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The genetic structure of the marine flatworm *Stylochoplana pusilla* (Rhabditophora: Polycladida) and its use of intertidal snails

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Abstract

Although marine phylogeographers have accumulated knowledge of the evolutionary history of various invertebrates, there is a large bias among the taxa regarding genetic data. The order Polycladida is a typical example for which little genetic information at population level is available. Here, we focused on the polyclad flatworm *Stylochoplana pusilla*, distributed in the Japanese Pacific coastal area. *Stylochoplana pusilla* is known to have commensal relationships with certain intertidal snails, using snails (mainly *Monodonta confusa*) as a refugee house. During low tide, *S. pusilla* hides in the mantle cavity of snails to protect themselves from desiccation and predation. Here, we investigated the genetic structure of *S. pusilla* using a mitochondrial Cytochrome Oxidase I marker and the species diversity of snails used by it. We found that *S. pusilla* has high genetic diversity of its populations. While *S. pusilla* showed a significant genetic differentiation among populations, it was relatively low. In addition, we also showed that *S. pusilla* used several intertidal snail species which inhabit various coastal environments. The present study suggests *S. pusilla* has sufficient dispersal ability to connect among its local populations. Also, the range of available snails for *S. pusilla* may help the connectivity among local populations. We provide important knowledge about this invertebrate taxon with a unique ecology, which has been insufficiently studied.

Introduction

Marine phylogeographers have been attempting to reveal the formation mechanisms of genetic connectivity responsible for regional fauna (Crandall *et al.*, 2019). Our knowledge of the genetic structures of various marine organisms has been greatly enhanced by recent advances in molecular methods. However, there is a large bias among the invertebrate taxa that have been genetically studied, and some of the neglected taxa play important roles in the marine ecosystem (Keyse *et al.*, 2014). To address this research gap, which makes it difficult to compare the various marine species, it will be necessary to shed light on the level of genetic diversity and differentiation of those invertebrate taxa that remain enigmatic.

The order Polycladida (Platyhelminthes: Rhabditophora) is a group of marine flatworms living in various environments (Newman & Cannon, 2003; Quiroga *et al.*, 2006; Oya & Kajihara, 2019). Polycladida is thought to have about 800 species (Martín-Durán & Egger, 2012). However, the potential species diversity of Polycladida seems to be high, since new polyclad species were described one after another by the 'energetic polycladologists' (a term coined by J. Bahia in Dittmann *et al.*, 2019: e.g. Bahia *et al.*, 2015; Bahia & Schrödl, 2016; Tsuyuki *et al.*, 2019). Besides, molecular studies have highlighted polyclad phylogenies (e.g. Litvaitis *et al.*, 2010; Aguado *et al.*, 2017; Bahia *et al.*, 2017; Tsunashima *et al.*, 2017). Despite such work, polyclad species are still not well studied compared with other marine animals (Keyse *et al.*, 2014; Dittmann *et al.*, 2019; Litvaitis *et al.*, 2019). Furthermore, the genetic diversity and evolutionary history of these polyclad species are relatively unknown due to a lack of population genetic studies.

While most of the polyclad species have a free-living lifestyle, some have an association with invertebrates: symbiosis in Ophiuroidea (Doignon *et al.*, 2003), commensalism in hermit crabs (Lytwyn & McDermott, 1976) and gastropods (Faubel *et al.*, 2007), parasitism in chitons (Kato, 1935). Around the Japanese Pacific coastal area, the commensal relationships between a polyclad species and the intertidal gastropod have been well studied (Kato, 1933; Fujiwara *et al.*, 2016).

Stylochoplana pusilla, distributed in the Japanese Pacific coastal area from southern Hokkaido to Kyushu, is found associated with certain intertidal snails (Kato, 1933, 1965). After hatching, *S. pusilla* passes through the planktonic larval phase (7 days) and settles (Deguchi *et al.*, 2009). Next, they begin to use intertidal snails as refuge sites in the intertidal zone. At low tide, they hide in the mantle cavity of the intertidal snails and protect themselves from desiccation and predation (Fujiwara *et al.*, 2016). In fact, once *S. pusilla* leaves the snail in the sublittoral zone, *S. pusilla* is soon preyed upon by predators such as a large flatworm species and gobies (Fujiwara *et al.*, 2014). However, *S. pusilla* does not use all intertidal snails equally. Fujiwara *et al.* (2014) demonstrated that *S. pusilla* showed a specific preference for

Monodonta confusa, although *S. pusilla* was found in several intertidal snails in Mutsu Bay, the northern part of Honshu, Japan. The range of available species is known to influence the genetic population structure of the user (Li *et al.*, 2014). However, to our knowledge, no population genetic studies have yet been conducted in polyclad species, including *S. pusilla*, which have a unique association with certain snails.

Here, we aimed to show the genetic population structure of a Polycladida species. Our model species is *S. pusilla.* Since data about its dispersal ability is needed to discuss the formation process of the population genetic structure, *S. pusilla* is a suitable study model. The genetic diversity and level of genetic differentiation among populations were estimated using the mitochondrial Cytochrome c Oxidase subunit I (COI) marker developed by Oya & Kajihara (2017), because the resolution of it was also suitable for the evaluation of the intraspecific variation. Also, we surveyed the intertidal snail species used by *S. pusilla*. The reason is that although Fujiwara *et al.* (2014) studied the preference of *S. pusilla* in Mutsu Bay, in the whole distribution area of *S. pusilla* it is unknown if and how *S. pusilla* makes use of snail species other than *M. confusa*; despite the distribution of snails potentially available for *S. pusilla* around the Japanese coastal area (Genus *Monodonta* and Genus *Tegula*: Sasaki, 2017; Yamazaki *et al.*, 2019). Our data will help to bridge the research gap of enigmatic invertebrate taxa that are insufficiently genetically studied.

Materials and methods

We collected *S. pusilla* samples from 17 locations in the Japanese mainland (Table 1, Figure 1). Our study area extends from the north of Honshu to Kyushu within about 2000 kilometres. We recorded what kinds of intertidal snails were used by *S. pusilla* in all locations. When sampling, the collected snails were placed into species-specific plastic bags containing seawater, and then checked for whether they were used by *S. pusilla*, which is known to go out into the seawater when snails are submerged (Fujiwara *et al.*, 2016). The collected *S. pusilla* were stored in 99.5% ethanol for subsequent molecular analyses.

The NucleoSpin® Tissue kit (TaKaRa, Shiga Pref., Japan) was used to extract DNA from the tissue, according to the manufacturer's instructions. Fragments of the COI gene were amplified using the primers Acotylea_COI_F (5′-ACTTTATTCTACTAAT CATAAGGATATAGG-3′) and Acotylea_COI_R (5′-CTTTCCT CTATAAAATGTTACT ATTTGAGA-3′) (Oya & Kajihara, 2017). Polymerase chain reaction (PCR) was performed for the COI gene following the protocol described in Oya & Kajihara (2017): 94°C for 5 min; 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min; 72°C for 7 min. The PCR products were subsequently purified using Exo-SAP-IT (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Cycle sequencing was performed using the PCR primers with the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA), and the products were directly sequenced from both directions using an ABI 3130xl automated sequencer (Applied Biosystems).

The forward and reverse sequences were assembled using CLUSTALW (Thompson *et al.*, 1994). We checked the validity of the sequences using a chromatogram viewer and the quality scores of each base using the software package 4Peaks (Griekspoor & Groothuis, 2005). The obtained sequences were aligned using MUSCLE v3.8 (Edgar, 2004).

To reconstruct the population genetic structure of *S. pusilla* using six locations where more than 10 individuals of *S. pusilla* were collected (locality numbers: 1, 5, 9, 10, 12 and 14), we calculated two genetic indices (haplotype and nucleotide diversity) and estimated the hierarchical analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) and pairwise F_{ST} among populations with 1000 permutations, using Arlequin v. 3.5 for statistical analyses (Excoffier & Lischer, 2010). To visualize the geographic distribution pattern of haplotypes obtained from all *S. pusilla* individuals, haplotype networks were reconstructed using a TCS network implemented in PopART (Leigh & Bryant, 2015).

Table 1. Genetic information of each population of *Stylochoplana pusilla*

No.	Sampling locality	N	nH	HD	ND
1	Aomori, Aomori	20	15	0.921	0.00454
2	Hachinohe, Aomori	3	3	–	–
3	Onagawa, Miyagi	3	3	–	–
4	Ishinomaki, Miyagi	2	2	–	–
5	Miura, Kanagawa	14	13	0.989	0.00652
6	Kozushima Island, Tokyo	1	1	–	–
7	Shimoda, Shizuoka	3	2	–	–
8	Toba, Mie	1	1	–	–
9	Shirahama, Wakayama	14	9	0.923	0.00461
10	Kurashiki, Okayama	18	13	0.948	0.00509
11	Sakaide, Kagawa	2	2	–	–
12	Hiji, Oita	10	9	0.978	0.01152
13	Ichikikushikino, Kagoshima	6	5	–	–
14	Amakusa, Kumamoto	14	11	0.934	0.00459
15	Omura, Nagasaki	1	1	–	–
16	Kitakyushu, Fukuoka	1	1	–	–
17	Matsue, Shimane	3	3	–	–
	Total	116			

N, number of individuals; nH, number of haplotypes; HD, Haplotype diversity; ND, Nucleotide diversity.
Six populations (locality numbers: 1, 5, 9, 10, 12 and 14) were used for population genetic analyses.

Results

The snails used by *S. pusilla* in the 17 locations we surveyed are shown in Table 2. Although *S. pusilla* mainly used *M. confusa* at all 17 sampling locations, it also used eight other snail species. *Stylochoplana pusilla* used three families (Trochidae, Tegulidae and Muricidae) and mainly used two genera, *Monodonta* and *Tegula*.

In total, 116 *S. pusilla* COI sequences were obtained, and the alignment length was 615 base pairs. We identified 72 haplotypes from the 116 sequences, and the data are available from GenBank (Supplemental Table S1; accession numbers: LC515251–LC515366). The haplotype diversity and nucleotide diversity of *S. pusilla* are shown in Table 1 (locality numbers: 1, 5, 9, 10, 12 and 14). The haplotype diversity of six populations was higher than 0.90 (0.921 (locality number 1: Aomori, Aomori) – 0.989 (locality number 5: Miura, Kanagawa)). Nucleotide diversity was 0.00454 (locality number 1: Aomori, Aomori) – 0.01152 (locality number 12: Hiji, Oita). The hierarchical AMOVA showed the existence of a genetic structure in six *S. pusilla* populations ($\Phi_{ST} = 0.056$, $P < 0.0001$; Table 3). Table 4 shows pairwise F_{ST} among the six populations of

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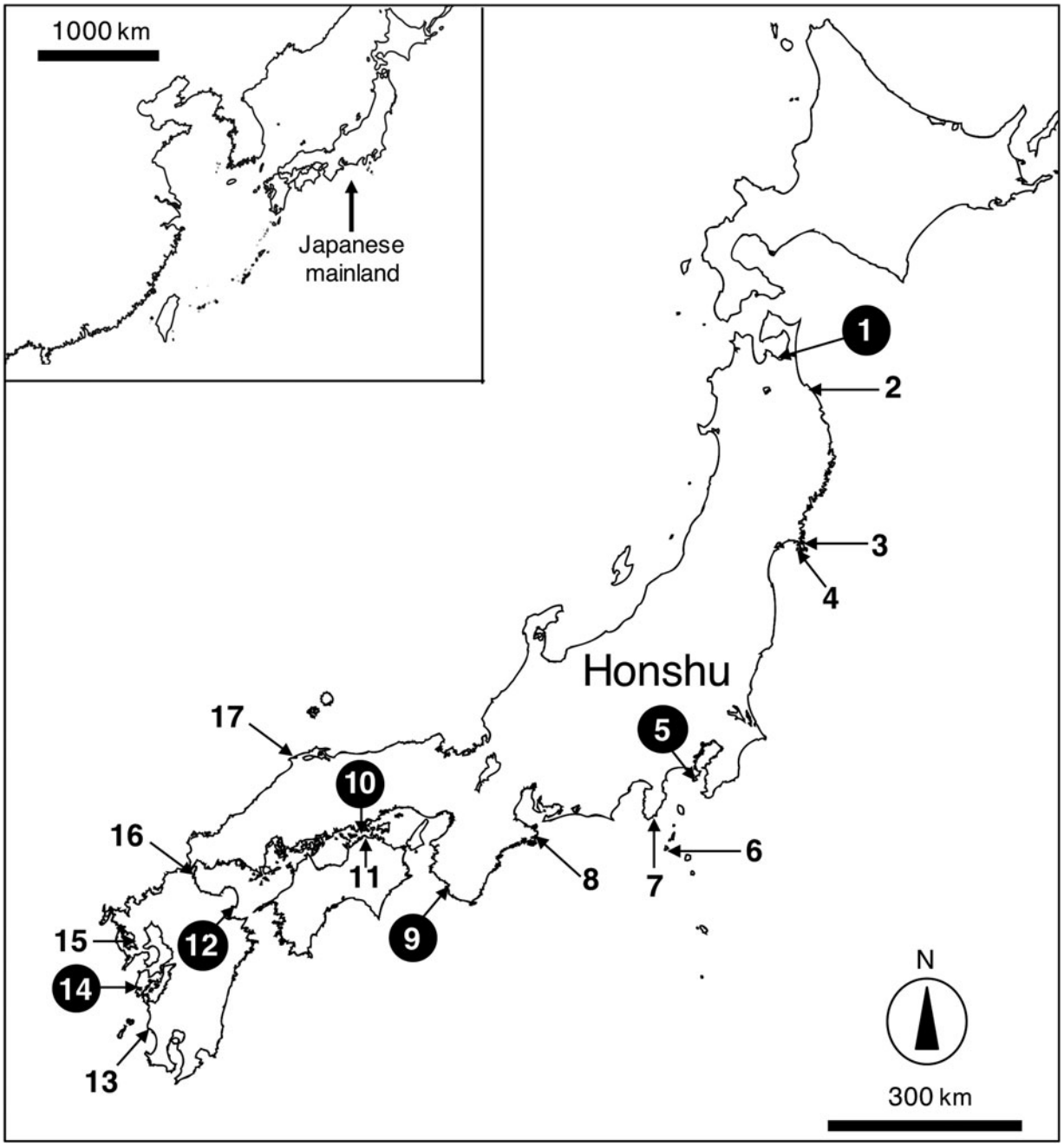

Fig. 1. Map of the survey and sampled locations. Numbers in black circles indicate the locations used for genetic population structure analyses (calculation of the genetic diversity indices, AMOVA and pairwise F_{ST} values).

Table 2. The host snails used by *Stylochoplana pusilla* in the 17 locations surveyed.

Species	Family	Locality number
Monodonta confusa	Trochidae	All 17 locations
M. labio	Trochidae	10, 11, 16
M. perplexa	Trochidae	4, 6
Tegula lischkei	Tegulidae	16
T. turbinatum	Tegulidae	1
T. xanthostigma	Tegulidae	6, 14
Tegula sp.	Tegulidae	11
T. rustica	Tegulidae	1, 11
Ceratostoma fournieri	Muricidae	3

Table 3. Result of hierarchical analysis of molecular variance (AMOVA) among the six populations (locality number: 1, 5, 9, 10, 12 and 14).

Source of variation	df	Fixation Index	Percentage of variation
Among population	5	0.056 ($P < 0.0001$)	5.56
Within population	84		94.44

S. pusilla. While the population of Hiji, Oita (locality number 12) was genetically differentiated from other populations, significant genetic differentiation among the population was not detected after Bonferroni correction. The haplotype network exhibited no clear geographic structure and showed a complex network, including some dominant haplotypes (Figure 2).

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Table 4. Pairwise F_{ST} among the six populations of *Stylochoplana pusilla*

Locality No.	1	5	9	10	12
5	0.0086	–			
9	0.0390	−0.0104	–		
10	0.0246	0.0391*	0.0686*	–	
12	0.1408*	0.0905	0.1306*	0.1380*	–
14	0.0018	−0.0043	0.0236	0.0079	0.1197*

*P < 0.05.

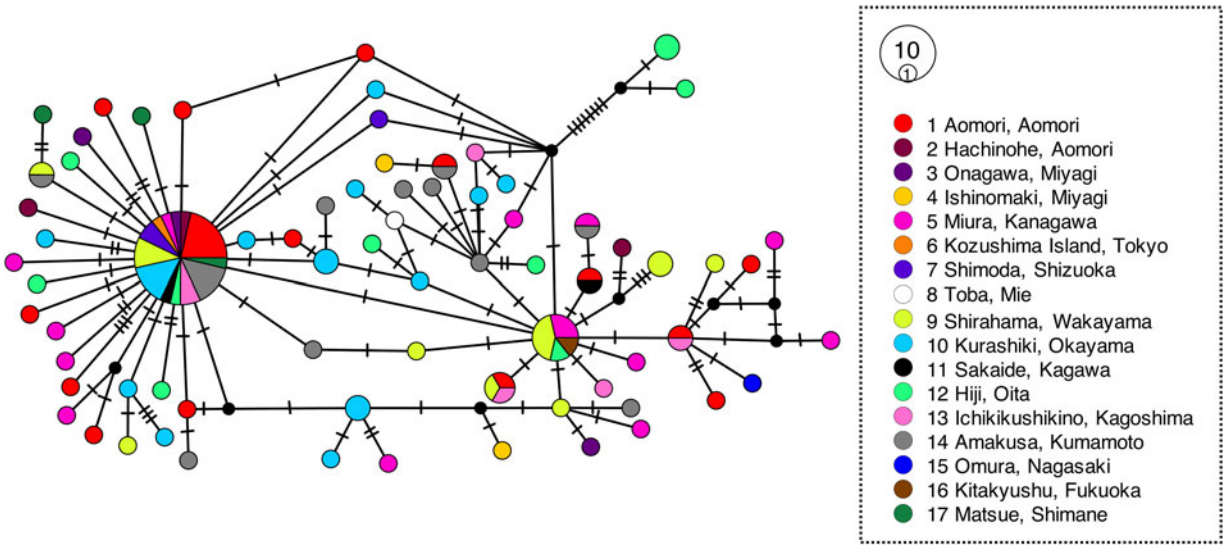

Fig. 2. Haplotype network inferred from 116 individuals and 72 haplotypes of *Stylochoplana pusilla*.

However, locality number 12 (Hiji, Oita) had two genetically distantly related haplotypes (12 nucleotide substitution).

Discussion

Our present study is the first aimed at clarifying the mitochondrial genetic structure of the polyclad species *S. pusilla*, which has a commensal association with the intertidal snail *M. confusa*. Although genetic differentiation among local populations of *S. pusilla* is significant (AMOVA), the level of differentiation is relatively low as no genetically differentiated population pairs were detected after Bonferroni correction and no geographic structure is observed. Also, high genetic diversity within populations is found in *S. pusilla*. However, some marine species such as fish and intertidal snails along the Japanese coast are genetically differentiated in accordance with geographic structure, such as splitting oceanic currents (fish: Hirase *et al.*, 2012; snails: Kojima *et al.*, 1997; Yamazaki *et al.*, 2017). In the case of *S. pusilla*, the low level of genetic differentiation indicates that it possesses sufficient dispersal ability to maintain the opportunities of gene flow among populations (7 days of planktonic larval duration: Deguchi *et al.*, 2009).

The present study also revealed that *S. pusilla* used several intertidal snail species in their whole distribution area. The snails used by *S. pusilla* are known to exhibit a variety of habitat preferences. For instance, *M. confusa*, the main available snail for *S. pusilla*, can inhabit wider coastal environments compared with other congeneric species (Takenouchi, 1985; Yamazaki *et al.*, 2017). Besides, *S. pusilla* used other intertidal snails such as Tegulidae and Muricidae. Among tegulid species, *Tegula xanthostigma* lives in an exposed shore while its sister species, *Tegula* sp., prefers sheltered habitats, like the inner bay (Yamazaki *et al.*, 2019). This suggests that *S. pusilla* can live in a wide range of coastal exposures due to the utilization of various snails. If populations of the available snail tend to be connected due to a wide range of available habitat, *S. pusilla* populations are also likely to be connected and weaken the levels of genetic differentiation among the populations. In general, species that can use various host species showed a lower level of genetic differentiation than host-specific species (Li *et al.*, 2014). In the marine environment, various types of ecological interactions have been reported, and commensal relationships have often been observed (e.g. Williams & McDermott, 2004). In the case of polyclad species, many are free-living, but some have commensal relationships with other marine organisms (Kato, 1933; Lytwyn & McDermott, 1976; Faubel *et al.*, 2007; Fujiwara *et al.*, 2016). However, to date, knowledge on the genetic structure of organisms that have an ecological relationship with other species is missing in marine taxa compared with terrestrial species. The present finding indicates that the association with other marine species might not prevent genetic connectivity among populations of *S. pusilla*. The wide range of available snail species may help the genetic connectivity among populations. To better understand the relationship between these lifestyles and genetic structure, we have to perform a comparative study using a polyclad species which has an association with marine invertebrates.

We detected the two distant haplotypes of Hiji, Oita (locality number 12). Although the lack of examples of population genetic studies in polyclad species makes it difficult to discuss the cause of these distantly related haplotypes, this implies ancestral polymorphisms (Dillon & Robinson, 2009). It is necessary to carry out genetic studies on other polyclad species using not only mitochondrial but also other highly variable nuclear markers, such as microsatellite DNA data and genome-wide SNPs.

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In conclusion, the present study showed the low level of genetic population structure of *S. pusilla* due to their sufficient dispersal ability. Also, a wide range of available snail species may help the above trend. At present, there are no population genetic studies of Polycladida. This study thus could be a framework for future genetic studies. Although detailed genetic and ecological studies are needed, the present study provides important knowledge to help bridge the gap of invertebrate taxa, which have been insufficiently studied.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0025315420000570.

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