

## First record of *Clavelina lepadiformis* (Clavelinidae: Aplousobrachia), an invasive Atlantic form, from Wakayama Prefecture, Japan

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### Abstract

The colonial ascidian *Clavelina lepadiformis* (Müller, 1776), commonly known as the light bulb tunicate, is of concern as an invasive species due to its extensive distribution from its original habitat in the northeast Atlantic. This study reports the first discovery of *C. lepadiformis* in Kushimoto, Wakayama Prefecture, Japan. Molecular phylogenetic analysis using the mitochondrial cytochrome oxidase c subunit I (COI) gene sequences indicated that *C. lepadiformis* from Kushimoto sample belonged to the Atlantic clade. The present study improves our understanding of the geographic distribution of this species in Japan, adds to our knowledge of its invasion dynamics within the country, and suggests the potential for ongoing environmental impacts. Continuous monitoring is needed to further elucidate the dispersal mechanisms and ecological impacts of this invasive species.

**Key words:** Tunicata; Urochordata; Pacific Ocean; Kumano Sea; intraspecific variation

### Introduction

The colonial ascidian *Clavelina lepadiformis* (Müller, 1776), commonly called the lightbulb tunicate, is known as an invader initially described from the type locality Norway (Müller 1776). Its original distribution area was thought to be from the Shetland Islands and Bergen, Norway in the north to the Bay of Biscay, the Mediterranean, and the Adriatic in the south (Berrill 1951). This species has been reported from all around the world: Portugal (Azores and Madeira) (Wirtz and Martins 1993; Wirtz 1998), South Africa (Knysna Estuary and Port Elizabeth) (Primo and Vazquez 2004; Robinson et al. 2005), Connecticut (Stonington) (Reinhardt et al. 2010), and Korea (Bangeojin, Daebyeon, Gampo, and Seogwipo) (Pyo and Shin 2011). A recent study of *C. lepadiformis* based on mitochondrial DNA sequence data (Tarjuelo et al. 2001) showed that, within the Mediterranean Sea, populations inhabiting harbors (interior form) were genetically distinct from those thriving in the open habitats (exterior form). Phylogenetic and phylogeographic analyses

supported that *C. lepadiformis* originated in the Atlantic and colonized the Mediterranean (Turon et al. 2003). The analyses also indicated that the interior form and the Atlantic form together comprise a single clade, known as the Atlantic clade (Turon et al. 2003). It was suggested that the interior and exterior forms, which are morphologically indistinguishable, might represent cryptic species (Turon et al. 2003). In this study, following Nishikawa (2017), I define the group characterized by the biological features—not only morphological but also ecological, genetic, and others—of the Atlantic and Mediterranean interior populations as the Atlantic form, and the group characterized by those of the Mediterranean exterior populations as the Mediterranean form.

In Japan, the Atlantic form of *C. lepadiformis* species complex *sensu* Turon et al. (2003) has been documented at three locations (Fig. 1). The Atlantic form invaded Orido Bay at least 27 years ago, according to label information for specimens of this species in the National Museum of Nature and Science, Tsukuba (cf. Nishikawa and Namikawa

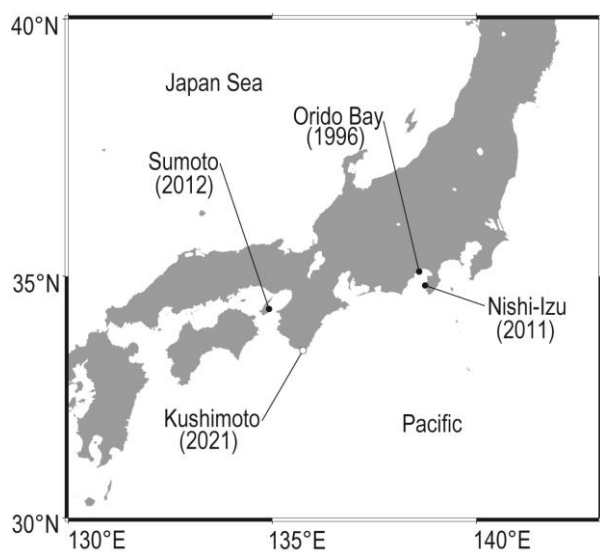


Fig. 1. Map indicating sites where *Clavelina lepadiformis* was recorded in Japan. Closed circles show sites where *C. lepadiformis* has been recorded in previous studies; the open circle marks Kushimoto, where the sample for this study was collected. The numbers under the site names indicate the years that the species was first detected at each site.

2018). Additional records include observations from Nishi-Izu, Suruga Bay, in 2011 (Nishikawa 2017) and collections made at Oishi Port in Sumoto, Awajishima Island, in 2012 (Nishikawa and Namikawa 2018). Conversely, the Mediterranean form has not yet been reported in Japan (Nishikawa 2017).

During a survey of marine invertebrate fauna conducted May 11–14, 2021 (Hasegawa et al. 2022) in Kushimoto, Wakayama Prefecture, Japan, a colony of this species was found in Fukuro Port (Fig. 1: 33°28'31.5" N; 135°46'28.7" E). Here, I present the first report of the invasive species *C. lepadiformis* from Wakayama Prefecture, Japan, with a phylogenetic analysis to confirm whether the colony is the Atlantic or Mediterranean form.

## Materials and Methods

### Sampling and mitochondrial COI sequencing

Zooids from a massive colony attached to a rope for mooring a boat were sampled by hand in Fukuro Port,

Kushimoto, Wakayama Prefecture, Japan on May 12, 2021. After they were anesthetized with menthol, one (designated as Kushimoto sample) of the zooids was preserved in 99% ethanol for DNA extraction. Other zooids were fixed in 10% formalin for morphological observations. The specimen has been deposited in the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo, Japan, under the ID: ICHUM 8847. Total DNA was extracted from a portion of thoracic tissue from a zooid following Hasegawa and Kajihara (2019). Amplification, purification, and sequencing of a partial sequence of the mitochondrial cytochrome oxidase *c* subunit I (COI) gene were conducted following Hasegawa and Kajihara (2024). The primer pair *dinF*/*Nux1R* (Brunetti et al. 2017) was used for amplifying a partial sequence of COI gene by PCR. PCR conditions were as follows: 94°C for 2 min; 35 cycles of 94°C for 45 sec, 46°C for 50 sec, and 72°C for 80 sec; with a final extension at 72°C for 5 min. The PCR product was purified by an enzymatic reaction: 24 mU/μL of Exonuclease I (TaKaRa, Kusatsu, Japan) and 4.9 mU/μL of Shrimp Alkaline Phosphatase (TaKaRa). Each dye terminator reaction was conducted using a BigDye Terminator ver. 3.1 Cycle Sequence Kit (Thermo Fisher Scientific) with either the primer *dinF* (Brunetti et al. 2017) or *Nux1R* (Brunetti et al. 2017). Capillary electrophoresis was performed with 3730 Genetic Analyzer (Thermo Fisher Scientific). Base calling was performed with GeneStudio Professional Edition ver. 2.2.0.0 (GeneStudio).

### Phylogenetic analysis

COI sequences of *Clavelina* species were obtained from the International Nucleotide Sequence Database Collaboration (INSDC) for phylogenetic analysis, along with the sequences of the sister taxon *Pycnoclavella*, which served as an outgroup, represented by *P. communis*, *P. flava*, and *P. producta* (Table 1). Among the registered sequences, those that did not produce long branches in the phylogenetic tree

Table 1. COI sequences for each *Clavelina* and *Pycnoclavella* species obtained from the International Nucleotide Sequence Database Collaboration (INSDC).

OTU	Accession number	Locality of <i>C. lepadiformis</i> samples	References
<i>Clavelina arafurensis</i> Tokioka, 1952	AM706463	—	Pérez-Portela and Turon (2008)
<i>Clavelina australis</i> (Herdman, 1899)	AM706464	—	Pérez-Portela and Turon (2008)
<i>Clavelina breve</i> C. Monniot, 1997	AM706465	—	Pérez-Portela and Turon (2008)
<i>Clavelina delavallei</i> (Zirpolo, 1925)	AY603105	—	Turon and Lopez-Legentil (2004)
<i>Clavelina lepadiformis</i> (Müller, 1776), interior form	AF368352	Spain (Mediterranean coast)	Tarjuelo et al. (2001)
<i>Clavelina lepadiformis</i> (Müller, 1776), exterior form	AF368353	Spain (Mediterranean coast)	Tarjuelo et al. (2001)
<i>Clavelina lepadiformis</i> (Müller, 1776), haplotype IX	AY211529	Azores Islands and Spain (Atlantic and Mediterranean coast)	Turon et al. (2003)
<i>Clavelina lepadiformis</i> (Müller, 1776), haplotype XII	AY211530	Sesimbra, Spain	Turon et al. (2003)
<i>Clavelina lepadiformis</i> (Müller, 1776), haplotype XIII	AY211531	Azores Islands	Turon et al. (2003)
<i>Clavelina lepadiformis</i> (Müller, 1776), haplotype CL5	HM012482	southeastern Connecticut	Reinhardt et al. (2010)
<i>Clavelina lepadiformis</i> (Müller, 1776), haplotype CL15	HM012483	southeastern Connecticut	Reinhardt et al. (2010)
<i>Clavelina lepadiformis</i> (Müller, 1776), Kushimoto	LC813233	Kushimoto	Present study
<i>Clavelina oblonga</i> Herdman, 1880	AY603106	—	Turon and Lopez-Legentil (2004)
<i>Pycnoclavella communis</i> Pérez-Portela et al., 2007	MW363028	—	Salonna et al. (2021)
<i>Pycnoclavella flava</i> (F. Monniot, 1988), haplotype Pfl1	AM706475	—	Pérez-Portela and Turon (2008)
<i>Pycnoclavella flava</i> (F. Monniot, 1988), haplotype Pfl3	AM706477	—	Pérez-Portela and Turon (2008)
<i>Pycnoclavella producta</i> (Milne Edwards, 1841)	AM706482	—	Pérez-Portela and Turon (2008)

were selected. The alignment was manually edited on MEGA ver. 11.0.13 (Tamura et al. 2021). P-distance and Kimura's 2-parameter (K2P) distance (Kimura 1980) between all the pairs of the species in the dataset were calculated with MEGA ver. 11.0.13 (Tamura et al. 2021). By using PartitionFinder ver. 2.1.1 (Lanfear et al. 2016), the best-fit substitution model was selected for each partition: TIM + I for the first codon positions, GTR + I for the second, and K81UF + G for the third. Maximum-likelihood (ML) analysis was conducted using IQ-TREE ver. 2.2.2.6 (Minh et al. 2020) with the above-mentioned partition scheme. Branch-supported values evaluating 1000 ultrafast (UF) bootstrap replicates were calculated by the ultrafast bootstrap method implemented in IQ-Tree ver. 2.2.2.6 (Minh et al. 2020). Bayesian inference was performed with MrBayes ver 3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003; Ronquist et al. 2012) for inferring a phylogenetic

relationship among the OTUs. Four Markov chains starting from a random tree were parallelly run with  $1.0 \times 10^6$  generations and sampling frequency every 100 generations. A first quarter of the total trees were discarded by a burn-in fraction. A consensus tree was obtained by using the "sumt" command; the posterior probabilities (PPs) for interior branches were calculated to assess the inference. Tracer ver. 1.6 (Rambaut et al. 2014) was used for evaluating run convergence.

## Results

The *Clavelina lepadiformis* formed a single clade in the phylogenetic tree, supported by a ultrafast (UF) bootstrap value of 86 and a posterior probability (PP) of 1.00 (Fig. 2); within this clade, it was divided into the Atlantic and Mediterranean forms. *Clavelina lepadiformis* from Kushimoto was included in the clade, which consisted solely of the haplotypes

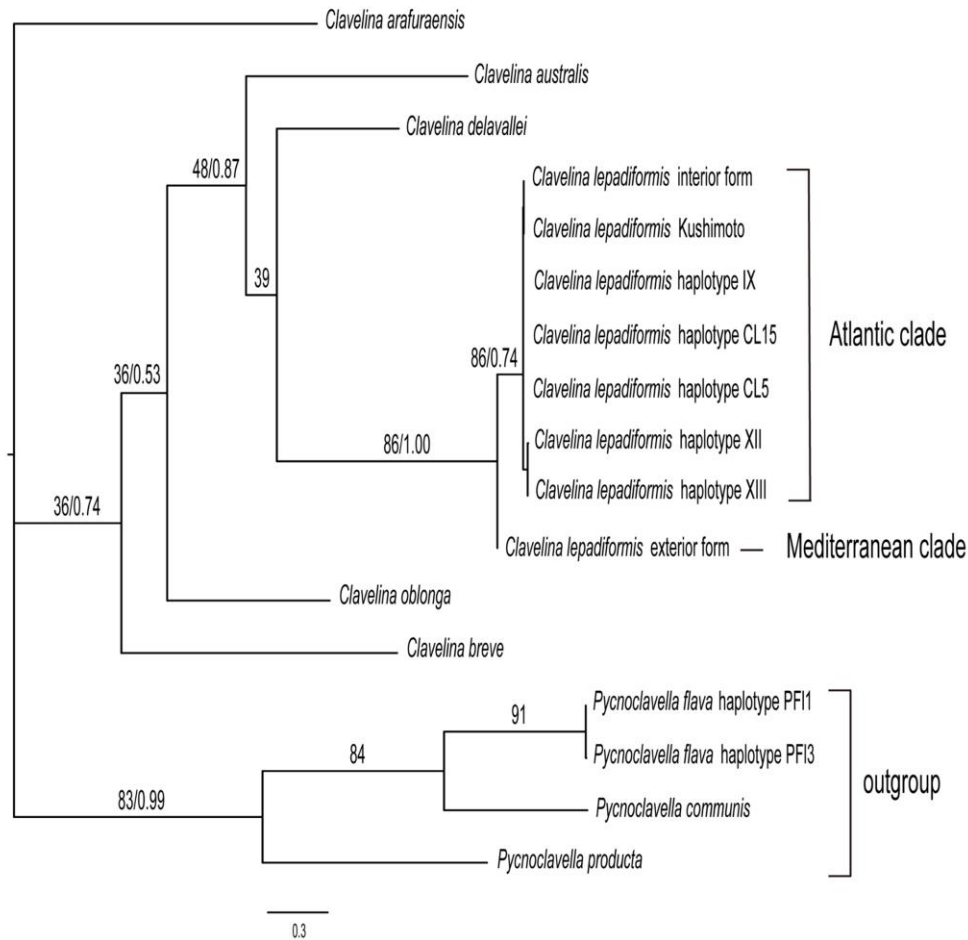


Fig. 2. ML tree indicating intraspecific and interspecific relationships among *Clavelina* species. Details of each OTU are described in Table 1.

regarded as Atlantic forms by Turon et al. (2003) and Reinhardt et al. (2010). This clade, comprising the species from Kushimoto and the Atlantic form, was supported by a UF bootstrap value of 86 and a PP of 0.74.

The genetic distances between the Kushimoto sample and the other five *Clavelina* species ranged from 14.63 to 17.89 % (mean 16.91%) in p-distance, and from 18.83 to 23.96 % (mean 22.41%) in K2P distance. As for intraspecific variation in *C. lepadiformis*, the genetic distances between the Kushimoto sample and the other haplotypes in the Atlantic clade ranged from 0.00 to 2.17 % (mean 0.81 %) in p-distance, and from 0.00 to 2.25 % (mean 0.84 %) in K2P distance. The p-distance and K2P distance between the Kushimoto sample and the Mediterranean exterior form were 5.68 % and 5.15 %, respectively.

## Discussion

Based on the topology of the phylogenetic tree (Fig. 2) and genetic distances, the Kushimoto sample was assigned to the Atlantic form. Nishikawa (2017) stated that at the 2013 Annual Meeting of the Zoological Society of Japan, a paper presentation (Nishikawa et al. 2013) showed that *C. lepadiformis* from Suruga Bay belonged to the Atlantic form, not the Mediterranean form. Although the data in the presentation from that time remains unpublished, if this is true, then the present study would be the second instance of the Atlantic form of *C. lepadiformis* being recorded in Japan. Including international reports, this would be the fourth instance reported molecularly as an Atlantic form, following Spain (Tarjuelo et al. 2001; Turon et al. 2003), Connecticut (Reinhardt et al. 2010), and Suruga Bay (Nishikawa 2017).

Nishikawa (2018) discussed the possible invasion routes for *C. lepadiformis*; however, since it has been discovered that the species complex is also distributed in Osaka Bay and the Kumano-nada Sea, pinpointing the invasion routes is challenging. There have also been reports of this species complex in Korea (Pyo and Shin 2011), suggesting the possibility of a widespread invasion in East Asia. This report indicates the need for continuous and extensive surveys of ascidian fauna to better understand the mechanisms of invasion and ecological consequences of *C. lepadiformis* in East Asia, particularly in Japan.

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