

***Placida babai* (Mollusca: Gastropoda: Sacoglossa) from Russian waters of the Sea of Japan**

***A. Yu. Chichvarkhin*^{1,2}, *I. A. Ekimova*^{2,3}, *K. P. Chalenko*²,
*D. Schepetov*⁴, *O. V. Chichvarkhina*¹, *Á. A. Valdés*⁵**

¹*A. V. Zhirmunsky Institute of Marine Biology, Far East Branch,
Russian Academy of Sciences, Vladivostok 690041, Russia
e-mail: anton.chichvarkhin@gmail.com*

²*Far Eastern Federal University, School of Natural Sciences,
Vladivostok 690950, Russia*

³*Lomonosov Moscow State University, Faculty of Biology, Moscow 119234, Russia*

⁴*Koltzov Institute of Developmental Biology, Russian Academy of Sciences,
Moscow 119334, Russia*

⁵*California State Polytechnic University, Department of Biological Sciences,
Pomona, California 91768, USA*

The sea slug previously referred to as *Placida dendritica* (Alder et Hancock, 1843) distributed in the Sea of Japan (East Sea), Yellow Sea, and adjacent waters of the Pacific Ocean has been confirmed as the distinct species from *P. dendritica*. Cytochrome *c* oxydase subunit I and 16S rRNA genes sequence data were used for species delimitation using either a tree method or ABGD delimitation approach. The *p*-distances between both species are similar to interspecific divergences between other Limapontiidae species. Morphologically both species possess less evident but nevertheless discernible and consistent differences in branching pattern of digestive tubules. The two names are potentially available in zoological literature to name this species but validity of one of them and their synonymy are in question, hence we are proposing to refer these slugs as *Placida babai* E. Marcus, 1982 until this problem will be completely untangled.

Key words: Limapontiidae, species delimitation, cytochrome oxydase I, 16S rRNA, DNA barcoding, *Placida dendritica*.

***Placida babai* (Mollusca: Gastropoda: Sacoglossa) в российских водах Японского моря**

***А.Ю. Чичвархин*^{1,2}, *И.А. Екимова*^{2,3}, *К.П. Чаленко*²,
*Д. Щепетов*⁴, *О.В. Чичвархина*¹, *А.А. Валдес*⁵**

¹*Институт биологии моря им. А.В. Жирмунского ДВО РАН,
Владивосток 690041, Россия
e-mail: anton.chichvarkhin@gmail.com*

²*Дальневосточный федеральный университет, Школа естественных наук,
Владивосток 690950, Россия*

³*Московский государственный университет имени М.В. Ломоносова,
Биологический факультет, Москва 119234, Россия*

⁴*Институт биологии развития им. Н.К. Кольцова РАН, Москва 119334, Россия*

⁵*Калифорнийский политехнический университет, Отделение биологических наук,
Помона, Калифорния 91768, США*

Показано, что морские слизни, обитающие в Японском, Желтом и прилегающих акваториях Тихого океана и известные ранее под названием *Placida dendritica* (Alder et Hancock, 1843) относятся к другому виду. Для подтверждения гипотезы о существовании второго псевдокриптического вида, нами был задействован делимитационный анализ частичных последовательностей генов COI и 16S рНК, используя как кладистический подход, так и метод ABGD. Нескорректированные дистанции между нуклеотидными последовательностями двух видов оказались сопоставимы с уровнями межвидовой дивергенции в семействе лимпонтиид. Морфологически эти виды менее различимы, однако хорошим признаком можно считать характер ветвления отростков пищеварительной системы. На данный момент, имеется два потенциально пригодных названия для тихоокеанского вида, однако пригодность и синонимия одного все еще находятся под вопросом, поэтому мы предлагаем именовать япономорский вид как *Placida babai* E. Marcus, 1982, по крайней мере до того момента, пока данный номенклатурный вопрос не будет окончательно разрешен.

Ключевые слова: Limapontiidae, разделение видов, cytochrome oxydase I, 16S рНК, ДНК-штрихкодирование, *Placida dendritica*.

The herbivorous sea slug species *Placida dendritica* (Alder et Hancock, 1843) has been reported from of all boreal and temperate waters in the Northern Hemisphere [Hamatani, 2000] and is also presumably found in the south Pacific [Burn 1966; Thompson 1973; Marshall, Willan, 1999]. This mollusk inhabits shallow sub-coastal waters, feeding on a wide range of green algae belonging to the genera *Cladophora*, *Bryopsis*, *Codium*, *Derbesia*, *Halimeda*, and *Pedobesia* [Baba, Hamatani, 1952; Baba, 1959; Bleakney, 1989, 1990; Shimadu et al., 2006; Trowbridge, 2002; Trowbridge et al., 2008]. The name *Placida babai* Ev. Marcus, 1982 was introduced for the animals initially collected in Sagami Bay on the eastern shore of Japan, as reported by Baba [1955], supporting the idea of Burn [1966] to split Atlantic and Pacific populations into different species [Marcus, 1982]. Moreover, Marcus proposed to use the name *P. babai* for animals from eastern Australia reported by Thompson [1973]. Few years after the original description, the anatomy of this species was described by Baba [1986]. In this paper, Baba [1986] rejected the name *P. dendritica* for Japanese populations but did not use the name *P. babai* (considering it as *nomen nudum* albeit Marcus's description met ICZN requirements), nor assigned a new name. As a postscript to his paper, Baba [1986] noted that Marcus had finally doubted the validity of *P. babai* because of high variability of radular teeth shape, which she denoted as the only character used to separate these species. After this publication, the name *P. babai* remains questioned and this species was referred to as «*Placida* sp. (sensu Baba, 1986)» [Hirano et al., 2006a, 2006b, 2006c]. Also, *Placida daguilaensis* Jensen, 1990 originally known from Hong Kong and Singapore [Jensen, 1990, 2015] found in the waters of Japan being sister species of *Placida* sp. (sensu Baba, 1986) [Hirano et al., 2006c]. However, the authors of this discovery are not sure whether *P. daguilaensis* is a re-description of *P. babai* or *Placida* sp. (sensu Baba, 1986) is conspecific with *P. babai*. The question is still not resolved because the specimens that Baba referred in his earlier publications, hence the types of *P. babai* are not found and probably lost.

Recently, we found three populations of presumably *P. babai* in the Russian waters of the Sea of Japan: one in southwestern Sakhalin Island and two in Peter the Great

Bay. Also, we obtained a morphologically similar sample from Boso Peninsula in Japan located relatively close to Sagami Bay, where the specimens described by Baba were collected. The slugs from Russia and Japan are morphologically undistinguishable from each other, as they are from those described by Baba. Contrary to North Atlantic slugs assigned to *P. dendritica*, all Pacific specimens possess a dense network of fine tubules of the digestive system penetrating the head, rhinophores, and foot, while the Atlantic slugs possess thick tubular stems never reaching the ventral portion of the foot and forming only 2–5 branches inside the rhinophores. In the Pacific specimens, the dense network of narrow digestive tubules penetrating the foot leaves no empty spaces in the head and the rhinophores, hence coloring them in nearly uniform green tan.

In this study, we examined the morphology of specimens collected in Japan, Russia and North America, and sequenced a portion of the mitochondrial gene of cytochrome *c* oxidase subunit I (*COI*) to compare it with the same fragments of *P. dendritica* from the Atlantic Ocean using DNA barcoding approaches.

Materials and methods

Live specimens were photographed with Nikon D300 digital camera with Nikkor 60/f2.8D lens. Underwater imaging was conducted using Sea&Sea MDX-D300 housing and two Sea&Sea YS-D1 strobes. The specimens were photographed individually, then immersed in 95% or 70% ethanol and preserved at ambient temperature before DNA or morphological analysis, respectively. The animals, their host algae, and egg masses were preserved in 95% ethanol and kept at ambient temperature before analysis. The list of used specimens and DNA sequences is presented in Table 1, collection locations for *P. dendritica* s.l. are shown in Fig. 1.

The external morphology was studied under a stereomicroscope. For the description of internal features preserved specimens were dissected under the stereomicroscope. The buccal mass of each specimen was extracted and soaked in 10% sodium hypochlorite solution for 1–2 minutes 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. Reproductive systems of different specimens were also examined and drawn using the stereomicroscope.

DNA was extracted using the Diatom™ DNA Prep 100 kit (Isogene Lab, Moscow, Russia) according to 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; Ekimova et al., 2016]. GenBank/NCBI accession 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

Table 1

List of specimens of the Limapontiidae used in the molecular genetic analysis

Species name	Voucher number	Origin	NCBI accession number	
			COI	16S
<i>Placida babai</i>	ie183	Vostok Bay, Russia	KU133299*	KU133308*
<i>P. babai</i>	ie184	Vostok Bay, Russia	KU133300*	KU133307*
<i>P. babai</i>	ie180	Nevelsk, Sakhalin Is., Russia	KU133297*	KU133306*
<i>P. babai</i>	ie181	Nevelsk, Sakhalin Is., Russia	KU133298*	KU133305*
<i>P. babai</i>	AC21-18	Vladivostok, Russia	KU133296*	KU133310*
<i>P. babai</i>	AC11-11	Chiba, Japan	KU133301*	KU133309*
<i>P. babai</i>		Yellow Sea, China	KC171014	KC171014
<i>P. dendritica</i>	AC15-17	Vancouver Is., Canada	KU133302*	–
<i>P. dendritica</i>	iewsbs	White Sea, Russia	KU133303*	–
<i>P. dendritica</i>	ie236	Barents Sea, Russia	KU140946*	–
<i>P. dendritica</i>	AC19-7	Falmouth, UK	KU133304*	KU133311*
<i>P. dendritica</i>		Tossa de Mar, Spain	GQ996663	EU140869
<i>P. dendritica</i>		Tossa de Mar, Spain	KF921396	EU140870
<i>P. dendritica</i>		Tossa de Mar, Spain	–	EU140871
<i>P. dendritica</i>		North Sea, Tjärnö, Sweden	–	AJ223399
<i>P. cremoniana</i>			HQ380201	–
<i>P. verticillata</i>			HQ380202	–
<i>P. kingstoni</i>			GU191063	–
<i>Stiliger ornatus</i>			AB501311	–
<i>Ercolania felina</i>			GU191060	–
<i>Limapontia senestra</i>			HQ380200	–
<i>Aplysiopsis minor</i>			AB501314	–
<i>Alderiopsis nigra</i>			KF921392	–

*Data obtained in this study.

and identification: comparing tree topologies, and Automatic Barcode Gap Discovery (ABGD). The *p*-distances (i.e., the proportion of variable positions) and Neighbour-Joining (NJ) [Saitou, Nei, 1987] gene trees were calculated using MEGA 6 software [Tamura et al., 2013]. ABGD method [Puillandre et al., 2012] is based on pairwise distances,

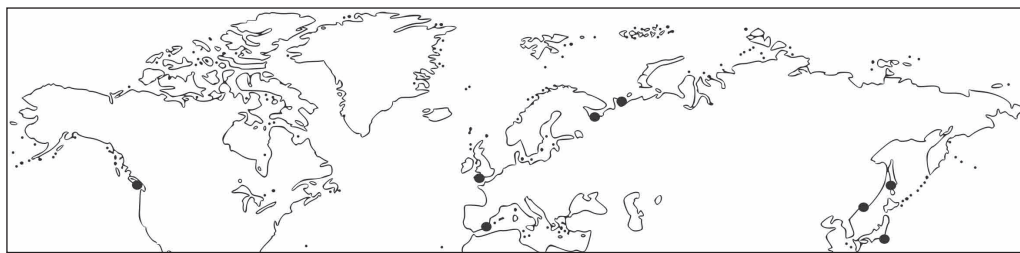


Fig. 1. Collecting locations of specimens used in this study.

detecting the breaks in the distribution referred to as the «barcode gap» [Herbert 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; Camara et al., 2014; Katugin *et al.*, 2015; Ekimova et al., 2016]. The ABGD program is available at the web-site <http://www.abi.snv.jussieu.fr/public/abgd/abgdweb.html>. We analyzed *COI* and *16S* alignments excluding the outgroups using either uncorrected *p*-distance. *P*max was increased to 0.15 and *X* (relative gap width) decreased to 1.0 for *COI*, while these settings were set as default for *16S*. Other settings remained as default for both fragments.

Results

Specimens examined. *Placida babai*: 66 specimens: 15–30 Aug. 2014, Vostok Bay, 1–2 m depth, leg. A. Chichvarkhin; 1 specimen: 25 July 2015, Vladivostok, Amursky Bay, 2 m depth, leg. K. Dudka; 2 specimens: 27 Aug. 2014, Nevelsk, Sakhalin, Russia, 0.1 m depth on rocky intertidal, leg. A. Chichvarkhin; 1 specimen: 6 May 2015, intertidal, Chiba, Japan, leg. Y. Fujita. *Placida* cf. *dendritica*: 2 specimens: July 2013, San Francisco, CA, USA, leg. Á. Valdés; 1 specimen: White Sea Biological Station, Lomonosov Moscow State University, intertidal, June 2013, leg. I. Ekimova; 8 specimens: White Sea Biological Station, Lomonosov Moscow State University, 5 m depth, July 2014, leg. T. Antokhina; 1 specimen: 2 July 2014, Falmouth, Cornwall, UK, leg. K. Bolton; 1 specimen: 5 Oct. 2014, Vancouver Isl., BC, Canada; 3 specimens: 20 Aug. 2015, Barents Sea, Zelenaya Bay, 7 m depth, leg. T. Antokhina.

External morphology (Fig. 2A–E). Body elongate, laterally compressed. Foot narrow, tail short. Mouth area muscular, without papillae. Dorsal appendages (cerata) conical, well developed, long. Rhinophores auriculate. Foot wider in head area, with oral lobe. Two dorsolateral eyes, each behind a rhinophore. Anal papillae long and narrow, located in anterior dorsal position. Anal opening on the dorsal side of the body, about one-third of body length from head. Reproductive openings lateral, on the right side behind the head. Branches of digestive system are thin, dense, entirely penetrate and fill the rhinophores, foot and papillae, leaving empty areas around the eyes and on ventral side of foot and body.



Fig. 2. Live animals: **A, D** – *Placida babai*, Sakhalin Isl.; **B, E** – *P. dendritica*, White Sea; **C** – *P. cf. dendritica*, California; radulae: **F, G** – *P. dendritica*, White Sea; **H, I** – *P. cf. dendritica*, California; **J, K** – *P. babai*, Sakhalin Isl.; **L** – *P. babai*, Vostok Bay. Scale bars: **A–E** – 5 mm; **F–I, K** – 40 μ m; **J, L** – 20 μ m.

C o l o u r (Fig. 2A–E). Background color translucent-white or pale or light yellow (Fig. 2A, D). Pale-white and dark green spots, stripes and dots on the head, papillae and

rhinophores, white spots denser distally. White gonads visible between papillae dorsally. Colour of the digestive system varies from brownish-yellow to dark green.

I n t e r n a l m o r p h o l o g y (Fig. 2F–L): Radular formula: (8–9)+(21–30)x0.1.0; 8+29x0.1.0 (Fig. 2J–L). Median tooth strong, more chesel-shaped rather than awl-shaped, some teeth tips rounded (Fig. 2L). Reproductive system pseudo-diaulic (three ducts: male duct, oviduct and vagina and two openings). Ootestis large and white made of large follicles. Hermaphroditic duct arises from ootestis and enter small ampulla with expansion in midline at one-third part of body, then divides into small oviduct and vas deferens. Vas deference wide, winding, with bilobed prostate near its beginning. Penis armed with short stylet. Albumen glands made of numerous lobules and placed laterally on both sides of body. Female duct bifurcates forming vagina and oviduct. Small oval bursa copulatrix locates on right side near body wall. Female genital aperture open posterior to penis on right side of body, which is located behind the head.

D i s t r i b u t i o n. Sea of Japan, Pacific shore of Japan, Yellow Sea. Probably has wider distribution.

H a b i t a t a n d e c o l o g y. Live animals were collected on the continental shore of the Sea of Japan in Vladivostok (Amurskiy Bay) and Vostok Bay at the depths of 1–2 m on *Bryopsis plumosa* (Hudson) C. Agardh, 1823 growing on artificial objects (ropes and abandoned pier constructions). In Nevelsk, Sakhalin Island they were found on *B. hypnoides* J.V. Lamouroux, 1809 at about 10 cm depth in a rocky intertidal. The report of feeding on *Ulva* in Peter the Great Bay by Martynov and Korshunova [2011] has never been reconfirmed.

Molecular analysis

We acquired 7 distinct *COI* haplotypes for the 15 specimens of the genus *Placida* from the Pacific, Atlantic, and Arctic oceans. ABGD analysis of *COI* dataset run with unbiased (simple) distance models revealed 4 clades each: one for Atlantic *P. dendritica*, two for White and Barents Seas and NE Pacific *P. dendritica*, respectively, and the fourth for NW Pacific *P. babai*. For *16S* fragment, ABGD analysis revealed the three groups: *P. dendritica* from Mediterranean, *P. dendritica* from western Sweden and Britain, and *P. babai* from Sea of Japan and Yellow Sea. The prior maximum distance ranged between 0.0010 and 0.0359 for both fragments. The NJ tree built using uncorrected *p*-distances between *COI* sequences (Fig. 3) revealed the same pattern with well supported by bootstrap *P. babai*+*P. dendritica* clade consisting of two distinct subclades attributed to each species while north-eastern Pacific and Arctic forms were closer to *P. dendritica*. All the members of *P. dendritica* clade are clearly distinct from the rest Limapontiidae outgroup. Uncorrected *p*-distances (Table 2) between *P. babai* and Atlantic *P. dendritica* ranged 12.3–13.3% for *COI* and 4.7–4.9% in *16S*. The most divergent from *P. babai* was White and Barents Seas form (13.4–13.4%, *COI*) while the least divergent was North American one (11.3–11.6%, *COI*). The distance between ingroup (*P. dendritica* and *P. babai*) and outgroup *COI* sequences *Placida* species was 18.3–21.9%. *P. babai*

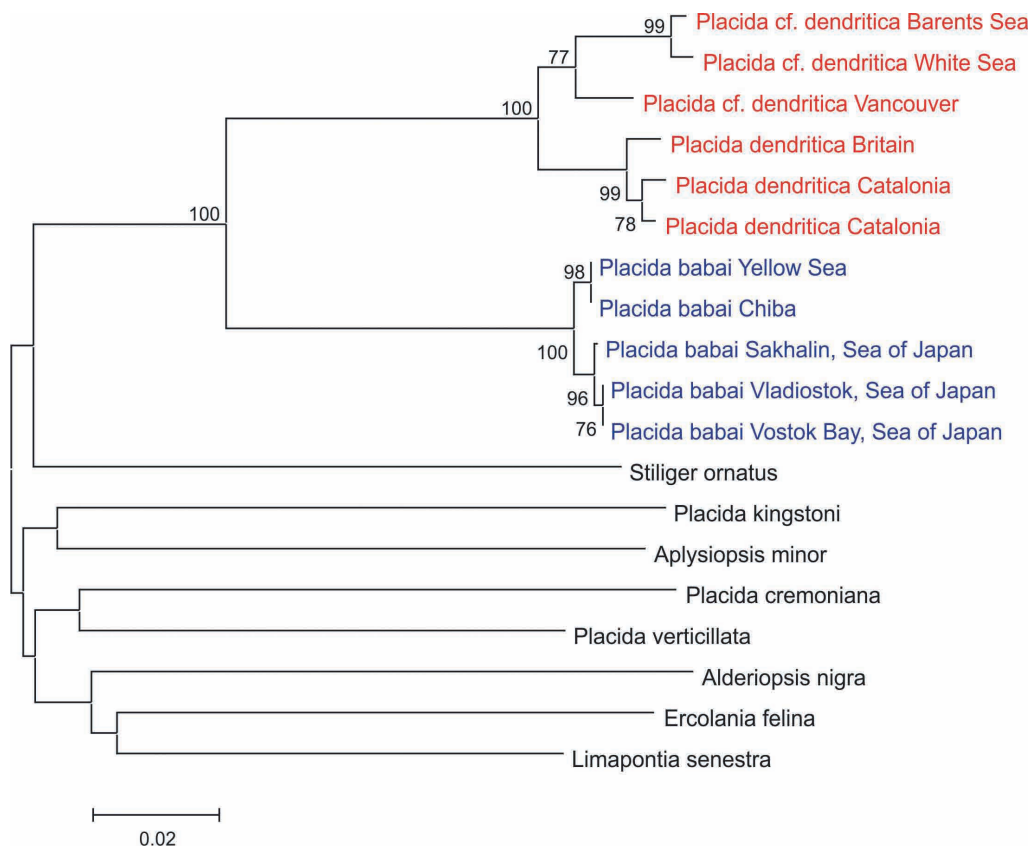


Fig. 3. Unrooted cytochrome oxydase subunit 1 NJ gene tree built using p-distances. Bootstrap support values (1000 replications) are marked at the internodes.

was almost invariable intraspecifically (0–0.8%) while the *p*-distance between *P. dendritica* s.l. ranged 0.8% between Mediterranean samples to 4.6% between North Sea and White/Barents Sea specimens (2.7% between White Sea and Canada). In *I6S* fragment, intraspecific distances were 0–0.3% for both species.

Discussion

The individuals of *P. babai* from the Russian waters of the Sea of Japan possess similar internal and external morphology with eastern Pacific, Arctic and Atlantic *P. dendritica*. Unlike the latter, *P. babai* possesses very fine network of digestive tubules penetrating almost entire foot (excluding ventral side), and entire the rhinophores making the body almost uniformly green (Fig. 2A, D), while in *P. dendritica* the tubules are thick, form 2–3 distinct stems in head and rhinophoral area, fill mainly distal portion of the rhinophores, never permeate to rhinophoral tips or foot edge (Fig. 2B). Young individuals of *P. babai* are in some degree similar to *P. dendritica*, although their fine branched

Table 2

Intraspecies and interspecies p -distances between *Placida* species.
Lower triangle – *COI*, upper triangle – *16S*

No.	Species, locality	1	2	3	4	5	6	7	8	9	10	11	12
1	<i>Placida babai</i> , Vladivostok												
2	<i>P. babai</i> , Vostok Bay	0.000		0.000	0.003	0.000	–	–	0.049	0.049	0.049	–	–
3	<i>P. babai</i> , Sakhalin	0.002	0.002		0.003	0.000	–	–	0.049	0.049	0.049	–	–
4	<i>P. babai</i> , Yellow sea	0.008	0.008	0.006		0.003	–	–	0.047	0.047	0.047	–	–
5	<i>P. babai</i> , Chiba	0.008	0.008	0.006	0.000		–	–	0.049	0.049	0.049	–	–
6	<i>P. dendritica</i> , Vancouver	0.117	0.117	0.116	0.113	0.113		–	–	–	–	–	–
7	<i>P. dendritica</i> , White/Barents seas	0.137	0.137	0.137	0.134	0.134	0.027		–	–	–	–	–
8	<i>P. dendritica</i> , Britain	0.133	0.133	0.132	0.131	0.131	0.041	0.035		0.000	0.003	–	–
9	<i>P. dendritica</i> , Sweden	–	–	–	–	–	–	–	–	0.003		–	–
10	<i>P. dendritica</i> , Catalonia	0.127	0.127	0.125	0.123	0.123	0.038	0.044	0.012	0.008		–	–
11	<i>P. cremoniana</i>	0.183	0.183	0.183	0.186	0.186	0.190	0.193	0.189	0.189	0.195		–
12	<i>P. verticillata</i>	0.191	0.191	0.190	0.190	0.190	0.196	0.195	0.193	0.193	0.198	0.170	
13	<i>P. kingstoni</i>	0.191	0.191	0.190	0.190	0.190	0.202	0.212	0.213	0.218	0.219	0.215	0.175

tubules are obvious. The specimens from north-eastern Pacific resemble *P. dendritica* but manifest an intermediate form possessing more branched network of thick tubules penetrating the foot like in *P. babai* (Fig. 2C). All examined Pacific specimens of *P. babai* and *P. dendritica* possess chisel-shaped radular teeth (Fig. 2H, J, L) while the slugs from White Sea possess awl-like teeth (Fig. 2F) – this contradicts Marcus's [1982] diagnosis for *P. babai* and *P. dendritica*, thus supports Baba's [1986] idea of the uselessness of this very polymorphic character for these species delimitation, and also supported with an observation of a dependence of radula morphology on *Placida* algal prey [Bleakne, 1990]. Internal morphology also fully coincided in these species and correspond to drawings by Baba [1986], we found no characters useful for unequivocal discrimination of these species. Although, Kumagai [2009] reported several anatomical differences between *Placida* sp. (sensu Baba [1986]) and *P. dendritica*. *Placida* sp. (sensu Baba [1986]) illustrated by Trowbridge et al. [2010, fig. 2E] resembles young/smaller individuals found in Vostok Bay with their incompletely developed network of digestive tubules and white pigmentation pattern.

Two independent molecular species delimitation approaches are in congruence with our new morphological data (patterns of coloration and tubules network) and thus, justify a separation of *P. dendritica* and *P. babai* inhabiting Russian waters of the Sea of Japan. The clades that comprise White and Barents Seas and North American forms may represent other cryptic species despite of similar morphology and low level genetic divergence between them and Atlantic specimens (3.5–4.6% in *COI*). Both distances and tree topologies have been recently shown of poor use as species delimitation tool possessing particular methodological perils [Will, Rubinoff, 2004; Meier et al., 2006; Wiemers, Fiedler 2007; Meier, 2008; Goldstein, DeSalle, 2011; Collins, Cruickshank 2012], in particular, disguising species boundaries with the «lack of barcode gap», thus these group revealed using more advanced ABGD approach may constitute one or more overlooked species, although this hypothesis requires further revision.

This study provides the evidence for two *Placida* species existence: *P. dendritica*, which inhabits the waters of the Northern Atlantic, and the other species from Russian part of the Sea of Japan, Yellow Sea, and Japan. Assigning any of two available names described from Asian Pacific region is not well substantiated yet because of questioned synonymy of *P. babai* and *P. daguilaensis* (C. Trowbridge, pers. comm.). Examination of the *P. babai* type material or exclusion of this name from zoological nomenclature along with molecular comparative analysis of Russian *Placida* sp. and *P. daguilaensis* is necessary. Therefore, we recommend referring *Placida* species from Russian waters of the Sea of Japan to as *Placida babai* E. Marcus, 1982 until future studies will untangle this question.

Few *Placida* taxa (*P. capensis* Macnae, 1954, *P. aoteana* Powell, 1937) described from different parts of the world were synonymized with *P. dendritica* [Bleakney, 1989; Marshall, Willan, 1999] but our study suggests that they may constitute distinct species of *P. dendritica* species group.

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