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| タイトル | Molecular and morphological discrimination between an invasive ascidian, Ascidiella aspersa, and its congener A. scabra (Urochordata: Ascidiacea) |
| 別タイトル | 侵入性のヨーロッパザラホヤ<I>Ascidiella aspersa</I>とその同属種<I>A. scabra</I>の分子と形態による識別 |
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# Molecular and Morphological Discrimination Between an Invasive Ascidian, *Ascidiella aspersa*, and Its Congener *A. scabra* (Urochordata: Ascidiacea)

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**The solitary ascidian *Ascidiella aspersa* (Müller, 1776) has sometimes been regarded as conspecific with *A. scabra* (Müller, 1776), although previous detailed morphological comparisons have indicated that the two are distinguishable by internal structures. Resolution of this taxonomic issue is important because *A. aspersa* has been known as a notoriously invasive ascidian, doing much damage to aquaculture e.g. in Hokkaido, Japan. We collected many specimens from European waters (including the Swedish coast, near the type localities of these two species) and Hokkaido, Japan (as an alien population) and made molecular phylogenetic analyses using the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, and found that in terms of COI sequences all the analyzed specimens were clustered into two distinct groups, one of which is morphologically referable to *A. aspersa* and the other to *A. scabra*. Thus, these two species should be regarded as distinct from each other.**

**Key words:** *Ascidiella aspersa*, *Ascidiella scabra*, invasive ascidian, molecular analysis, morphology, species, taxonomy

## INTRODUCTION

Precise identification of specimens is a primary and crucial basis for biological studies related to populations from the wild, including alien species. In September 2008, a solitary ascidian was found densely covering cultured scallops in Funka (= Uchiura) Bay on the Pacific coast of Southwest Hokkaido, northern Japan, severely damaging the aquaculture activities. It was originally considered to be a common native ascidian, *Ascidia zara* Oka, 1935, but later one of us (TN) identified it as *Ascidiella aspersa* (Müller, 1776) on the basis of morphological information (Kanamori et al., 2012). This seems to be the first record of this invasive ascidian in the northern Pacific Ocean. At that time, this species was distinguished from a congener, *A. scabra* (Müller, 1776), based on differences in internal morphological features (Nishikawa and Otani, 2004). However, Hartmeyer (1924) treated *A. scabra* as a junior synonym of *A. aspersa*, while Harant and Vernières (1933) and Ärnbäck-Christie-Linde (1934) regarded *A. scabra* as merely a form or variety, respectively, of *A. aspersa*, and more recently Sanamyan (2012) treated *A. scabra* as a junior synonym of *A. aspersa* in the Ascidiacea World Database, though Sanamyan and Monniot (2013a, b) accepted both as valid. This disagreement should be resolved, especially given the fact that *A. aspersa* is regarded as a notorious invasive species in the Global Invasive Species Database (2013). Any species-level differences in ecological traits could necessitate different approaches to the control of individual invasive species. This paper aims to test whether these two nominal species are distinct using DNA information, and then to confirm the reliability of the morphological differences that have been suggested as diagnostic characters to distinguish one from

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the other.

## TAXONOMIC BACKGROUND

*Ascidiella aspersa* and *A. scabra* were originally described as separate species by Müller (1776) as the binomina *Ascidia aspersa* and *Ascidia scabra*, respectively, each accompanied by a brief Latin description. Their detailed descriptions were given by Müller (1784), with their geographical distribution as "in sinu Christiansandensi" [= in the Bay of Kristiansand, Norway, in the Skagerrak strait] for the former species, and "in sinu Christianiensi Norvegiae" [= in the Bay of Norwegian Christiania, presently Oslo Bay, also in the Skagerrak] for the latter. Therefore, their type localities are the Norwegian coasts of the Skagerrak. The genus *Ascidiella* is considered to be unique within the family Ascidiidae in the absence of secondary branchial papillae on the internal longitudinal vessels (e.g., Berrill, 1950). After a long history of taxonomic and nomenclatural confusion, the genus is now considered to consist of three species, *A. aspersa*, *A. scabra*, and *A. senegalensis* Michaelsen, 1914, while the former two have occasionally been regarded as synonymous (see above).

Lindsay and Thompson (1930) made a significant contribution to the morphological distinction between *A. aspersa* and *A. scabra* through comparative studies of various characters among numerous European specimens. They concluded that *A. aspersa* has "more longitudinal vessels than tentacles", while *A. scabra* has "fewer longitudinal vessels than tentacles" (p. 32), insofar as specimens longer than 10 mm are concerned, and further that the outer follicle cells of the egg were markedly larger and far fewer in *A. aspersa* (28–32 cells in optical section) than in *A. scabra* (with 70–80 cells). Eggs of *A. aspersa* float in seawater, while those of *A. scabra* sink (Berrill, 1928). These differences were accepted by taxonomists such as Berrill (1950), Millar (1966, 1970), etc. As noted by Nishikawa and Otani (2004), the number of longitudinal vessels is evidently that of the vessels on each side of the branchial basket, instead of their total number, because "the longitudinal bars [= vessels] were enumerated in a transverse direction in the right half of the branchial sac" (Lindsay and Thompson, 1930, p. 11). As the follicle cell features are unavailable in immature, non-reproductive, or poorly preserved specimens, emphasis should be put on the number of oral tentacles in total and that of longitudinal vessels on each side.

## MATERIALS AND METHODS

### Materials

Specimens of the genus *Ascidiella*, 50 in total, were collected from: near Kristineberg (Fiskebäckskil), west coast of Sweden in the south-eastern Skagerrak, by JB on 19 and 21 July 2011 (eight individuals from the same hauls, labeled Sweden001 to 008); Plymouth, England, by JB on 22 September 2010 (12, Plymouth001 to 012); Vilanova i Geltrú, Mediterranean coast of Spain, by MC Pineda on 21 July 2010 (11, Spain001 to 011); Arenys de Mar, Mediterranean coast of Spain, by XT on 27 July 2010 (nine, Spain012 to 020); Blanes, Mediterranean coast of Spain, by XT on 17 February 2006 (two, Spain021 to 022); and off Yakumo-cho, Funka Bay, Hokkaido, Japan, by KB on 19 January 2010 (eight, Hokkaido001 to 008). The specimens, except those from Hokkaido, were preserved whole in 99.5% ethanol, while those from Hokkaido were kept cold and fixed with ca. 8% seawater-formalin, after small pieces of tissue had been sampled and preserved in ethanol for DNA analysis. For molecular comparison, six specimens of *Ascidia zara* were collected from Matsushima Bay, Miyagi Prefecture, Japan, and preserved in 99.5% ethanol.

### Morphological examination and analysis

Body length was measured with a vernier caliper before dissection. Using a stereoscopic microscope, the inner structure of branchial sac was observed to confirm the generic affiliation and then to count all the oral tentacles (including minute ones) and the inner longitudinal vessels on each side. The inner surface of branchial sac was lightly stained with Delafield's haematoxylin when necessary. Statistical analyses were made on the correlations between the number of oral tentacles and the body length using Spearman rank-order correlation, and on the difference between the number of longitudinal vessels on each side using Wilcoxon signed-rank test, using the statistical software package R, version 2.15.0 (R Development Core Team 2012).

### DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from mantle and branchial sac of each specimen using the QuickGene DNA tissue kit S (DT-S) and the QuickGene-Mini80 (Fuji Film, Japan).

For the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, DNA fragments were amplified by polymerase chain reactions (PCRs) using a newly designed primer set based on the sequence in GenBank (accession number AY116600), namely Asc.COI-ForC: 5′-TTATTCGGGTTGAGTTATCTCA-3′ and Asc.COI-RevC: 5′-TCAAAAAGAAGCATAGTAATAGC-3′ for the genus *Ascidiella*, or using the previously published primer set LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′ and HCO2198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (Folmer et al., 1994) for *Ascidia zara*. The latter fragment covered a 658-base COI partial sequence, which included the region amplified by the former primer set (484 bp).

PCRs were performed in a 20-μl total reaction volume with 1.0 μl of each primer (10 μM), 2.0-μl dNTPs (2.5 mM each), 2.0-μl 10×Ex Taq Buffer (TaKaRa Bio), 0.1-μl ExTaq Hot Start DNA polymerase (5 U/μl, TaKaRa Bio), 12.9-μl distilled water and 1.0-μl template DNA. A single soak at 94°C for 5 min was followed by 40 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min, then 72°C for 7 min on a GeneAmp PCR System 9700 (Applied Biosystems). Amplified PCR products were purified with an ExoStar enzymatic PCR purification system (GE Healthcare). Purified products were sequenced on an ABI 3130XL sequencer (Applied Biosystems) using the ABI Prism BigDye Terminator v3.1 (Applied Biosystems).

### Molecular phylogenetic analysis

We sequenced and aligned 484 nt of partial mitochondrial COI for the specimens of *Ascidiella* and *Ascidia zara*, together with sequences of *A. aspersa* in GenBank as AY116600 from Roscoff, France (Stach and Turbeville, 2002), JQ742948 and JQ742949 from South Korea (Pyo et al., 2012), and HF548561 from Venice, Italy (Rubinstein et al., 2013), as well as those of some species of the genus *Phallusia* in GenBank. The sequences of *Ascidiella* and *A. zara* from the 56 specimens analyzed here and the four thus cited from GenBank were classified into 24 haplotypes numbered from 1 to 24 in order of frequency in each cluster (Table 1); HF548561 was assigned to the number 8 (shared with the Sweden021 specimen), JQ742948 to 15, JQ742949 to 16, and AY 116600 to 17. Sequence alignment was carried out using Clustal W in MEGA5 (Tamura et al., 2011). MEGA5 was also used to choose the best-fit model for the data set with the Bayesian information criterion.

Using *Styela clava* (Styelidae) and *Halocynthia roretzi* (Pyuridae) as outgroups, phylogenetic trees were generated by the maxi-

**Table 1.** Specimens of the genus *Ascidiella* used for the present analysis, with accession number of COI sequence, haplotype designation, body length, and some meristic characters in internal structure; "?" or "+" indicate that a value is uncertain or is an incomplete count, respectively.

| Species Name | Specimen Number | DNA Accession Number of COI | Haplotype designation | Locality | Body Length (mm) | Number of tentacles | Number of longitudinal vessels on left and right sides |
|---|---|---|---|---|---|---|---|
| *A. scabra* | Sweden001 | AB794962 | 19 | Kristineberg, west coast of Sweden | 20 | 40 | 31 and 36 |
| *A. scabra* | Sweden004 | AB794963 | 18 | | 24.5 | 50 | 29 and 38 |
| *A. scabra* | Sweden005 | AB794964 | 18 | | 24 | 50 | 24 and 30 |
| *A. scabra* | Sweden006 | AB794965 | 18 | | 28 | 30 | 25 and 31 |
| *A. scabra* | Sweden007 | AB794966 | 18 | | 26 | 33 | 25 and 37 |
| *A. scabra* | Sweden008 | AB794967 | 18 | | 19 | 40 | 25 and 30 |
| *A. aspersa* | Sweden002 | AB794654 | 4 | | 27 | 28 | 33 and 42 |
| *A. aspersa* | Sweden003 | AB794655 | 6 | | 48 | 23 | 36 and 46 |
| *A. aspersa* | Plymouth001 | AB794942 | 1 | Plymouth, England | 65 | 13 | 34 and 39 |
| *A. aspersa* | Plymouth002 | AB794943 | 1 | | 50 | 17 | 34 and 37 |
| *A. aspersa* | Plymouth003 | AB794944 | 1 | | 35 | 17 | 32 and 40 |
| *A. aspersa* | Plymouth004 | AB794945 | 4 | | 35 | 20 | 38 and 41 |
| *A. aspersa* | Plymouth005 | AB794946 | 1 | | 36 | 16 | 30 and 35 |
| *A. aspersa* | Plymouth006 | AB794947 | 4 | | 26.5 | 18 | 30 and 38 |
| *A. aspersa* | Plymouth007 | AB794948 | 1 | | 32 | 15 | 31 and 36? |
| *A. aspersa* | Plymouth008 | AB794949 | 1 | | 23 | 18 | 31 and 38 |
| *A. aspersa* | Plymouth009 | AB794950 | 1 | | 33 | 21 | 32 and 37 |
| *A. aspersa* | Plymouth010 | AB794951 | 1 | | 38 | 22 | 31 and 37 |
| *A. aspersa* | Plymouth011 | AB794952 | 13 | | 33 | 17 | 30 and 40 |
| *A. aspersa* | Plymouth012 | AB794953 | 14 | | 20 | 16 | 29 and 35 |
| *A. aspersa* | Spain001 | AB794920 | 9 | Vilanova i la Geltrú, Mediterranean coast of Spain | 32 | 18 | 32 and 40 |
| *A. aspersa* | Spain002 | AB794921 | 2 | | 24.5 | 21 | 28 and 37 |
| *A. aspersa* | Spain003 | AB794922 | 10 | | 21.5 | 30 | 30 and 35 |
| *A. aspersa* | Spain004 | AB794923 | 3 | | 17 | 27 | 23+ and 22+ |
| *A. aspersa* | Spain005 | AB794924 | 5 | | 40 | 14 | 31 and 33 |
| *A. aspersa* | Spain006 | AB794925 | 1 | | 33.5 | 22 | 29 and 32 |
| *A. aspersa* | Spain007 | AB794926 | 7 | | 30.5 | 23 | 30 and 36 |
| *A. aspersa* | Spain008 | AB794927 | 3 | | 28 | 18 | 26 and 33 |
| *A. aspersa* | Spain009 | AB794928 | 5 | | 27.5 | 22 | 30 and 40 |
| *A. aspersa* | Spain010 | AB794929 | 5 | | 25 | 27 | 29 and 38 |
| *A. aspersa* | Spain011 | AB794930 | 2 | | 17.5 | 22 | 28 and 34 |
| *A. aspersa* | Spain012 | AB794931 | 1 | Arenys de Mar, Mediterranean coast of Spain | 21.5 | 23 | 26 and 40 |
| *A. aspersa* | Spain013 | AB794932 | 2 | | 25 | 21 | 15+ and 20+ |
| *A. aspersa* | Spain014 | AB794933 | 2 | | 15 | 14 | 23 and 31 |
| *A. aspersa* | Spain015 | AB794934 | 5 | | 15 | 18 | 22 and 28+ |
| *A. aspersa* | Spain016 | AB794935 | 2 | | 25 | 17 | 30 and 34 |
| *A. aspersa* | Spain017 | AB794936 | 7 | | 25 | 20 | 29 and 32 |
| *A. aspersa* | Spain018 | AB794937 | 11 | | 21.5 | 18 | 30 and 38 |
| *A. aspersa* | Spain019 | AB794938 | 1 | | 17.5 | 20 | 29 and 35 |
| *A. aspersa* | Spain020 | AB794939 | 12 | | 15 | 16 | 30 and 32 |
| *A. aspersa* | Spain021 | AB794940 | 8 | Blanes Harbour, Mediterranean coast of Spain | 36.5 | 28 | 41 and 48 |
| *A. aspersa* | Spain022 | AB794941 | 2 | | 30 | 26 | 38 and 41 |
| *A. aspersa* | Hokkaido001 | AB794912 | 1 | Off Yakumo-cho, Funka Bay, Hokkaido, Japan | 68 | 22 | 28 and 45 |
| *A. aspersa* | Hokkaido002 | AB794913 | 6 | | 78 | 21 | 30 and 42 |
| *A. aspersa* | Hokkaido003 | AB794914 | 3 | | 64 | 22 | 34 and 38 |
| *A. aspersa* | Hokkaido004 | AB794915 | 1 | | 64 | 18 | 27 and 36 |
| *A. aspersa* | Hokkaido005 | AB794916 | 1 | | 64 | 20 | 33 and 38 |
| *A. aspersa* | Hokkaido006 | AB794917 | 4 | | 67 | 22 | 35 and 40 |
| *A. aspersa* | Hokkaido007 | AB794918 | 1 | | 73 | 20 | 30 and 35 |
| *A. aspersa* | Hokkaido008 | AB794919 | 3 | | 63 | 19 | 32 and 38 |

mum likelihood (ML) method implemented in MEGA5, based on the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985). A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+*G*, parameter = 0.528), and the rate variation model allowed for some sites to be evolutionarily invariable (+[*I*], 19.87% sites). We employed a heuristic tree search method by

the nearest-neighbor-interchange (local rearrangement with a neighbor-joining tree as a starting tree) for the ML tree. Bootstrap probabilities were obtained from 1000 replications of sequence resampling.

## RESULTS

### Phylogenetic and genetic analysis

The log likelihood value of the ML tree for COI was –3142.54. The ML tree indicated five distinct primary clusters, including those of *Phallusia mammillata*, *Ascidia zara* and the outgroups, supported by 100% bootstrap probability (Fig. 1). All the *Ascidiella* specimens analyzed were included in two of the five distinct clusters, and further constituted a secondary cluster supported also by 100% probability. The larger cluster of *Ascidiella* contained the haplotypes 1–17 from the *Ascidiella* specimens of Sweden 002 and 003, Spain 001–022, Plymouth 001–012, and Hokkaido 001–008, as well as the sequence data of AY116600, JQ742948, JQ742949, and HF548561 in GenBank. The smaller cluster of *Ascidiella* was composed of the haplotypes 18 and 19 from the *Ascidiella* specimens of Sweden 001, 004–008, and the third cluster was composed of the haplotypes 20–24 from the *Ascidia zara* specimens of Matsushima001–006. The sequences of *P. mammillata*, *P. ingeria*, and *P. fumigata* from GenBank formed a paraphyletic group, though the bootstrap values of respective nodes were rather low, except that of *P. mammillata* itself.

In the COI tree, the larger cluster of *Ascidiella* was genetically distant from the smaller cluster by 71 to 75 bases (14.7 to 15.5%) among the 484 bases aligned. Within the larger cluster, the genetic variation ranged from 0 to 19 bases (0 to 3.93%) among the 484 bases aligned; when the Korean OTU (JQ742948) was removed, the remaining variation ranged from 0 to only 6 bases (0 to 1.24%). Genetic distances between the Korean (JQ742948) and other OTUs were from 15 to 19 bases. On the other hand, within the smaller cluster, the genetic variation ranged from 0 to 1 base (0 to 0.02%) among the 484 bases aligned.

Thus, all the analysed *Ascidiella* specimens were grouped on the basis of COI into two distinct clusters, well separated from *Ascidia* and *Phallusia* specimens. Notably, sympatric Swedish specimens were sorted into the two distinct clusters. Accordingly, these two clusters represent different species of the same genus.

### Morphological analysis of *Ascidiella* specimens and their specific affiliation

Table 1 shows the body length, the total number of oral tentacles, and the number of inner longitudinal vessels on each side, and these data (excluding four specimens from which definite counts could not be obtained) are plotted on Fig. 2. These morphological data indicate a marked difference in the number of tentacles (Fig. 2A) between the members of the larger and smaller clusters of *Ascidiella* detected

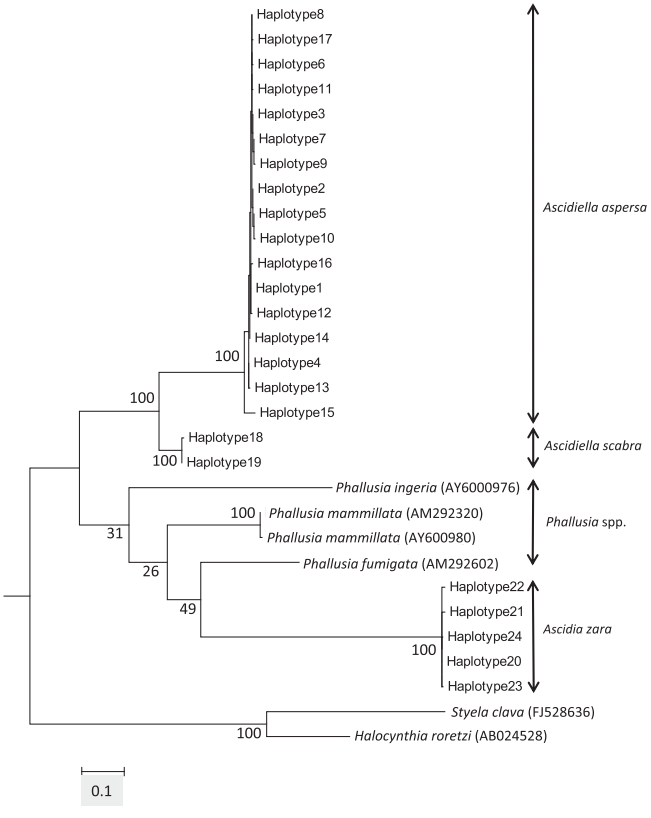


**Fig. 1.** The ML tree of *Ascidiella aspersa, A. scabra*, and allies, inferred from a mitochondrial COI dataset. The ascidians *Styela clava* (GenBank FJ528636) and *Halocynthia roretzi* (GenBank, AB024528) were chosen as outgroups. Numbers on branches are the bootstrap probabilities (%) from 1000 replications. The scale bar indicates the number of substitutions per site.

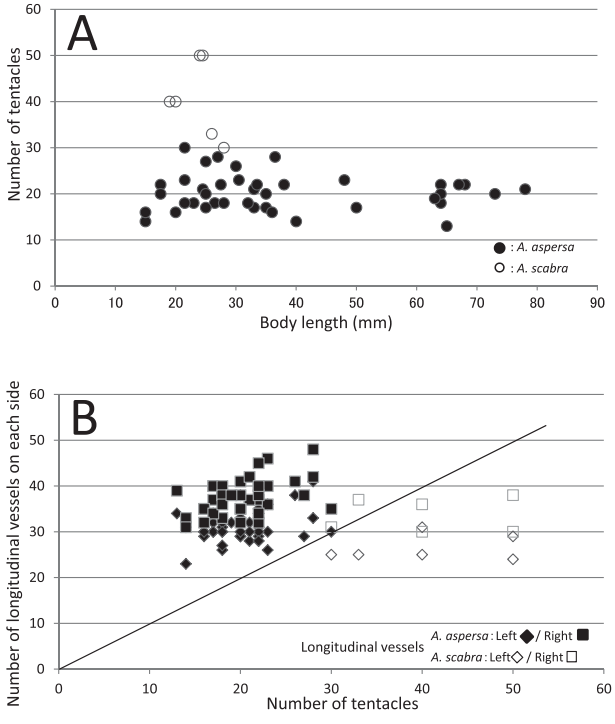


**Fig. 2.** Morphological comparisons between *A. aspersa* and *A. scabra*, identified from the COI dataset as the larger and smaller clusters of *Ascidiella* specimens (see Fig. 1). **(A)** Relationship between body length and number of oral tentacles. **(B)** Relationship between total number of oral tentacles and that of longitudinal vessels on each side, with a straight line showing equality between these numbers.

in the COI tree (Fig. 1), indicating that the larger cluster has fewer tentacles than longitudinal vessels on either side, while the smaller cluster generally has more tentacles than longitudinal vessels on either side (with exceptions relating to longitudinal vessel counts only on the right side) (Fig. 2B). Following the traditional taxonomy of the present genus, it appears valid to identify the larger cluster as *A. aspersa* and the smaller as *A. scabra*, each cluster including OTUs derived from the Swedish coast of the Skagerrak, adjacent to the Norwegian coast that represents the type localities of these species. Re-examination of the name-bearing type specimens of these two species is desirable, but unfortunately we have no information of their whereabouts.

The data show no correlation between body length and the number of oral tentacles for either species (Spearman rank-order correlation: *A. scabra*, $r_s = -0.500$, $P = 0.312$, $n = 6$, body length-range 19–28 mm; *A. aspersa*, $r_s = 0.006$, $P = 0.968$, $n = 44$, body length-range 15–78 mm). For both species, the number of longitudinal vessels is consistently greater on the right side of the branchial basket than on the left (Wilcoxon signed rank test: *A. scabra*, $V = 0$, $P < 0.05$, $n = 6$; *A. aspersa*, $V = 0$, $P < 0.001$, $n = 40$), as noted for *A. aspersa* by Tatián et al. (2010).

## DISCUSSION

The present study confirms the reliability of differences in the total number of oral tentacles relative to that of inner longitudinal vessels on each side to distinguish *A. aspersa* morphologically from *A. scabra.* The lower number of longitudinal vessels on the left side compared to the right appears to make the count on the left more reliable for separating the species, at least in the present data set.

Nishikawa and Otani (2004) claimed an ephemeral occurrence of *A. scabra* in Nagasaki, Japan, probably in 1861, based on the actual examination of museum specimens and their identification on the basis of the excess of tentacles over longitudinal vessels. The present study has validated their claim, which indicates that *A. scabra*, together with *A. aspersa* as mentioned above, has a potential ability to become invasive; this might possibly have happened but remained unrecognized due to misidentification. Comparative studies of various biological traits will be necessary between these species, as initiated by Berrill (1928), not only for better understanding of their speciation, but also for better practice to control alien populations of the genus *Ascidiella*.

Traditionally, the size and shape of the outer follicle cells has also been noted to distinguish the two species. In the present study this character could not be examined for technical reasons, but Kanamori et al. (2012) found the follicle cells in the Japanese (Hokkaido) population comparable in size and shape to the previous descriptions of *A. aspersa* given by Berrill (1928) and Lindsay and Thompson (1930).

Many previous records of "*A. aspersa*", including alien populations, must be reassessed in the future based on the specific differences in internal morphology confirmed in this study, because the reports lack any reference to these morphological criteria. Exceptions include Brewin's (1946) report of *A. aspersa* in New Zealand, from which its introduction to the southern half of the Pacific around 1945 seems beyond doubt, and the records by Tatián et al. (2010) from Argentina (SW Atlantic) starting in 1962. In the Pacific, native populations of the genus *Ascidiella* have been represented only by *Ascidiella incrustans* Herdman, 1898 and *Ascidiella griffini* Herdman, 1898 recorded from Puget Sound in the Northeast Pacific. These species are, however, not assignable to the genus *Ascidiella* in the modern sense, because their original descriptions by Herdman (1898) clearly show the existence of secondary papillae, as unambiguously depicted in figures 6 and 3 of plate 12 of the descriptions, respectively. Therefore, it appears that the indigenous ascidian fauna of the Pacific completely lacks the genus *Ascidiella*.

In the northern Pacific, other than our present record from Japan, the undoubted occurrence of *A. aspersa* in South Korea has been confirmed by comparing Pyo et al.'s (2012) COI data with ours, although Pyo et al. (2012) did not provide any detail of the internal structure of their specimens. The specimens were first collected in October 2010 at eight out of 26 harbors or ports along the entire South Korean coast during their survey, which started in August 2009, and therefore, it follows that the first documented occurrence in South Korea is almost two years later than that in Japan. The pathways and processes involved in the invasion of the boreal waters of Korea and Japan in the NW Pacific by *A. aspersa* remain to be elucidated.

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