 8, 9, 13, 14}	$ø_{ct}$ = 0.42859*
5	Group 1 {10} Group 2 {12} Group 3 {5, 6} Group 4 {7, 8, 11} Group 5 {1, 2, 3, 4, 9, 13, 14}	$ø_{ct}$ = 0.42952*

The *k*-values represent number of groups hypothesized. The populations included in each SAMOVA group are described using the population code in Table 1.

**P*-value is <0.05.

In addition, nucleotide diversity (π) in southern Japan and Korea (populations 5–13, $\pi = 0.005852 \pm 0.003107$) was larger than that in northern Japan (populations 1–4, $\pi = 0.002594 \pm 0.001504$). These results might reflect the parthenogenetic reproduction occurring in the populations in northern Japan.

Isolation by distance was tested to evaluate the frequency of long-distance dispersal by rafting; frequent long-distance dispersal should decrease the correlation between genetic differentiations and geographic distance. Isolation by distance was tested using IBDWS ver. 3.14 (Jensen et al. 2005). The correlations between geographic distance and the genetic distance among populations (measure based on Phist) were performed for either normal or logarithmic axis, with 10,000 randomizations, for the whole Pacific and Seto Inland Sea populations in Japan (1, 3, 4, 5, 6, 7, 10, and 11), excluding one population (8) with only one specimen for molecular study.

Cutleria species exhibit a heteromorphic life history alternating between dioecious gametophytes with macroscopic erect thalli and minute crustose (*Aglaozonia* type) sporophytes (Falkenberg 1879, Kitayama et al. 1992). Theoretically, meiosis in the unilocular zoidangia (meiosporangia) results in equal numbers of male and female unizoids (meiospores), and hence the sex ratio of resultant gametophytes is 1:1. To examine the actual sex ratio of each local population, thin sections of the gametangial portion of erect thalli (gametophytes) were examined under a dissecting microscope (Stemi SV6, Carl Zeiss AG, Oberkochen, Germany), and the sex of the plurilocular gametangia was determined. The following specimens were examined: Japan (102 individuals from 14 localities), Korea (two individuals from one locality), and California (15 individuals from two localities). The ratios of female and male gametophytes in these populations are shown in Figure 1 together with a summary of the life-history patterns as described below (i.e., sexual vs. asexual). Both female and male gametophytes were recorded in populations where more than two individuals were examined, except for Sado Island (female:male = 0:10) in the Sea of Japan and Netanai/Ohma (f:m = 11:1) facing the Tsugaru Strait, and Santa Catalina Island, California, USA (f:m = 15:0). At Netanai/Ohma, as reported in Kitayama et al. (1992), only female gametophytes (10) were found at Netanai, and the single male individual was collected at Ohma. It is unlikely that male gametophytes ever dominate in field populations, because the parthenogenetic development of male gametes is very rare (Kitayama et al. 1992). Therefore, the occurrence of solely male gametophytes at Sado Island is considered an artifact of sampling. In contrast, the dominance of female gametophytes at Netanai/Ohma and Santa Catalina Island reflects dominance of an asexual direct type of life history as found in our culture study.

For the culture study, the following specimens were used (Table 1): Japan (11 individuals from four localities) and Santa Catalina Island (five individuals from two localities). For examining the occurrence of parthenogenetic reproduction (i.e., occurrence of erect thalli from the prostrate disks [morphological gametophytes]), female gametes were isolated from fertile female gametangia and cultured in polystyrene petri dishes. In culture, the development of the prostrate thalli was observed for 3 months under 15°C long day (16:8 light:dark [L:D]) culture conditions (Kitayama et al. 1992).

In culture, three strains from Netanai, Tsugaru Strait, as well as five strains from Santa Catalina Island developed into small disks within several days after the settlement of the female gametes, and uniseriate erect filaments developed directly from the disk. The erect filaments became parenchymatous by trichothallic growth and within 3 months developed into large erect thalli comparable to field specimens. The erect thalli formed female gametangia on the thallus and multiplied by parthenogenesis. The ability of female gametes to fuse with male gametes was not examined. In contrast, other strains developed into *Aglaozonia*-type disks attaining up to 5 mm in diameter. Therefore, the Netanai and Santa Catalina populations have an asexual (direct type) life history with parthenogenetic female gametes, as reported by Kitayama et al. (1992), whereas other populations exhibit a sexual, heteromorphic life history. It is noteworthy that only parthenogenetic populations of *C. cylindrica* and *C. multifida* became established outside their native range.

On the basis of these results, we conclude that the disjunct population of *C. cylindrica* in California originated from a relatively recent introduction from Japan, most likely from the parthenogenetic population in the Tsugaru Strait. The mechanism for the introduction of *C. cylindrica* to California remains unknown. The presence of another Japanese macroalga, *Sargassum filicinum*, was observed in Long Beach Harbor on the California mainland in 2003; in Baja California, Mexico, in 2005; and on Santa Catalina Island in 2006 (Aguilar-Rosas et al. 2007, Miller et al. 2007). As with *S. filicinum*, it is likely that *C. cylindrica* was introduced to the mainland and then was secondarily transported to Santa Catalina Island, possibly as a hitchhiker on boat hulls.

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