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Comparison of *Symbiodinium* dinoflagellate flora in sea slug populations of the *Pteraeolidia ianthina* complex

Makiko Yorifuji[1,2,*], Hirohiko Takeshima[1], Kohji Mabuchi[1], Toshiki Watanabe[1,‡], Mutsumi Nishida[1,3]

[1]**Department of Marine Bioscience, Atmosphere and Ocean Research Institute, The University of Tokyo, Kashiwanoha, Kashiwa, Chiba 277-8564, Japan**

[2]***Present address:*** **Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, 3422 Sesoko, Motobu, Okinawa 905-0227, Japan**

[3]***Present address:*** **University of the Ryukyus, 1 Senbaru, Nishihara, Okinawa 903-0213, Japan**

ABSTRACT: Symbiotic dinoflagellates of the genus *Symbiodinium* (zooxanthellae) harbored by a nudibranch mollusk, *Pteraeolidia ianthina* complex, were investigated from host individuals collected at 10 locations in the tropic to temperate waters of the northwestern Pacific. The *P. ianthina* complex is widely distributed throughout the Indo-Pacific Ocean and, in the northwestern Pacific, contains 2 genetically highly diverged cryptic species with variable genetic structuring. Information on such genetic composition and structuring of the host is useful for assessing whether the relationship between the host and symbiont flora is determined by the genotype of host organisms or by environmental factors. The diversity of the harbored *Symbiodinium* was analyzed using the sequence differences of internal transcribed spacer region 2 in the nuclear ribosomal gene, chloroplast ribosomal gene large subunit 23S Domain V, and mitochondrial cytochrome *b* gene. Symbionts of clades A, C, and D were detected in this nudibranch host. Among them, 6 types were identified (1 clade A, 3 clade C, and 2 clade D types); 2 of them are suggested to be novel. The occurrence of each *Symbiodinium* type differed among the geographical locations, and hence the co-occurrence pattern (i.e. composition) of the symbionts also varied geographically. The geographical pattern of symbiont composition was correlated with the genetic structuring of the host as well as climatic environmental factors. Therefore, these results suggest that both environmental conditions and the host mollusk's genetics affect the symbiont composition.

KEY WORDS: *Symbiodinium* · Zooxanthellae · Geographic variation · Sea slug · *Pteraeolidia* · Northwestern Pacific

INTRODUCTION

Many marine invertebrates, including cnidarians and mollusks, have symbiotic relationships with unicellular dinoflagellates of the genus *Symbiodinium* (commonly known as zooxanthellae). This symbiotic relationship is essential for the majority of the host organisms, and it is an important component underpinning coral reef ecosystems (Taylor 1973, Muscatine & Porter 1977, Trench 1993). Through recent molecular phylogenetic analyses, the genus *Symbiodinium* has been found to contain 9 genetically highly diverged groups: clades A through I (Pochon & Gates 2010). Subsequently, these have been fur-

*Corresponding author: makikoyorifuji@gmail.com
‡Deceased

ther subdivided into numerous subclades or types (e.g. Coffroth & Santos 2005). Some of the clades/types of *Symbiodinium* exhibit different physiological responses to water temperature and/or solar irradiance, and accordingly, the stress tolerance and resilience of hosts and the harbored symbionts (holobionts) also vary (Kinzie et al. 2001, Rowan 2004, Tchernov et al. 2004, Sampayo et al. 2008, Suggett et al. 2008).

Variations in *Symbiodinium* have been investigated for various host species. Studies conducted in the last decade have reported that the composition of their symbiont types varies geographically, even within a single host species (LaJeunesse & Trench 2000, Burnett 2002, Reimer et al. 2006, Lien et al. 2007, 2013, Macdonald et al. 2008, Finney et al. 2010, LaJeunesse et al. 2010). Such geographic variation in *Symbiodinium* may suggest environmental adaptation by these holobionts. In particular, the physiological characteristics of holobionts (e.g. tolerance to high or low water temperature and high solar irradiance) are generally assumed to change with symbiont composition in response to prevailing environmental conditions (e.g. LaJeunesse et al. 2010).

Another factor—the host identity—also contributes to the geographical variation in *Symbiodinium* symbiosis. Recent population genetic studies have provided evidence for the presence of highly genetically structured populations in benthic marine organisms (e.g. Avise 2000). Furthermore, morphologically very similar (i.e. cryptic) species have been repeatedly discovered using molecular techniques (Knowlton 1993, Avise 2000, Miglietta et al. 2011). Therefore, geographic variation in harbored *Symbiodinium* may be dependent on genetic (i.e. phylogenetic) differences of the host animal populations. Several studies have recently been conducted on both host genetics and symbiotic algae for some cnidarian symbiotic systems. Correlations between host genetic identity and symbiont flora were reported for the coral genus *Pocillopora* in the tropical eastern Pacific (Pinzón & LaJeunesse 2011); *Montastraea annularis* species complex in the Florida Keys, USA (but not in Exuma Cays, Bahamas; Thornhill et al. 2010a); *Orbicella annularis* species complex, genus *Siderastrea* and *Agaricia* in the Greater Caribbean (Thornhill et al. 2014); sea anemone genus *Aiptasia* (Thornhill et al. 2013); and *Oulastrea crispata* in the west Pacific (Lien et al. 2013); but see Barshis et al. (2010) and Baums et al. (2010) for studies that have not found clear relationships.

In this study, *Symbiodinium*-type composition (i.e. flora) was analyzed for a species of nudibranch mollusk, *Pteraeolidia ianthina* (Angas, 1864). The geographic variation in the symbiont of this mollusk has been reported in the southwestern Pacific (Loh et al. 2006); however, the genetic identity of the host and correlation to its symbiont flora has not yet been evaluated. On the other hand, genetic composition and genetic population structuring of this molluscan species have been well studied in the northwestern Pacific waters: 2 genetically distinct cryptic species have been identified and further genetic population structuring has been discovered in one of the species (Yorifuji et al. 2012). We analyzed *Symbiodinium* flora for individuals of the *P. ianthina* complex collected in the tropic to temperate waters of the northwestern Pacific. Furthermore, the similarity of the *Symbiodinium* flora was compared with genetic relationships among the host populations.

MATERIALS AND METHODS

Samples

In total, 283 individuals of the *Pteraeolidia ianthina* complex collected from 10 localities in the tropic to temperate waters of the northwestern Pacific were analyzed (Fig. 1, Table 1). All sea slugs that were analyzed here were the same individuals as those analyzed in our previous study on genetic structure

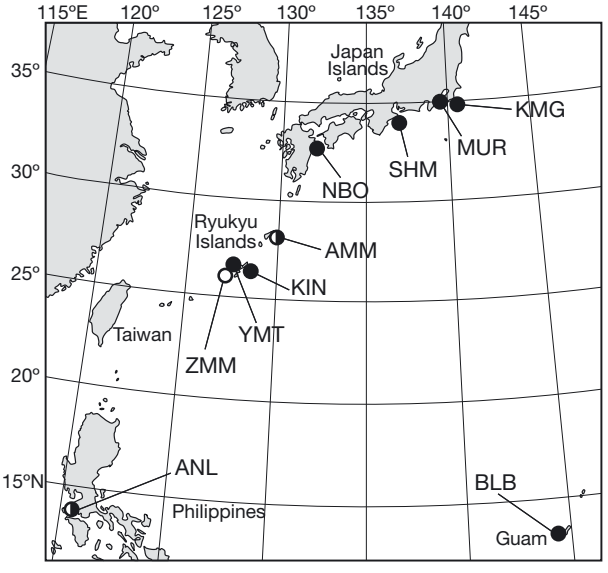

Fig. 1. Locations of the sampled *Pteraeolidia ianthina* complex. Circles denote the 2 cryptic species detected from the locations (solid: species A; half-solid: both species A and B; open: species B). Details of the location abbreviations and species are listed in Table 1

Table 1. Collection data and sample size of the *Pteraeolidia ianthina* complex. Host species designation was referenced from Yorifuji et al. (2012)

Sample abbrev.	Collection location			Collection depth (m)	Collection date (yr and mo)	No. of host individuals	
	Location name	Latitude °N	Longitude °E			Species A	Species B
KMG	Kamogawa, Chiba, Japan	35.12	140.18	0–3	2007 Jun	22	–
MUR-1	Miura, Kanagawa, Japan	35.15	139.61	0–3	2007 May	30	–
MUR-2	Miura, Kanagawa, Japan	35.15	139.61	0–3	2007 Aug	26	–
SHM	Shima, Mie, Japan	34.26	136.78	0–6	2008 Oct	29	–
NBO	Nobeoka, Miyazaki, Japan	32.65	131.77	0–15	2008 Dec	30	–
AMM	Amami, Kagoshima, Japan	28.40	129.62	0–10	2009 Jan	30	6
KIN	Kin, Okinawa, Japan	26.45	127.91	0–5	2008 Dec	30	–
YMT-1	Yomitan, Okinawa, Japan	26.44	127.71	0–5	2007 May	30	–
YMT-2	Yomitan, Okinawa, Japan	26.44	127.71	0–5	2007 Nov	16	–
ZMM	Zamami, Okinawa, Japan	26.18	127.28	3–6	2009 Apr	–	11
ANL	Anilao, Batangas, Philippines	13.72	120.86	5–20	2009 May	9	9
BLB	Bile Bay, Merizo, Guam	13.26	144.66	2–5	2009 Jun, Jul	5	–

(Yorifuji et al. 2012), except for those newly collected from 2 locations (MUR and YMT) in a different season (Table 1). For specimens newly included in this study, the methods of tissue fixation and DNA extraction were similar to those used by Yorifuji et al. (2012). The genetic assignments of host species (species A/B) were also performed as described in our previous report.

PCR and denaturing gradient gel electrophoresis (DGGE) of *Symbiodinium* nuclear ribosomal DNA

Clades/types of the genus *Symbiodinium* were identified by sequence differences of the internal transcribed spacer 2 region of nrRNA genes (nrITS2). DGGE was used to separate PCR products according to the method of LaJeunesse & Trench (2000). The nrITS2 region was amplified using the primer pair ITSint-for2/ITS2CLAMP (LaJeunesse & Trench 2000) and amplification conditions described by LaJeunesse et al. (2003). PCR was performed in a total volume of 20 µl with TaKaRa Ex*Taq* (Takara Bio); the PCR cocktail was prepared according to the manufacturer's instructions. The amplified nrITS2 PCR products were separated by DGGE using a Bio-Rad DCode System (Bio-Rad Laboratories) as described by LaJeunesse & Trench (2000) with slight modification. Briefly, the denaturing gradient concentrations, running time, and voltage were modified for optimal resolution of the samples that were used in this study: 8% polyacrylamide gels with an internal gradient of 20 to 75% denaturants (urea and formamide) were run at 90 V for 15 h at a constant temperature of 60°C.

TA cloning and sequencing of DNA from representative DGGE bands

DGGE bands showing identical mobilities have the same sequences (LaJeunesse 2002, Thornhill et al. 2010b, LaJeunesse & Thornhill 2011); thus, several host individuals were selected (31 individuals in total) using the DGGE fingerprints of their symbionts and sequenced to identify the clades and types of harbored *Symbiodinium* spp. In the majority of the previous studies that analyzed *Symbiodinium* diversity using DGGE, DNA was extracted from the DGGE bands and sequenced (e.g. LaJeunesse 2002). This method was developed for host individuals predominantly bearing 1 or 2 types of *Symbiodinium.* However, a co-occurrence of 3 or 4 symbiont types was frequently observed within a host individual in this study. When several types and clades of symbionts co-occurred, DGGE fingerprints became complicated, and the sequences that were obtained from a band were unclear. Therefore, PCR products of the *Symbiodinium* were verified by cloning and sequencing as described below.

First, a region including part of the 5.8S and 28S rRNA genes along with ITS2 was amplified using primers ITSint-for2 (LaJeunesse & Trench 2000) and ITS2 rev (reverse primer lacking the GC clamp). PCR was performed in a total volume of 20 µl with TaKaRa Ex*Taq* polymerase according to the manufacturer's instructions. The thermal cycling profile for amplification was as previously described (LaJeunesse et al. 2003). Next, the PCR products were purified using a MinElute® PCR Purification kit (Qiagen) and subsequently cloned using the pGEM-T Easy Vector system (Promega) and com-

petent DH5-α cells (Toyobo). The presence of recombinant colonies was screened by direct PCR with vector-specific primers, in which vector-inserted DNA (including the ITS2 region) was amplified in a total volume of 10 µl using TaKaRa Ex*Taq* polymerase according to the manufacturer's instructions. The thermal cycling profile was the same as that used for inserts (LaJeunesse et al. 2003).

For sequencing, direct PCR products of the recombinant clones were purified using ExoSAP-IT (USB Corp) and subsequently used for cycle sequencing reactions using BigDye® Terminator v.3.1 (Applied Biosystems) with the primers used for the vector-inserted DNA. The thermal cycling profile was followed as per the manufacturer's instructions. The reaction products were sequenced using an ABI 3130xl automated capillary DNA sequencer (Applied Biosystems). The sequences were edited and assembled using ATSQ v.6.0.1 (Genetyx). The DNA sequences obtained in this study have been deposited in DDBJ under accession numbers AB778572 to AB778765. Finally, to check the correlation between DGGE fingerprints and the final sequences obtained, PCR products from the recombinant clones were subjected to DGGE along with raw (not cloned) PCR products, and the mobility of the inserts was examined. The PCR and DGGE conditions were the same as those described above.

Phylogenetic analyses and identification of *Symbiodinium* types

For all sequences obtained, the boundaries between the 5.8S rRNA gene, ITS2 region, and 28S rRNA gene were identified by comparing them with other *Symbiodinium* sequences from the DNA databases (GenBank/EMBL/DDBJ). The multi-copied rRNA gene may contain minor variant sequences, and in some cases these sequences are obtained via cloning. In addition, cloning itself can occasionally induce sequence errors, and these minor sequences can be difficult to distinguish (Thornhill et al. 2007, LaJeunesse & Pinzón 2007, LaJeunesse & Thornhill 2011). Thus, sequences were used for further analyses only when more than 2 identical sequences were obtained from the same host individual. The authenticity of DGGE bands was also checked by a basic local alignment search tool (BLAST) search of DNA databases using sequences of the nrITS2 region. The nrITS2 region of each DGGE band was assigned to a specific clade on the basis of phylogenetic analysis with known sequences obtained from the GeoSymbio database (Franklin et al. 2012), which provides alignments of *Symbiodinium* nrITS2 sequences that have been published from 1982 to the present. The type was then determined for each clade: the nrITS2 sequences of each clade were aligned again with sequences obtained in this study; alignment was performed according to the aligned sequences provided by the GeoSymbio database. Phylogenetic analysis by the maximum-likelihood (ML) method was performed using RAxML v.7.2.6 (Stamatakis 2006). The model of evolution for this analysis was determined as GTR+Γ using Akaike's information criterion (AIC; Akaike 1974) implemented in Kakusan 4 (Tanabe 2011). Finally, rapid bootstrap analyses were performed 1000 times to assess the reliability of the nodes in the constructed phylogenetic tree. Because clade C *Symbiodinium* is a diverse group and many nrITS2 sequences have been reported, the number of operational taxonomic units (OTUs) in the phylogenetic analysis of clade C was reduced to 23, according to clusters defined by Correa & Baker (2009).

Symbiodinium type recognition with chloroplast and mitochondrial gene markers

Because intragenomic variation has been reported for nrITS2 in *Symbiodinium* (LaJeunesse & Thornhill 2011), type recognition was also performed with chloroplast and mitochondrial gene markers. A total of 11 samples representing typical DGGE profiles were selected and analyzed. The chloroplast ribosomal gene large subunit Domain V (cp23S) and mitochondrial cytochrome *b* gene (mtCyt*b*) fragments were amplified using the primer pairs 23S4F/23S7R (Pochon et al. 2006) and Dinocob1F/Dinocob1R (Zhang et al. 2005), respectively, using the thermal cycling profiles described by the primer designers. PCR was performed in a total volume of 20 µl with TaKaRa Ex*Taq*; the PCR cocktail was prepared according to the manufacturer's instructions. For 3 samples, which were considered to have a single type of *Symbiodinium* from their DGGE profiles, the PCR products of cp23S and mtCyt*b* were directly sequenced. For the other samples, PCR products were subsequently cloned and then sequenced. Conditions for TA cloning and sequencing were as described above; however, the amplification conditions were maintained according to the inserted DNA fragments. The obtained sequences were edited and assembled using ATSQ v.6.0.1, and the correlations between the 3 gene

markers were verified. The DNA sequences obtained for cp23S and mtCyt*b* in this study have been deposited in DDBJ under accession numbers AB971141–AB971157 (see Table 2).

Statistical analyses

Cluster analysis was performed to evaluate the similarity of *Symbiodinium* flora in each species/population of the *P. ianthina* complex, which consisted of 2 genetically highly diverged species (A and B) and 3 regional population groups in species A (Yorifuji et al. 2012). The infection frequencies of the *Symbiodinium* types and composition of the symbionts were used as indicators of the symbiont flora. The infection frequency of each type was calculated on the basis of the number of host individuals that were infected in a population. The symbiont composition in each host individual was represented by the DGGE profile (band pattern) obtained. The number of host individuals in each population that harbored the same symbionts was recorded by the DGGE profiles, and their frequency of occurrence in each population was calculated. Two sets of Euclidian distance between populations were calculated from these 2 frequencies, and a hierarchical cluster analysis was conducted with the unweighted pair-group method using the arithmetic mean (UPGMA) on the basis of each set of the Euclidian distances. The topology of the obtained UPGMA dendrogram was compared with that of the tree for the host slug populations, which shows the genetic relationships of slug populations. Differences in the occurrence of each *Symbiodinium* type and species/populations of the host were also evaluated by Fisher's exact test. The above-mentioned statistical analyses were performed using the statistical package R v.3.0.2 (www.r-project.org).

RESULTS

Symbiodinium clades/types detected

A total of 9 major bands (a to i) were identified from the DGGE profiles for *Symbiodinium* harbored by the *Pteraeolidia ianthina* complex (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m521p091_supp.pdf). Bands were assigned to clade A (band a), clade C (bands b to e), or clade D (bands f to i) on the basis of phylogenetic analysis with known sequences. Type grouping of sequences from the bands of each clade was performed through phylogenetic analysis of ITS2 sequences and the correlation between cp23S and mtCyt*b* sequences, as described below (Fig. 2).

Clade A: sequences of the clones corresponding to band a were identical to those of A1 sensu LaJeunesse (2001) (Fig. 2a). These sequences were also identical to that obtained from *P. ianthina* in Hayama Bay, temperate Japan (HpiH-1; Ishikura et al. 2004). A single type of clade A sequence was obtained for both cp23S and mtCyt*b* markers (Table 2). No identi-

Table 2. DGGE bands and *Symbiodinium* types indicated by nrITS2, cp23S, and mtCyt*b*

Clade	A	C				D			
Type in this study	A1	C3*		C1	C_{Pi}-2	D_{Pi}-1	D1*		
DGGE band	a	b	d	c	e	f	g	h	i
nrITS2	A1	C3	C_{Pi}-1	C1	C_{Pi}-2	D_{Pi}-1	D1	D4	D5
cp23S	i	ii	ii	ii	ii	iii	iv	iv	iv
mtCytb	I	II	II	III	IV	V	V	V	V

i: [AB971141], 99% identical (1 nucleotide [nt] difference) to *Symbiodinium* sp. A13 (JN558027; Pochon et al. 2012);
ii: [AB971142–4]‡, 100% identical to *Symbiodinium* sp. C1 (FN298482; Pochon et al. 2010);
iii: [AB971145], 98% identical (15 nt difference) to *Symbiodinium* sp. D1 (JN558007; Pochon et al. 2012);
iv: [AB971146–7]‡, 100% identical to *Symbiodinium* sp. D1 (JN558007; Pochon et al. 2012);
I: [AB971148], 99% identical (5 nt difference) to *Symbiodinium* sp. CCMP832 (AY456112; Zhang et al. 2005);
II: [AB971149–51]‡, 99% identical (1 nt difference) to *Symbiodinium* sp. C78a (FJ529540; Sampayo et al. 2009);
III: [AB971152-53]‡, 100% identical to *Symbiodinium goreaui* (EU130574; Zhang et al. 2008)/*Symbiodinium* sp. C1 (FJ529534; Sampayo et al. 2009);
IV: [AB971154], 99% identical (2 nt difference) to *Symbiodinium* sp. C78a (FJ529540; Sampayo et al. 2009);
V: [AB971155–7]‡, 100% identical to *Symbiodinium* sp. 1 TCLJ-2013 (KF193520; T. C. LaJeunesse unpubl. data).

‡When the same sequence was detected from multiple locations, the sequence type was assigned as multiple accession numbers

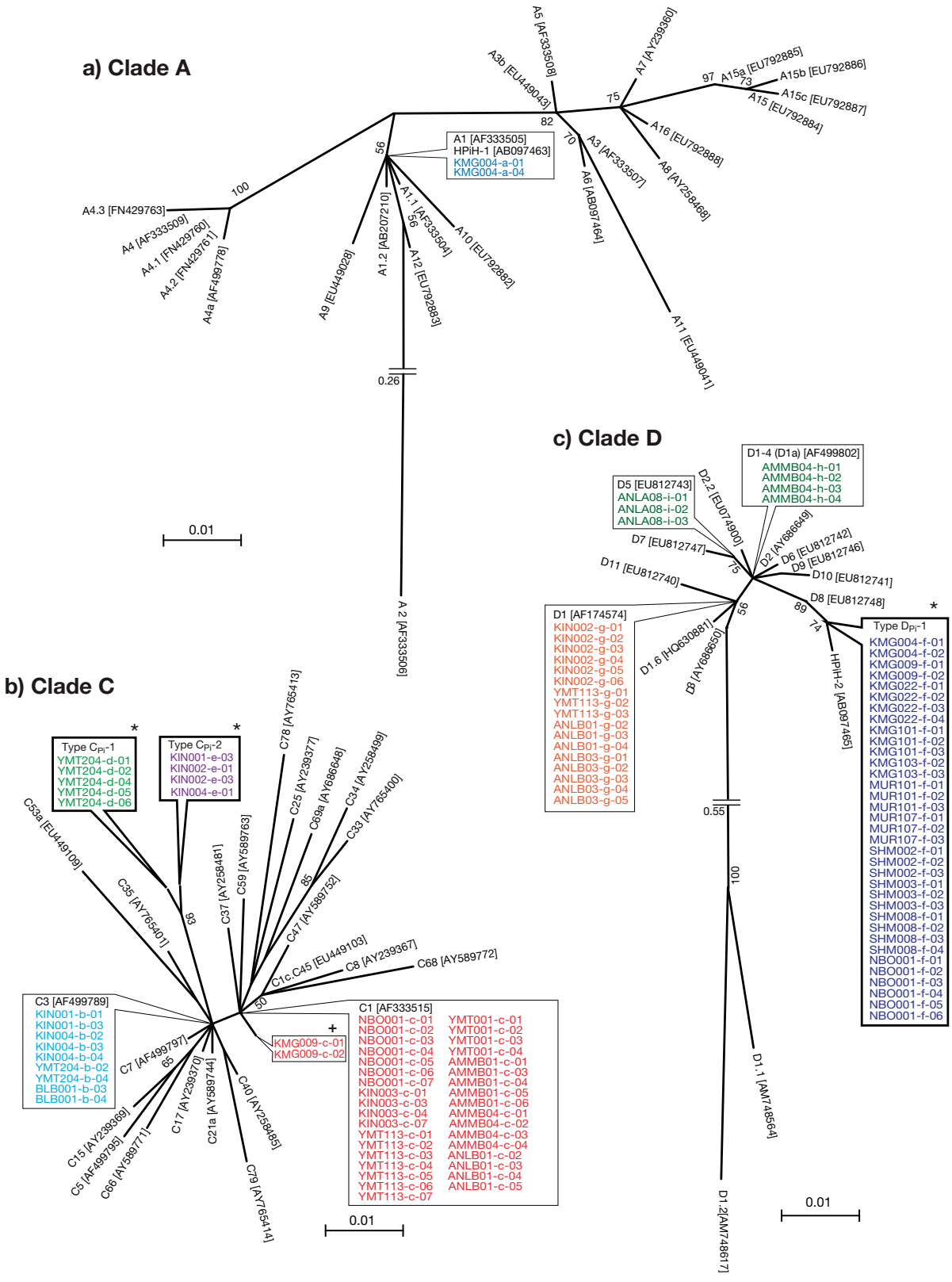

Fig. 2. Phylogenetic relationships among *Symbiodinium* using the maximum-likelihood (ML) method based on the ITS2 region and partial 5.8S and 28S rRNA gene sequence data for (a) clade A *Symbiodinium* (264 bp), (b) clade C *Symbiodinium* (291 bp), and (c) clade D *Symbiodinium* (314 bp). Numbers at some nodes indicate bootstrap values (>50%) based on 1000 replicates for internal branch support. Some operational taxonomic units (OTUs) in black letters have been obtained from DNA databases, and their accession numbers are shown in parentheses; others in colored letters represent clone sequences obtained in this study, with names designated in the following manner: [host individual code]-[band code]-[clone number]. (*) indicates novel types; (+) indicates band c clones obtained in low frequency

cal sequence for cp23S and mtCyt*b* has been reported thus far.

Clade C: all sequences of band b clones were identical to those of C3, which were reported by LaJeunesse (2005) (Fig. 2b). The sequences of the majority (35 out of 37) of the band c clones were identical to those of C1, which were reported by LaJeunesse (2001) (Fig. 2b). Two sequences of the band c clones obtained from the KMG individual were not identical to C1 (1 nucleotide [nt] difference; Fig. 2b): *Symbiodinium* with this sequence may be naturally present at a low frequency, or it may be an intragenomic variation of C1; however, because of the low frequency, we omitted this sequence from further analyses in this study. No identical sequences have been previously reported for those obtained from bands d and e; thus, we defined them as novel types and named them C_{Pi}-1 and C_{Pi}-2, respectively (Pi: abbreviation for the species name of the host sea slug; Fig. 2b). A single type of clade C sequence was obtained from the cp23S marker, which was identical to *Symbiodinium* sp. C1 (sensu Pochon et al. 2010; Table 2). In contrast, from the mtCyt*b* marker, 3 sequences were obtained: II, III, and IV (Table 2), which corresponded to C3 + C_{Pi}-1, C1, and C_{Pi}-2 of nrITS2, respectively. The III sequence of mtCyt*b* was identical to *Symbiodinium goreaui* (sensu Zhang et al. 2008; Table 2); however, no identical sequences were reported for II and IV. The novel nrITS2 sequences of C_{Pi}-1 and C_{Pi}-2 were highly similar (Fig. 2), and the corresponding DGGE bands d and e were always observed together (see Table S1 in the Supplement). However, distinct mtCyt*b* sequences (II and IV) were obtained from hosts showing band d and bands d + e in DGGE; thus, we designated the nrITS2 sequences of C_{Pi}-1 and C_{Pi}-2 as distinct *Symbiodinium* types (Table 2). According to the data from 3 gene markers, bands b and d (C3 + C_{Pi}-1) were designated as C3*, band c as C1, and band d as C_{Pi}-2, as represented by the name of nrITS2 type.

Clade D: sequences of the band f clones were also concluded to be of a novel type; thus we designated them as D_{Pi}-1 (Fig. 2c). Among clade D sequences reported thus far, *Symbiodinium* HpiH-2 from the same host sea slug in temperate Japan (Ishikura et al. 2004) and D8 from the scleractinian coral *Oulastrea* spp. in the west Pacific (LaJeunesse et al. 2010, Lien et al. 2013) differ from D_{Pi}-1 by only 1 nt, and hence, they clustered together in the phylogenetic tree (Fig. 2c). Sequences of clones corresponding to DGGE band g were identical to D1 (LaJeunesse 2001). Those corresponding to bands h and i were identical to D4 and D5 (LaJeunesse et al. 2010), respectively. The co-occurrence pattern of bands g, h, and i (i.e. types D1, D4, and D5, respectively, in Table S1; also see Fig. S1) may represent types D1-4 and D4-5, which have co-dominant nrITS2 sequences, D1 + D4 or D4 + D5, within their genome (LaJeunesse et al. 2010, LaJeunesse & Thornhill 2011). Note that the thickness of these DGGE bands differed between host individuals, even those in the same location (see lower panel of Fig. S1), which may indicate the presence of separated types of D1, D4, and D5. A single type of clade D sequence was obtained from the mtCyt*b* marker, which was identical to *Symbiodinium* sp. 1 TCLJ-2013 (Table 2, T. C. LaJeunesse unpubl. data). In contrast, 2 types of clade D sequences were obtained from the cp23S marker (named iii and iv), which corresponded to D_{Pi}-1 and D1 + D4 + D5, respectively (Table 2). No identical sequences have been reported for sequence iii, but sequence iv is identical to *Symbiodinium* sp. D1 (sensu Pochon et al. 2012; Table 2). According to the data from the 3 gene markers, band f was designated as D_{Pi}-1, and bands g, h, and i (D1 + D4 + D5) were designated as D1*.

Occurrence of each *Symbiodinium* type in host populations

The occurrence of the above-mentioned *Symbiodinium* types varied geographically. Although types C1 and C3* were detected from all 3 climate zones investigated, the others showed regional characteristics: types A1 and D_{Pi}-1 were isolated only from temperate locations, whereas types C_{Pi}-2 and D1* were identified in subtropical and tropical locations (Table 3). As for between-host species differences (species A and B), *Symbiodinium* types A1, C_{Pi}-2, and D_{Pi}-1 were detected only from species A individuals; type C3* was also detected predominantly from species A (Table 3). In contrast, types C1 and D1* were detected from both *P. ianthina* A and B (Table 3). When this comparison was restricted to the populations from subtropical and tropical regions, where both species co-occurred (Fig. 1, Table 1), the infection frequencies of types C3* and C_{Pi}-2 were higher in species A ($p < 0.05$), whereas those of C1 and D1* were higher in species B (Fisher's exact test, $p < 0.005$; see Table 3 for infection frequencies).

Regarding differences within a host species (i.e. the difference between 3 regional population groups of *P. ianthina* A; Table 3), types A1 and D_{Pi}-1 were detected only from temperate Japan, C_{Pi}-2 was detected only from Ryukyus + Philippines (specifi-

Table 3. Frequency and number of infected host individuals (in parentheses) by *Symbiodinium* types. *Various sequences of nrITS2 were gathered (C3+C_{Pi}-1, D1+D4+D5) according to the sequence profiles of cp23S and mtCyt*b* (see Table 2)

Samples	No. of host individuals	*Symbiodinium* clade and type					
		A	C			D	
		A1	C1	C3*	C_{Pi}-2	D_{Pi}-1	D1*
Host species A							
Temperate Japan population							
A-KMG	22	72.7 (16)	81.8 (18)	4.5 (1)	–	90.9 (20)	–
A-MUR-1	30	10.0 (3)	13.3 (4)	13.3 (4)	–	100.0 (30)	–
A-MUR-2	26	26.9 (7)	23.1 (6)	50.0 (13)	–	96.2 (25)	–
A-SHM	29	10.3 (3)	89.7 (26)	6.9 (2)	–	75.7 (22)	–
A-NBO	30	–	100.0 (30)	–	–	53.3 (16)	–
Ryukyus + Philippines populations							
A-AMM	30	–	90.0 (27)	40.0 (12)	–	–	3.3 (1)
A-KIN	30	–	36.7 (11)	63.3 (19)	63.3 (19)	–	23.3 (7)
A-YMT-1	30	–	100.0 (30)	–	–	–	3.3 (1)
A-YMT-2	16	–	50.0 (8)	68.8 (11)	–	–	–
A-ANL	9	–	77.8 (7)	44.4 (4)	–	–	77.8 (7)
Guam population							
A-BLB	5	–	80.0 (4)	40.0 (2)	–	–	20.0 (1)
Host species B							
B-AMM	6	–	100.0 (6)	–	–	–	66.7 (4)
B-ZMM	11	–	100.0 (11)	–	–	–	45.5 (5)
B-ANL	9	–	100.0 (9)	11.1 (1)	–	–	55.6 (5)

cally, only from the KIN population), and D1* was detected among the Ryukyus + Philippines and Guam populations. In contrast, types C1 and C3* were detected from all 3 regional population groups (Table 3). Among these population groups, the infection frequency of all types was significantly different ($p < 0.005$), except that of type C1 (Fisher's exact test, $p = 0.144$).

Composition of *Symbiodinium* types within host individuals

The co-occurrence pattern (i.e. composition) of *Symbiodinium* within host individuals was also different among types (Tables 4 & S1). Types C1, C3*, and D_{Pi}-1 were detected alone in some individuals, although no such cases were found in other types. Types C1 and C3* co-occurred with each of the clades A, C, and D, within host individuals, whereas clades A1 and D_{Pi}-1 co-occurred only with C1 and C3* but not with C_{Pi}-2 and D1* and vice versa.

More than half (152 of 283) of the host slug individuals had multiple symbiont types with varied compositions based on the geographical location (Tables 4 & S1). In temperate locations, host individuals with D_{Pi}-1 were dominant, where D_{Pi}-1 was generally observed along with either A1 or clade C types (C1, C3*, and C_{Pi}-2) or both. In contrast, in subtropical and tropical locations, all individuals harbored one or more clade C types, and some individuals also harbored D1*.

Comparison of *Symbiodinium* flora in host populations

Symbiodinium flora among the host slug populations were compared through cluster analysis based on the infection frequency (Table 3, Fig. S2a) and composition (Table 4, Fig. S2b) of 8 symbiont types. In both of the cluster dendrograms obtained from the 2 types of infection states of *Symbiodinium* types, 2 major clusters formed (Fig. S2). One cluster contained temperate populations of host *P. ianthina* A (Fig. S2, shaded blue), and the other contained subtropical and tropical populations of host species A and B (Fig. S2). Therefore, the *Symbiodinium* flora of subtropical and tropical populations of *P. ianthina* A (shaded gray and green) were more similar to those of species B (shaded red) compared with those of the temperate populations of species A. When these cluster analyses were performed with data of the *Symbiodinium* nrITS2 sequence types (Tables S1 & S2),

Table 4. Co-occurrence of *Symbiodinium* types in each host individual: their occurrence (%) and number of host individuals (in parentheses). *Various sequences of nrITS2 were gathered (C3+C_{Pi}-1, D1+D4+D5) according to the sequence profiles of cp23S and mtCyt*b* (see Table 2)

Samples	No. of host individuals	Composition of *Symbiodinium* types														
		A1 C1	A1 C1 D_{Pi}-1	A1 C3* D_{Pi}-1	A1 D_{Pi}-1	C1 D_{Pi}-1	C3* D_{Pi}-1	D_{Pi}-1	C1	C1 C3*	C3*	C3* C_{Pi}-2	C1 D1*	C1 C3* D1*	C3* D1*	C3* C_{Pi}-2 D1
Host species A																
Temperate Japan population																
A–KMG	22	9.1 (2)	50.0 (11)	–	13.6 (3)	22.7 (5)	4.5 (1)	–	–	–	–	–	–	–	–	–
A–MUR–1	30	–	–	–	10.0 (3)	13.3 (4)	13.3 (4)	63.3 (19)	–	–	–	–	–	–	–	–
A–MUR–2	26	–	7.7 (2)	15.4 (4)	3.8 (1)	11.5 (3)	34.6 (9)	23.1 (6)	3.8 (1)	–	–	–	–	–	–	–
A–SHM	29	–	10.3 (3)	–	–	55.2 (16)	6.9 (2)	3.4 (1)	24.1 (7)	–	–	–	–	–	–	–
A–NBO	30	–	–	–	–	53.3 (16)	–	–	46.7 (14)	–	–	–	–	–	–	–
Ryukyus + Philippines populations																
A–AMM	30	–	–	–	–	–	–	–	60.0 (18)	33.3 (10)	6.7 (2)	–	–	–	3.3 (1)	–
A–KIN	30	–	–	–	–	–	–	–	36.7 (11)	–	–	40.0 (12)	–	–	–	23.3 (7)
A–YMT–1	30	–	–	–	–	–	–	–	96.7 (29)	–	–	–	3.3 (1)	–	–	–
A–YMT–2	16	–	–	–	–	–	–	–	31.3 (5)	18.7 (3)	50.0 (8)	–	–	–	–	–
A–ANL	9	–	–	–	–	–	–	–	22.2 (2)	–	–	–	33.3 (3)	22.2 (2)	22.2 (2)	–
Guam population																
A–BLB	5	–	–	–	–	–	–	–	40.0 (2)	20.0 (1)	20.0 (1)	–	20.0 (1)	–	–	–
Host species B																
B–AMM	6	–	–	–	–	–	–	–	33.3 (2)	–	–	–	66.7 (4)	–	–	–
B–ZMM	11	–	–	–	–	–	–	–	54.5 (6)	–	–	–	45.5 (5)	–	–	–
B–ANL	9	–	–	–	–	–	–	–	44.4 (4)	–	–	–	44.4 (4)	11.1 (1)	–	–

the topologies of the obtained cluster dendrograms (Fig. S3) were predominantly similar to those obtained by the collected *Symbiodinium* types (Tables 3 & 4).

Symbiont flora of host species A and B in AMM and ANL, where the 2 species co-occur, were not clustered together irrespective of the data compared (Fig. S2). However, Fisher's exact test indicated marginally or no significant differences between the symbiont flora of the 2 host species in AMM and ANL ($p = 0.08$ to 1.00, based on the infection frequency of each type), except for the infection frequency of D1* in AMM ($p < 0.001$).

Some samples were collected twice at the same location (MUR and YMT) in the same year. The first and second samples in MUR clustered together in both the dendrograms, whereas those of YMT did not (Fig. S2). No significant differences in infection frequency were detected in MUR ($p = 0.16$ to 0.49), except for those of type C3* ($p < 0.005$). Conversely, the frequencies were significantly different in YMT ($p < 0.005$), except for those of type D1* ($p = 1$, Fisher's exact test).

Genetic structuring of the host and *Symbiodinium* flora

We compared the dendrogram showing the similarity of the *Symbiodinium* flora with that showing the genetic similarity of the host populations (Fig. 3). The former dendrogram was slightly modified from those shown in Fig. S2b because the data of the samples collected twice in the 2 locations were merged. Several important aspects can be elucidated from this comparison. First, although hosts *P. ianthina* A and B are distinct species in the same genus with considerable genetic differences, the symbiont flora of species B (observed only in the subtropics and tropics; Fig. 3, shaded red) was highly similar to that of species A in the subtropics and tropics (Fig. 3, shaded gray and green). Second, among the 3 regional population groups of *P. ianthina* A, the Guam population (Fig. 3, shaded green) was genetically highly differentiated compared with the other 2, as its symbiont flora was located deep within in a cluster containing the Ryukyus + Philippines populations (Fig. 3, shaded gray). Finally, although the local

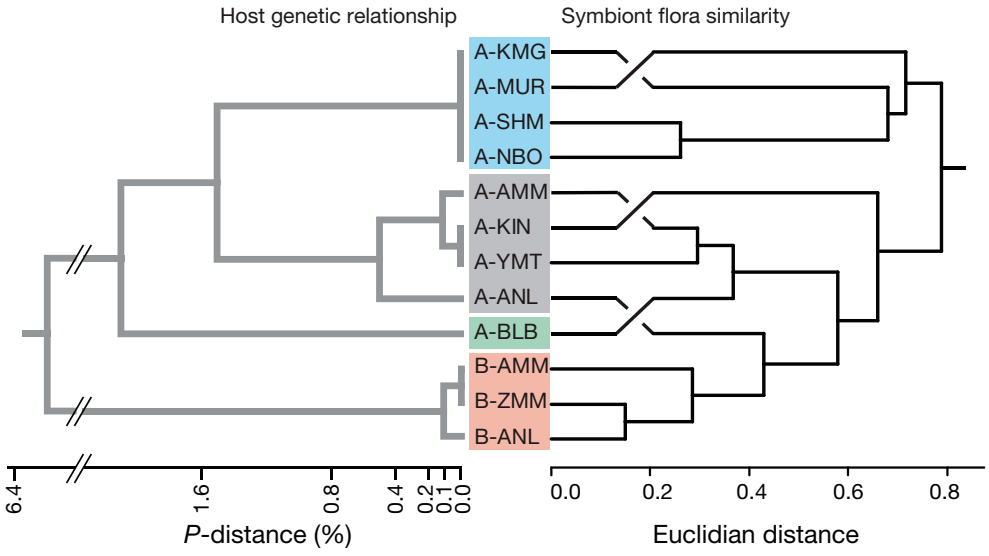

Fig. 3. Comparison of the host genetic relationship and similarity of symbiotic *Symbiodinium* composition. Host genetic relationship is depicted by the unweighted pair-group method with arithmetic mean (UPGMA) tree based on *P*-distance between populations (left; originally constructed by Yorifuji et al. 2012). The similarity of the detected symbionts is illustrated by the cluster dendrogram based on the data of *Symbiodinium* type composition observed in each population (right; similar to Fig. S2b in the Supplement at www.int-res.com/articles/suppl/m521p091_supp.pdf; original data shown in Table 4. The data of localities surveyed twice [A-MUR and A-YMT] was combined). Order of OTU follows the tree of host genetic relationship shown on the left. Shading colors indicate differences in host species/populations

populations of the species A host in temperate Japan were genetically homogenous, their symbiont flora were quite different (Fig. 3, shaded blue).

DISCUSSION

Symbiodinium harbored by the *Pteraeolidia ianthina* complex

Among the known clades (A to I) of *Symbiodinium*, clades A, C, and D were detected from the *Pteraeolidia ianthina* complex in the northwestern Pacific (Tables 2–4). As for the *Symbiodinium* types, 6 nrITS2 sequences that were detected in this study (A1, C1, C3, D1, D4, and D5) have previously been detected from other host organisms in various parts of the world (LaJeunesse 2001, 2005, LaJeunesse et al. 2010, Stat & Gates 2011). A1 is a commonly observed type of the worldwide observed clade A (LaJeunesse et al. 2009). C1, sensu LaJeunesse (2001) is the most common type of clade C observed worldwide (e.g. LaJeunesse 2005), although C3 is also very common (e.g. LaJeunesse 2005). The abundance of clade D *Symbiodinium* depends on the region (Stat & Gates 2011), but the previously identified 3 clade D types D1, D4, and D5 are present in many locations worldwide (LaJeunesse 2002, LaJeunesse et al. 2010, LaJeunesse & Thornhill 2011). These data apparently suggest that the *P. ianthina* complex shares symbiont members with other host animals, such as scleractinian corals. Note that recent studies using genetic markers with a finer resolution than that of nrITS2 revealed hidden diversity in *Symbiodinium*, and these studies found some host-specific lineages in types that were previously thought to be cosmopolitan (Thornhill et al. 2013, 2014). The types observed in our study (with the exception of C1 and D1*) were characterized by novel sequences in any of the 3 gene markers (Table 2), which may indicate that they are actually novel *Symbiodinium* types specific to this nudibranch host.

Symbionts of this sea slug complex were previously investigated using individuals in the Southern Hemisphere, and clades A, B, C, and D were detected (LaJeunesse et al. 2004, Loh et al. 2006). Clades A, C, and D are commonly observed in the sea slugs of both the Northern and Southern hemispheres. A marked difference between the results of the present and previous studies is that clade B, which was previously detected in the temperate east coast of Australia (Loh et al. 2006), was not observed in this study in the northwestern Pacific. This difference may be caused by genetic differences between the host indi-

viduals and/or the *Symbiodinium* flora prevailing in the environment (see next section).

Relationships between the population structure of hosts and their symbiont flora

In this study, the genetic identity of *Symbiodinium* was investigated, and the observed frequencies in each population were calculated into the symbiont flora similarity by cluster analyses (Figs. 3 & S2). This similarity was subsequently compared with the population genetic structure of the host sea slug. Because *P. ianthina* is simultaneously hermaphrodite (Thompson 1976), the sex difference between host individuals need not be considered when comparing symbiont flora. Furthermore, this sea slug is an annual species and recruits concurrently (Fukuta 2001); thus, all individuals in a population are of the same generation.

First, it is particularly important that highly variable symbiont floras were observed among genetically homogenous host populations in the temperate region (Fig. 3). This result indicates that the differences in symbiont floras were caused by varying environmental factors among different geographical locations. Geographically variable *Symbiodinium* floras were previously reported from genetically homogenous or close host scleractinian corals (Baums et al. 2010, Thornhill et al. 2010a). Second, genetically differentiated host species or populations harbored similar symbiont floras in the subtropics and tropics. Therefore, symbiont similarity is strongly influenced by environmental factors. Environmental conditions (e.g. fluctuations in water temperature) are more stable in the subtropics and tropics than in temperate regions (e.g. the annual sea surface temperature, SST, differences are approximately 11°C in KMG and 2°C in ANL, calculated from data obtained from the database of the Japan Meteorological Agency). Such conditions may affect the similarity in *Symbiodinium* composition.

Host–symbiont association is extremely complicated and several other factors remain to be elucidated or investigated. Note that the environmental factors we discuss here have several possible candidates in addition to the above-mentioned SST. For example, *Symbiodinium* flora in some cnidarian hosts differs under solar irradiance gradation (Rowan et al. 1997, Baker 2003, Coffroth & Santos 2005 and references therein). Alternatively, environmental *Symbiodinium* flora (i.e. free-living *Symbiodinium* and symbiotic dinoflagellates in other host organisms in the same habitat) may vary according to location, and symbiotic algal flora of the sea slug may reflect this fact. The environmental *Symbiodinium* flora has been recently investigated (Coffroth et al. 2006, Hirose et al. 2008, Littman et al. 2008, Manning & Gates 2008, Pochon et al. 2010, Reimer et al. 2010, Takabayashi et al. 2012, Yamashita et al. 2013), and these studies do indicate that the flora seems to vary with location. Thus, further studies should be conducted to examine these possibilities.

Host preference and/or suitability for symbionts

In addition to demonstrating the strong effects of environmental factors on symbiotic *Symbiodinium* flora, the results of this study have shown that the genetic differences of hosts also affect their symbiont flora. Inspection of the dendrogram of symbiont flora of host species A and B revealed that symbiont floras of sympatric populations of distinct species (in AMM and ANL) were less similar to those of allopatric populations of the same species, i.e. the symbiont flora of species B sea slug resembled each other and thus clustered together (Figs. 3 & S2). These results may indicate that genetic factors of the host can affect symbiont compositions. Indeed, several studies have reported a correlation between cnidarian host genotype and the diversity of their symbiont flora (Thornhill et al. 2010a, 2013, Pinzón & LaJeunesse 2011, Lien et al. 2013).

Furthermore, note that the occurrence of some *Symbiodinium* types was restricted or strongly biased to a specific host species (Tables 2 & 3). In this study, types A1 and D_{Pi}-1 were detected only from temperate Japan, whereas type A1 in particular has been observed in many other parts of the world (LaJeunesse et al. 2009). Our unpublished infection experiment showed that susceptibility of the host sea slug to this type A1 varied between individuals from subtropical and temperate locations (genetic assignment of the host is suggested to be species A, but this was unknown during the experiment). This implies a difference in the sea slug's preference and/or suitability for these symbiont types.

These differences in preference and/or suitability of symbiont types may also be present between the 2 slug species A and B. The type C3* and C_{Pi}-2 must have been strongly biased toward species A (Table 3). These 2 types in clade C symbionts have been detected from the sea slug's prey hydroid, *Myrionema amboinense*. The nudibranch mollusk was observed to acquire *Symbiodinium* by feeding on the

hydroid (M. Yorifuji pers. obs.). However, virtually no species B sea slugs harbored these types of symbiont even in AMM, the location from which the hydroid was also collected (M. Yorifuji pers. obs.). Therefore, the 2 species of host nudibranch may have different preferences for prey organisms and/or different suitability (susceptibility) for these *Symbiodinium* types. The acquisition of *Symbiodinium* from prey organisms was also reported from the nudibranch mollusk *Phyllodesmium lizardensis*, which feeds on octocoral of the genus *Heteroxenia* (FitzPatrick et al. 2012). This nudibranch harbors *Symbiodinium* types other than that of their prey (FitzPatrick et al. 2012), as observed for the *P. ianthina* complex in this study. Therefore, nudibranchs may have flexibility in symbiont acquisition, and additional factors may affect the differences in symbiont flora.

In conclusion, *Symbiodinium* flora in the host nudibranch mollusk *P. ianthina* complex showed geographic variation, which was influenced by the prevailing environmental conditions, and to some extent by genetic differences between hosts.

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