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SHORT COMMUNICATION

First record of nudibranch mollusk *Onchidoris muricata* (O. F. Müller, 1776) (Mollusca, Gastropoda, Heterobranchia) in the Sea of Japan and its ephemeral population associated with unusual prey

Anton Chichvarkhin[1,2] · **Olga Chichvarkhina**[1] · **Irina Ekimova**[2,3] · **Karina Chalenko**[2]

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Abstract The nudibranch mollusk *Onchidoris muricata* known from boreal waters of the western Pacific, North American Pacific shore, and both coasts of the northern Atlantic was first recorded from the Sea of Japan (East Sea). This discovery extends its distribution range southwards along the Asian Pacific coast from cool temperate waters of Kamchatka and Kommander's Islands to cool temperate sub-zone of the Sea of Japan. Three discovered populations in the northern Sea of Japan are confirmed to be conspecific with other Pacific and Atlantic populations of *O. muricata* because of external and radular morphology along with low interpopulation genetic variation (<3 %) found between studied COI gene sequences, while intraspecific distances between *Onchidoris* species range from 12.9 to 19.7 %. One of the discovered population constitutes an ephemeral settlement and is associated with the bryzoan *Microporina articulata*, an unusual prey for the Onchidorididae. Despite observing reproducing *O. muricata* adult individuals sheltered in *M. articulata* colonies, the nudibranch population remains unstable and became extinct within one season; however, it can be resurrected due to larvae migration from adjacent waters using ecological plasticity of this species as intrinsic resource for distribution area extension.

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✉ Anton Chichvarkhin
anton.chichvarkhin@gmail.com

[1] A.V. Zhirmunsky Institute of Marine Biology, Russian Academy of Sciences, Palchevskogo 17, Vladivostok 690041, Russia

[2] School of Natural Sciences, Far Eastern Federal University, Sukhanova 8, Vladivostok 690950, Russia

[3] Biological Faculty, Moscow State University, Leninskiye Gory 1–12, 119234 Moscow, Russia

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Introduction

There are 15 species of the Russian fauna of sea slugs of the family Onchidoridiidae, while six of them are known from the Sea of Japan (Martynov and Korshunova 2011; Martynov 2013). Distribution range of one of these species, *Onchidoris muricata* (O. F. Müller, 1776), was known as restricted to the waters of Bering Sea, where it is sympatric with similar but clearly distinct *O. macropompa* Martynov, Korshunova, Sanamyan and Sanamyan, 2009. Despite their superficial similarity, both species can be clearly distinguished with oral bulb anatomy and masticatory edge of first lateral radular teeth that are smooth in *O. macropompa* but carry array of denticles in *O. muricata* (Thompson and Brown 1984; Millen 1985; Martynov et al. 2009). Martynov et al. (2009) referred to *O. muricata* from the Sea of Japan, but this information is not substantiated with any literature data or collections other than a note by Smolyar (1981), which he earlier considered to be a misidentification of *Knoutsodonta (Adalaria) jannae* (Martynov 1999). In his recent publications, Martynov did not refer to *O. muricata* from this sea (Martynov and Korshunova 2011; Martynov 2013). *O. muricata* is also known from cool temperate northeastern Pacific (where its distribution range extends along Aleutian to Oregonian Provinces) and northern Atlantic (Thompson and Brown 1984; Millen 1985; Behrens and Hermosillo 2005; Martynov et al. 2009), where this species feeds on a range of encrusting bryozoans (Behrens and Hermosillo 2005; Martynov and Korshunova 2011).

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Fig. 1 Surveyed area map: **1** Kievka Bay, **2** Rudnaya Bay, **3** Nevelsk, **4** Rausu (unconfirmed distribution after Nakano 2004)

O. bilamellata (Linnaeus, 1767) is the only Onchidoridid species known form the Sea of Japan. This species can be recognized by its peculiar radula morphology, body coloration, and shape of notum tubercles, which are bud- or mushroom-like in *O. muricata* and *O. macropompa* (Martynov et al. 2009; Martynov and Korshunova 2011) but finger-like in *O. bilamellata*. Also, these species can be identified by the means of DNA barcoding using molecular markers (Hallas and Gosliner 2015).

Although anatomical characters can be successfully used in Onchidoridid identification, we anticipate their (pseudo-)cryptic species occurrence. Recently, several cryptic species were detected within other Opisthobranchia groups (Stout et al. 2010; Carmona et al. 2013; Churchill et al. 2014; Cooke et al. 2014; Ekimova et al. 2015; Hallas and Gosliner 2015; Lindsay et al. 2016; Breslau et al. 2016). Over the last decade, DNA barcoding has become a useful tool to quickly and reliably identify known species, and to aid in discovery of cryptic species based on the nucleotide sequence of usually one short DNA fragment (Hebert et al. 2003, 2004a, b; Ward et al. 2005; Barr et al. 2009; Chen et al. 2011). A use of a standardized fragment of cytochrome c oxidase subunit I (COI) in the mitochondrial DNA (mtDNA) was first advocated by Hebert et al. (2003). There is an ample evidence that sequences from COI (e.g., the DNA barcodes), have demonstrated their ability to identify animal species in many cases, including both terrestrial and aquatic taxa (e.g., Hebert et al. 2004a, b; Ward et al. 2005; Feng et al. 2011). The efficacy of COI-based barcoding in the discovery of cryptic species has also been documented in a number of animal taxa (e.g., Hebert et al. 2004a; Hebert and Gregory 2005; Chen et al. 2011). To date, this fragment has been successfully sequenced and analyzed in many species, and has proved its usefulness in identifying species and highlighting potentially overlooked species (Allcock et al. 2010; Undheim et al. 2010), including Opisthobranch mollusks (e.g., Carmona et al. 2013; Padula et al. 2014; Ekimova et al. 2015; Hallas and Gosliner 2015; Lindsay et al. 2016; Breslau et al. 2016).

Despite broad benefits of DNA barcoding, a number of problems arise when using only DNA barcoding for species delimitation (e.g., Rubinoff et al. 2006; Will et al. 2005; Dasmahapatra

Table 1 List of specimens of the *Onchidoris* specimens/sequences used in the molecular genetic analysis

Species name	Voucher number	Origin	COI NCBI accession number
Onchidoris muricata	AC16-18	Russia: Rudnaya Bay	KX951691*
Onchidoris muricata	AC16-19	Russia: Rudnaya Bay	KX951692*
Onchidoris muricata	AC17-24	Russia: Rudnaya Bay	KX951693*
Onchidoris muricata	AC17-40	Russia: Rudnaya Bay	KX951694*
Onchidoris muricata	AC19-30	Russia: Nevelsk, Sakhalin Is.	KX951697*
Onchidoris muricata	AC19-31	Russia: Kievka Bay	KX951695*
Onchidoris muricata	AC19-32	Russia: Kievka Bay	KX951696*
Onchidoris muricata	MT07703	North Sea	KR084489
Onchidoris muricata	10BCMOL-00318	Canada: British Columbia	KF643468
Onchidoris muricata	CASIZ 184185A	New Hampshire	KM219681
Onchidoris muricata	CASIZ 181312	USA: California	KM219680
Onchidoris muricata	n/a	Sweden	AJ223271
Onchidoris bilamellata	MT09252	North Sea	KR084801
Onchidoris sp. 1	10NBMOL-10020	Canada: New Brunswick	KF644026
Onchidoris sp. 2	CASIZ 101555	USA: California	KP340408
Onchidoris evincta	CASIZ 187758A	USA: Washington	KP340390
Onchidoris evincta	CASIZ 186817	USA: Washington	KP340389
Onchidoris proxima	CASIZ 183931A	USA: Maine	KM219677
Onchidoris proxima	CASIZ 183921A	USA: Maine	KM219676

*data obtained in this study

et al. 2010; Collins and Cruickshank 2012). These shortcomings include: misidentificaton of voucher specimens (Will and Rubinoff 2004; Becker et al. 2011), confusing the terms 'species identification' vs 'species delimitation' or 'species discovery' (DeSalle et al. 2005; Brower 2006; DeSalle 2006; Goldstein and DeSalle 2011), inappropriate use of NJ trees (Will and Rubinoff 2004; Meier et al. 2006; Meier 2008; Goldstein and DeSalle 2011) and bootstrap resampling values (Lowenstein et al. 2010; Collins et al. 2012; Zhang et al. 2012), misinterpreting the barcoding gap (Wiemers and Fiedler 2007; Collins and Cruickshank 2012), wrong use of fixed distance thresholds (Zhang et al. 2012), use of corrected (i.e., biased) distances (Srivathsan and Meier 2011), and conflating tested hypotheses (Meier 2008; Goldstein and DeSalle 2011). Recently, several novel methodological approaches appeared to avoid some of them (e.g., Meier et al. 2006; Pons et al. 2006; Sarkar et al. 2008; Puillandre et al. 2012).

The primary aim of this study is to identify the specimens belonging to the genus *Onchidoris* from three populations discovered in the Sea of Japan by the means of anatomical and improved DNA barcoding methodology as mentioned above. Therefore, our aim is to support or reject a hypothesis of unusually southern distribution of this species in Asia, as well as its ability to feed on bryozoan species *Microporina articulata* that was not previously recognized as *Onchidoris*'s prey.

Material and methods

Observations and sample collections were committed by SCUBA-diving in 2012–2015 during May through middle October in Rudnaya and Kievka Bays on northwestern shore of the Sea of Japan, and in the vicinity of the town of Nevelsk on the northeastern coast of this sea in Sakhalin Island, Russia (Fig. 1). Underwater images were taken with Nikon D300 and D810 cameras with Nikkor 105/2.8G lens and appropriate

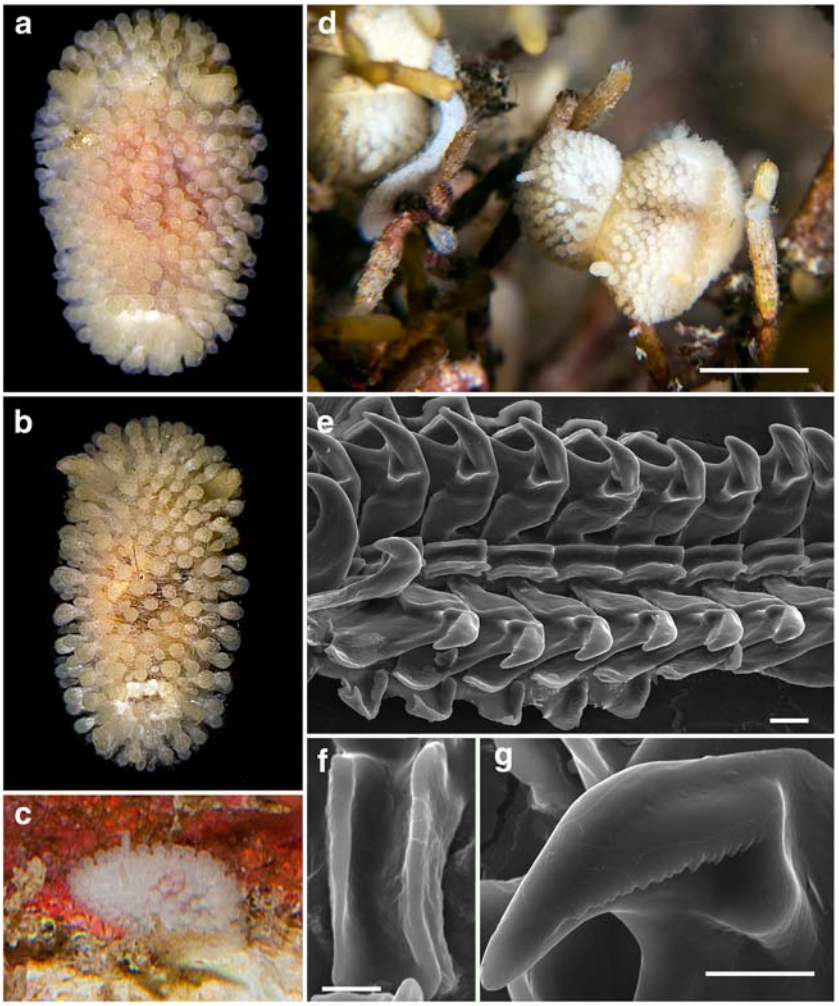

Fig. 2 *O. muricata* in the Sea of Japan. **a** Rudnaya Bay, 11 mm. **b** Kievka Bay, 9 mm. **c** Nevelsk, 7 mm. **d** Adult slugs and their egg masses on *M. articulata*, Rudnaya Bay, 18 m depth, May 16, 2015 (*scale bar* 10 mm). **e** Radula general view, Rudnaya Bay (*scale bar* 20 mkm). **f** Rachidian tooth (*scale bar* 10 mkm). **g** First lateral tooth (*scale bar* 10 mkm)

models of underwater housings and two Sea&Sea YS-D1 strobes.

Collected material:

Senkina Shapka pinnacle, south of Rudnaya Bay 16–19 m depth, 12 exemplars (5–11 mm), 16/05/2015, A. Chichvarkhin collected
2 km south of Nevelsk, Sakhalin Is., 4 m depth: 1 exemplar (8 mm), 26/08/2014, A. Chichvarkhin collected
Skaly Is., Kievka Bay 8 m depth: 2 exemplars (8–9 mm) 29/07/2015, A. Chichvarkhin collected

All specimens have been preserved in 96 % ethanol and deposited in the Museum of A.V. Zhirmunsky Institute of Marine Biology, Russian Academy of Sciences.

Anatomy was studied under a stereomicroscope. The buccal mass of each specimen was extracted and introduced into a 10 % sodium hypochlorite solution for 1–10 min to dissolve connective and muscle tissue, leaving only the radula. The chromium or gold-coated radulae were examined and photographed using scanning electron microscopes JEOL JSM or EVO-40 Zeiss.

DNA was extracted using the Diatom™ DNA Prep 100 kit (Isogene Lab, Moscow, Russia) according to the manufacturer's protocol. Partial sequence for mitochondrial Cytochrome *c* oxydase subunit I gene (COI) was used in this study. The primers used to amplify the fragments of mitochondrial genes for Cytochrome *c* oxydase, as well as PCR and sequencing conditions, were as described earlier (Chichvarkhin et al. 2015, 2016a, b; Ekimova et al. 2015). GenBank / NCBI access numbers of the sequences used in this study are presented in Table 1. Sequences were checked and aligned by eye using BioEdit software (Hall 1999). We used two methods for species delimitation and identification: comparing tree topologies, and Automatic Barcode Gap Discovery (ABGD). The *p*-distances (i.e., the proportion of variable positions), standard errors (SE), and neighbour-joining (NJ) (Saitou and Nei 1987) gene trees were calculated using MEGA 6 software (Tamura et al. 2013). The ABGD method (Puillandre et al. 2012) is based on pairwise distances, detecting the breaks in the distribution referred to as the "barcode gap" (Hebert et al. 2003) without any prior species hypothesis. It is commonly used for species delimitation analyses, including the latest works on molluscan taxa (Jörger et al. 2012; Barco et al. 2013; Krug et al. 2013; Cámara et al. 2014; Ekimova et al. 2015; Katugin et al. 2015). The ABGD program is available at the web-site http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html. We analyzed COI alignment using uncorrected *p*-distance. The X (relative gap width) was set to 1.5; the other settings remained as default for both fragments.

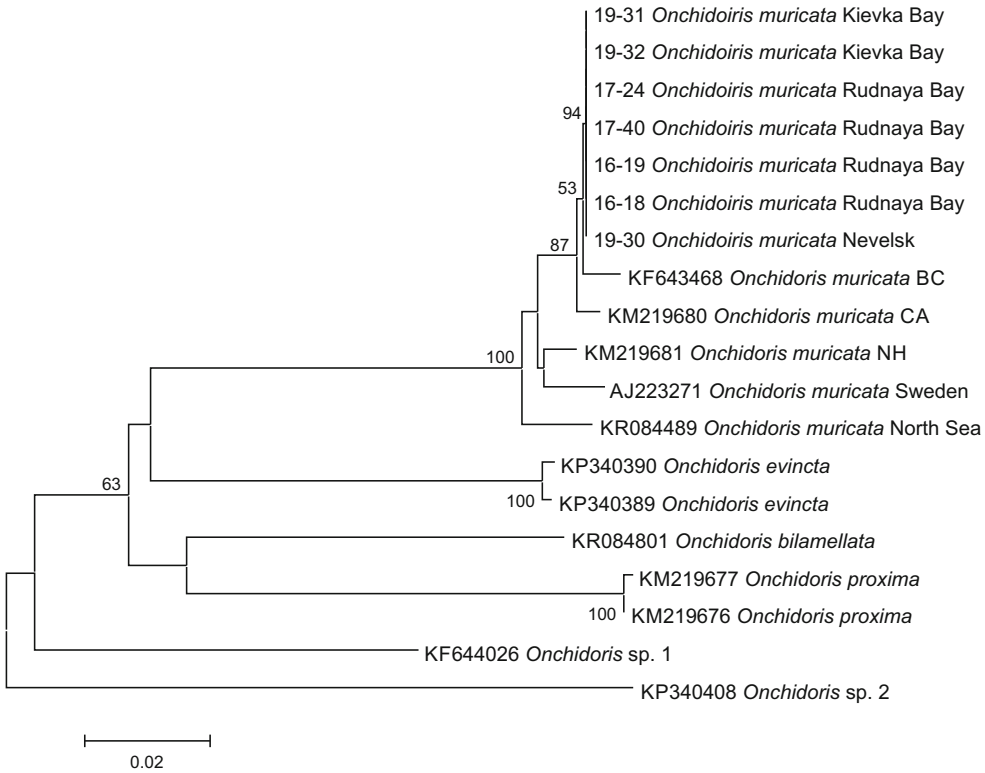

Fig. 3 Uncorrected distance based neighbour-joining gene tree of *Onchidoris* partial COI gene sequences. Bootstrap indices above 50 % shown at the internodes, 1,000 replicates

Table 2 Genetic divergence of *Onchidoris* COI sequences

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	*Onchidoris muricata* North Sea		0.006	0.006	0.007	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.014	0.015	0.016	0.014	0.014	0.015	0.015
2	*Onchidoris muricata* BC	0.022		0.006	0.004	0.007	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.015	0.015	0.017	0.014	0.014	0.015	0.015
3	*Onchidoris muricata* NH	0.019	0.020		0.006	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.015	0.015	0.016	0.014	0.014	0.015	0.015
4	*Onchidoris muricata* CA	0.027	0.012	0.019		0.006	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.014	0.015	0.016	0.014	0.014	0.015	0.015
5	*Onchidoris muricata* Sweden	0.024	0.026	0.015	0.020		0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.014	0.014	0.016	0.014	0.014	0.015	0.015
6	*Onchidoiris muricata* Rudnaya	0.022	0.007	0.014	0.005	0.019		0.000	0.000	0.000	0.000	0.000	0.000	0.014	0.015	0.016	0.014	0.014	0.015	0.015
7	*Onchidoiris muricata* Rudnaya	0.022	0.007	0.014	0.005	0.019	0.000		0.000	0.000	0.000	0.000	0.000	0.014	0.015	0.016	0.014	0.014	0.015	0.015
8	*Onchidoiris muricata* Rudnaya	0.022	0.007	0.014	0.005	0.019	0.000	0.000		0.000	0.000	0.000	0.000	0.014	0.015	0.016	0.014	0.014	0.015	0.015
9	*Onchidoiris muricata* Rudnaya	0.022	0.007	0.014	0.005	0.019	0.000	0.000	0.000		0.000	0.000	0.000	0.014	0.015	0.016	0.014	0.014	0.015	0.015
10	*Onchidoiris muricata* Kievka	0.022	0.007	0.014	0.005	0.019	0.000	0.000	0.000	0.000		0.000	0.000	0.014	0.015	0.016	0.014	0.014	0.015	0.015
11	*Onchidoiris muricata* Kievka	0.022	0.007	0.014	0.005	0.019	0.000	0.000	0.000	0.000	0.000		0.000	0.014	0.015	0.016	0.014	0.014	0.015	0.015
12	*Onchidoiris muricata* Nevelsk	0.022	0.007	0.014	0.005	0.019	0.000	0.000	0.000	0.000	0.000	0.000		0.014	0.015	0.016	0.014	0.014	0.015	0.015
13	*Onchidoris bilamellata*	0.143	0.146	0.146	0.139	0.143	0.143	0.143	0.143	0.143	0.143	0.143	0.143		0.015	0.016	0.015	0.015	0.014	0.014
14	*Onchidoris* sp. 1	0.150	0.150	0.146	0.146	0.143	0.146	0.146	0.146	0.146	0.146	0.146	0.146	0.163		0.015	0.015	0.015	0.015	0.016
15	*Onchidoris* sp. 2	0.199	0.202	0.199	0.197	0.197	0.197	0.197	0.197	0.197	0.197	0.197	0.197	0.180	0.167		0.016	0.016	0.016	0.016
16	*Onchidoris evincta*	0.134	0.139	0.138	0.131	0.134	0.136	0.136	0.136	0.136	0.136	0.136	0.136	0.146	0.163	0.177		0.002	0.014	0.014
17	*Onchidoris evincta*	0.136	0.138	0.139	0.129	0.136	0.134	0.134	0.134	0.134	0.134	0.134	0.134	0.146	0.165	0.179	0.003		0.014	0.014
18	*Onchidoris proxima*	0.155	0.163	0.158	0.153	0.158	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.133	0.170	0.180	0.131	0.133		0.002
19	*Onchidoris proxima*	0.153	0.162	0.156	0.151	0.156	0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.131	0.172	0.179	0.129	0.131	0.002	

Lower left triangle: uncorrected *p*-distances; upper right triangle: standard errors (SE)

Results and discussion

A few *O. muricata*-like slugs were collected in Nevelsk, Sakhalin 46° 67'N 141° 85'E (Fig. 2c) and Kievka Bay 42° 85'N 133° 68'E (Fig. 2b) during summer time at water temperature of 11–14 °C. A bryozoan host that they feed on was not detected, although several unidentified encrusting bryozoan species (i.e., potential hosts) were found in these areas. Feeding on encrusting bryozoans is a well-documented fact for most Onchidoridids including *O. muricata* (Behrens and Hermosillo 2005; Martynov and Korshunova 2011), hence such a choice may be expected for these populations. In contrast, the numerous individuals found at Rudnaya Bay 44° 32'N 135° 84'E (Fig. 2a, d) (we described this site earlier in Chichvarkhin et al. 2015, 2016a, b) were occupying ball-shaped bushy colonies of *Microporina articulata* (Fabricius, 1821) (Cheliostomatida: Microporoidea) bryozoan. Almost all the colonies were inhabited with the slugs, estimated to be up to 28 specimens per colony. The colonies of *M. articulata* were also inhabited with few other Opisthobranchia species grazing on them including *Janolus fuscus* O'Donoghue, 1924, *Trinchesia ornate* (Baba, 1937), *Cuthonella soboli* Martynov, 1992 as we recorded this earlier (Chichvarkhin et al. 2016a, b), but in May 2015 *O. muricata* was the most abundant *M. articulata* dweller.

Unlike encrusting bryozoan species that Onchidoridids normally feed on, *M. articulata* colonies are large (10–25 cm across diameter), possessing larger biomass (i.e., food supply) and playing the role of shelter for many invertebrate species. Peak numbers of *O. muricata* as well as their white short ribbon-shaped egg masses (10–25 mm long) (Fig. 2d) were found in Rudnaya Bay in mid-May 2015 at a water temperature of +4 °C. In June 2015, population density decreased, while the slugs completely disappeared in October when *M. articulata* colonies became smaller, partially dead, and depressed. We never found this slug species at the same site before in 2012–2014 except for two individuals recorded in June 2013. This observation suggests unstable ephemeral state of this population, which may be temporarily established because of larvae supply from adjacent waters. In fact, a sea slug with bud-like tubercles similar to *O. muricata* is illustrated from Rausu, northern Hokkaido as *'Adalaria* sp., species 191' (Nakano 2004). This site is relatively close to Nevelsk, and its Opisthobranchia fauna comprises many species that were recently recorded in Russian waters for the first time from Rudnaya Bay (Chichvarkhin et al. 2015, 2016a, b). Hence, larvae supply by the currents from Hokkaido or Sakhalin may explain the existence of genetically similar ephemeral sea slug settlement, although local environment or host properties do not provide stability of newly established population, while pelagic larvae are probably washed southwards from the parental site by the Primorskoye current.

Identification. Body length of collected specimens was 5 to 11 mm. The foot, notum, and rhinophores coloration is white or light creamy, semi-translucent. The notum is covered with numerous bud-like tubercles (Fig. 2a–c). The rhinophores, with 8–11 lamellae, can be hidden into rhinophoral cavities. The gills are white, gill cavity absent. Radula 22x1.1.1.1.1 (Fig. 2e–g) with 16–20 denticles on masticatory edge of the first lateral tooth (Fig. 2g) are characteristic for *O. muricata* (Martynov et al. 2009). Sequenced 658-bp fragments of COI gene were identical in all populations of the Sea of Japan and quite similar (*p*-distance <3 %) to eastern Pacific and Atlantic populations, while interspecific distances between *Onchidoris* species ranged from 12.9 to 19.7 % (Fig. 3, Table 2). Conducted species delimitation ABGD analysis detected five groups in our dataset corresponding to *O. muricata* (all populations); *O. bilamellata* (Linnaeus, 1767), *O. proxima* (Alder & Hancock, 1854), *O. evincta* (Millen, 2006), and both *O.* sp. In this way, our study confirms identity of discovered Onchidoridid populations as *O. muricata*, and occurrence of this species in northern waters of the Sea of Japan. The populations discovered in this sea are found to be conspecific with western Pacific and Atlantic populations of this species. Such significant southward extension of known distribution area from Asian boreal (Bering Sea) to temperate waters, along with its ability to establish temporary ephemeral settlements, may predict future findings of *O. muricata* in the Sea of Okhotsk, Kurile Islands, Japan, and Korean Peninsula. Ephemeral settlements of reproducing adults associated with alternative (albeit probably imperfect) host or environment may constitute a foothold for new generation larvae resettlement, promoting extension and stability of the entire distribution area occupied by this species. However, discovery of new species in the Sea of Japan can be more likely explained by insufficient knowledge about coastal marine biodiversity than by novel distribution area extension. Indeed, such 'extensions' caused by more detailed fauna exploration are a known phenomenon for Opisthobranchia of the northwestern Pacific (Goddard and Foster 2002; Chichvarkhin et al. 2015, 2016a, b).

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