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# DNA variation and symbiotic associations in phenotypically diverse sea urchin *Strongylocentrotus intermedius*

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***Strongylocentrotus intermedius*** **(A. Agassiz, 1863) is an economically important sea urchin inhabiting the northwest Pacific region of Asia. The northern Primorye (Sea of Japan) populations of *S. intermedius* consist of two sympatric morphological forms, "usual" (U) and "gray" (G). The two forms are significantly different in morphology and preferred bathymetric distribution, the G form prevailing in deeper-water settlements. We have analyzed the genetic composition of the *S. intermedius* forms using the nucleotide sequences of the mitochondrial gene encoding the cytochrome *c* oxidase subunit I and the nuclear gene encoding bindin to evaluate the possibility of cryptic species within *S. intermedius*. We have examined the presence of symbiont microorganisms by means of 16S rRNA sequences. The nucleotide sequence divergence between the morphological forms is low: 0.74% and 0.70% for cytochrome *c* oxidase subunit I and nuclear gene encoding bindin, respectively, which is significantly below average intrageneric sequence divergence among *Strongylocentrotus* species. We thus have found no genetic evidence of cryptic species within *S. intermedius*. Phylogenetic analysis shows that the bacteria symbionts of *S. intermedius* belong to the phylum *Bacteroidetes*, but the U and G forms predominantly harbor highly divergent bacterial lineages belonging to two different taxonomic classes, *Flavobacteria* and *Sphingobacteria*. We propose that the U and G forms of *S. intermedius* represent distinct ecomorphological adaptations to contrasting shallow- and deep-water marine environments and might be considered incipient species. We also propose that the symbiotic bacteria likely play an important role in the evolution of morphological divergence of *S. intermedius*.**

Bacteroidetes | DNA polymorphism | incipient speciation | marine adaptation | sympatric morphological forms

The intermediate (short-spined) sea urchin *Strongylocentrotus intermedius* (A. Agassiz. 1863) inhabits a wide range of the northwest Pacific region of Asia: the Sea of Japan, Sea of Okhotsk, east coast of Kamtchatka, Southern Kuril Islands, and coast of Japan (1, 2). The full distribution pattern remains uncertain as a result of the difficulty of reliable species identification (2). The species occurs from the littoral and upper sublittoral zone to a depth of 25 m (3); occasional specimens from the west coast of Japan islands have been dredged from depths as great as 150 to 225 m (1).

The body of *S. intermedius* is variable in color. Within the same locality, it can be deep green, reddish, brown, lilac, and white; moreover, the basal and apical parts of spines as well as the primary, secondary, and miliary spines, in adults as well as in juveniles, are frequently variable in color. A milk-white color of spines prevails in deep-water settlements (15–25 m). This color variant is known as the gray (G) morphological form to distinguish it from the "usual" (U) form that mostly occurs in shallow-water settlements (5–10 m). The spines of juvenile individuals (age, 1–3 y) belonging to the G form are completely white, but in mature individuals the spines (especially primary spines) are milk-white or pinkish white. The spines of U individuals occur in a variety of colors: green, brown, red, and lilac. There are also clear-cut differences between the U and G forms in the length of spines and the thickness of testa. The spines of the U form are relatively short; the length, as a rule, does not exceed one third of the radius of the testa. The spines of the G form are longer, reaching and frequently exceeding two thirds of the testa radius. The testa is significantly thicker in the U form than in the G form. The morphological differences between the U and G forms of *S. intermedius* are stable and easily recognizable (Fig. 1), and they are systematically reported for the northern Primorye coast region (V.A.P., unpublished data).

Little is known about the population genetics of *S. intermedius*; the available data are limited to allozyme polymorphisms (4–6). There are no genetic data concerning the differences between morphological forms of *S. intermedius*; the taxonomical status of the U and G forms remains undetermined.

In this article, we investigate nucleotide polymorphism in a fragment of the mitochondrial gene encoding *COI* and *BND* in phenotypically diverse *S. intermedius*, seeking to clarify the taxonomic status of the U and G forms and to evaluate the possibility of cryptic species within *S. intermedius*. We have also investigated 16S rRNA sequences in symbiotic bacteria of *S. intermedius*. Bacterial symbionts of marine organisms have diverse and important roles in nutrition, defense, recognition, and other host functions (e.g., 7–9) that indirectly may also promote evolutionary changes in their hosts. We have found that the U and G morphological forms are genetically very similar and therefore may not be thought of as distinct biological species. However, the two forms harbor symbionts of two different and strongly divergent bacterial lineages belonging to the phylum *Bacteroidetes*; moreover, the concordant difference in bacterial composition between the forms is maintained in three distantly located sea urchin settlements. The data on symbiotic bacteria obtained for another sea urchin species, *S. nudus*, suggest that the difference in bacterial composition between the *S. intermedius* forms might not simply reflect the different habitats (i.e., settlement depth) of the U and G forms and could play an important role in the morphological and potentially genetic divergence of *S. intermedius*.

## Results

**Nucleotide Diversity, Divergence, and Species Identity.** We have sequenced two gene regions in 12 *S. intermedius* individuals, six of each of the U and G forms, collected at depths of 5 to 10 m and 15 to 20 m, respectively, in a settlement near Zolotoi Cape, in the northern Primorye coastal region of the Sea of Japan. One gene region includes 1,056 bp of the mitochondrial *COI* gene; the second region includes 1,809 bp of the nuclear *BND* gene.

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The authors declare no conflict of interest.

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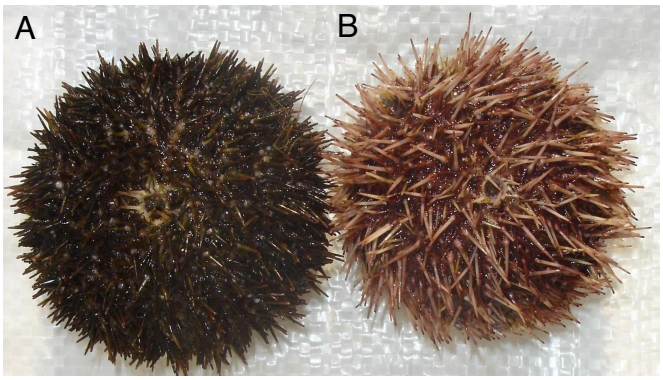


**Fig. 1.** The U (*A*) and G (*B*) morphological forms of *Strongylocentrotus intermedius*.

Supporting information (SI) Fig. S1 shows the 69 polymorphic nucleotide-substitution sites, 29 in *COI* and 40 in *BND*. In addition, there are nine length polymorphisms in the intron of the *BND* gene [see also *SI Text*]; no length polymorphisms are found in the *COI* gene. For comparison we have sequenced the same two genes in two additional species of closely related sea urchins: *S. pallidus* and *S. polyacanthus*; the *BND* and *COI* sequences of *S. purpuratus* and *Hemicentrotus pulcherrimus* (this species is phylogenetically placed within the genus *Strongylocentrotus*; refs. 10 and 11) were obtained from GenBank.

The estimates of nucleotide diversity for all sequences of *S. intermedius*, and for the morphological forms or haplotype lineages separately, are presented in Table 1. In the pooled sample, the total and nonsynonymous nucleotide diversity is similar for the *BND* ($\pi_{total} = 0.0070$; $\pi_{nsys} = 0.0025$) and *COI* ($\pi_{total} = 0.0077$; $\pi_{nsys} = 0.0025$) genes, but the level of synonymous polymorphism is 3.4 times less in *BND* ($\pi_{syn} = 0.0067$) than in *COI* ($\pi_{syn} = 0.0227$). The difference is highly significant by coalescent simulations ($P < 0.001$). The U and G morphological forms have significantly different levels of *BND* variability, especially in the coding region:

**Table 1. *COI* and *BND* nucleotide diversity in the two morphological forms of the sea urchin *Strongylocentrotus intermedius***

| Gene | U | G | Lineage 1 | Lineage 2 | Full sample |
|---|---|---|---|---|---|
| *COI* | | | | | |
| Sequences | 6 | 6 | 7 | 5 | 12 |
| Polymorphic sites | 16 | 22 | 10 | 13 | 29 |
| $\pi_{syn}$ | 0.0207 | 0.0272 | 0.0064 | 0.0075 | 0.0227 |
| $\pi_{nsyn}$ | 0.0021 | 0.0030 | 0.0015 | 0.0041 | 0.0025 |
| $\pi_{total}$ | 0.0068 | 0.0091 | 0.0027 | 0.0049 | 0.0077 |
| $K_{total}$ | 0.0844 | 0.0855 | 0.0835 | 0.0871 | 0.0850 |
| *BND* | | | | | |
| Sequences | 6 | 6 | 8 | 3 | 12 |
| Polymorphic sites | 35 | 20 | 8 | 23 | 38 |
| $\pi_{syn}$ | 0.0113 | 0.0025 | 0 | 0.0147 | 0.0067 |
| $\pi_{nsyn}$ | 0.0039 | 0.0013 | 0.0016 | 0.0047 | 0.0025 |
| $\pi_{cod}$ | 0.0057 | 0.0015 | 0.0012 | 0.0071 | 0.0035 |
| $\pi_{total}$ | 0.0097 | 0.0050 | 0.0015 | 0.0104 | 0.0070 |
| $K_{total}$ | 0.0276 | 0.0288 | 0.0276 | 0.0280 | 0.0280 |

*N* is the number of sequences. *S* is the number of polymorphic sites. $\pi$ is the average number of nucleotide differences per site among all pairs of sequences (12) obtained for the synonymous ($\pi_{syn}$), nonsynonymous ($\pi_{nsyn}$), coding ($\pi_{cod}$), and total ($\pi_{total}$) number of sites. $K_{total}$ is the average proportions of nucleotide differences between *S. intermedius* and *S. pallidus*. Nucleotide variability is calculated for the full sample, as well as separately for the two morphological forms and for the two haplotype lineages (see *Results*). The segregating sites associated with indels are excluded from the $\pi$ and *K* calculations.

for the U form, the $\pi_{cod}$ was 0.0057, 3.8 times larger than for the G form, which had a $\pi_{cod}$ of 0.0015. For the *COI* gene, the difference between the U and G forms is small and statistically not significant ($P > 0.05$). The total divergence between *S. intermedius* and *S. pallidus* is 3.0 times higher for *COI* than for *BND* ($K_{total} = 0.0850$ and 0.0280; Table 1); the same tendency is found for comparisons with the other three sea urchin species (data not shown).

There is some haplotype structure in the *COI* and *BND* genes that is not related to the color forms of *S. intermedius* (Fig. 2). For the *COI* gene there are two sets of haplotypes (lineages 1 and 2 in Table 1). The two haplotype sets are distinguished by nine synonymous substitutions (see Fig. S1 and *SI Text*); the difference between the lineages is highly significant ($F_{st} = 0.6706$, $P < 0.01$). Strong haplotype structure is also observed for the *BND* gene in *S. intermedius* (Fig. S1). The lineages differ by seven synonymous and intronic substitutions, a difference that is statistically significant ($F_{st} = 0.5634$, $P < 0.01$).

Fig. 2 displays a neighbor-joining tree of the *COI* and *BND* sequences of *S. intermedius*. Both trees show the sequences from the two color forms intermingled. For the *COI* gene, the $F_{st}$ is −0.0570 ($P = 0.7587$); total sequence divergence ($D_{xy}$) is 0.0074. For the *BND* gene, the $F_{st}$ is −0.0515 ($P = 0.7440$); total sequence divergence ($D_{xy}$) is 0.0070. The average intrageneric divergences among the *S. intermedius*, *S. purpuratus*, *S. pallidus*, *S. polyacanthus*, and *Hemicentrotus pulcherrimus* are 10.02% for *COI* and 5.55% for *BND*, whereas the average sequence divergences between the U and G forms of *S. intermedius* are 0.74% for *COI* and 0.70% for *BND*. These data suggest that the U and G morphological forms of *S. intermedius* are not distinct species (or at least that they have become reproductively isolated only very recently, without opportunity for much genetic differentiation, except perhaps in genes directly responsible for reproductive isolation).

**Phylogenetic Affiliation of the Bacterial 16S rRNA Clones.** We have investigated the bacterial infective agents present in two sea urchin species: *S. intermedius* and *S. nudus*. Bacteria of the genus *Tenacibaculum* (13) have been reported to be agents of the *S. intermedius* gonad spotting disease (14). To amplify the gene from representatives of the genus *Tenacibaculum* and related groups, we designed primers located in a conserved region of the 16S rRNA bacterial gene. Partial bacterial 16S rRNA genes (1,360 bp) are readily amplified from the gonad tissue in all three sea urchin species; other tissues tested (different intestinal sections and Lantern of Aristotle muscles) resulted in weak and inconsistent amplifications of the *Bacteroidetes* 16S rRNA. We have cloned and sequenced the amplified fragments of the bacterial 16S rRNA gene for 49 individuals of *S. intermedius* from three distant localities (see *Materials and Methods*) and nine individuals of *S. nudus*. Putative chimeras were identified with the Bellerophon program (15). The non-chimeric sequences (total of 229 clones) were used for phylogenetic analysis.

A BLAST search of each clone found close matches with multiple bacteria belonging to the phylum *Bacteroidetes*. Most of the inferred microorganisms are members of two classes: *Flavobacteria* and *Sphingobacteria*; two additional sequences were associated with the class *Bacteroidetes*.

The prevalent *Flavobacteria* phylotype [we use the term "phylotype" (i.e., phylogenetic type)] to refer to clusters of related 16S rRNA gene sequences characterized by levels of pair-wise sequence identity of ≥97%) matches most closely with the genus *Elizabethkingia* (family *Flavobacteriaceae*) described from human clinical specimens and aquatic environments (reviewed in ref. 16). The other *Flavobacteria* clones form a pretty divergent cluster of sequences matching with the different representatives of the family *Flavobacteriaceae* (reviewed in ref. 17). We have used two programs available on the Web, RDP Classifier (18) and Greengenes (19), to uncover the bacterial affinities of our *Flavobacteria* clones. These are closely related to the bacteria from the genera *Capnocytophaga*,

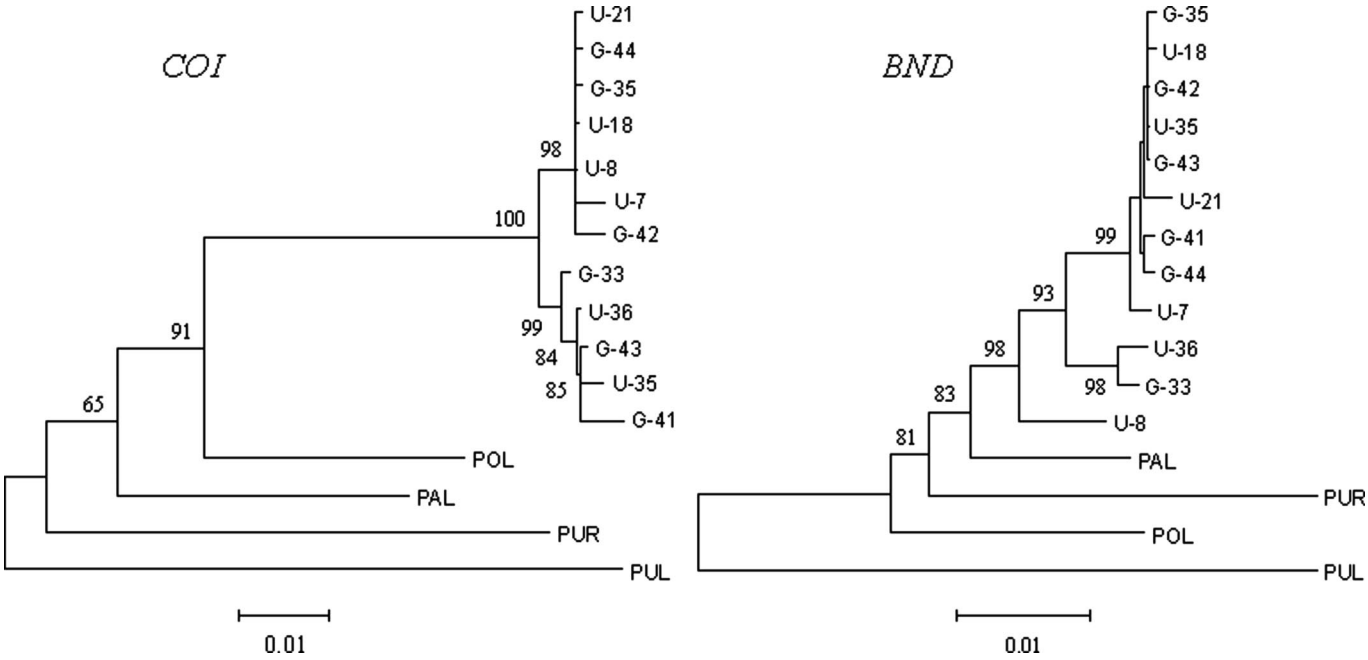


**Fig. 2.** Neighbor-joining tree of the *COI* and *BND* sequences of S*trongylocentrotus*, based on Kimura 2-parameter distance. The numbers at the nodes are bootstrap percent probability values based on 10,000 replications. Some *BND* and *COI* sequences are obtained from GeneBank with their accession numbers: *S. purpuratus*, *COI*, NC_001453 (44) and *BND*, AF077309 (10); *Hemicentrotus pulcherrimus*, *COI*, AF525453 (11) and *BND*, AF077319 (10). The sequences of *COI* and *BND* in *S. polyacanthus* and *S. pallidus* are from Balakirev and Ayala (accession numbers EU700089-EU700091 and EU700092-EU700094). POL = *S. polyacanthus*; PAL = *S. pallidus*; PUR = *S. purpuratus*; PUL = *Hemicentrotus pulcherrimus*.

*Lutibacter*, *Flavobacterium*, *Bizionia*, *Formosa*, and *Winogradskyella* (Kimura 2-parameter distances are within the range of 0.6%–5.4% of sequence divergence).

The *Sphingobacteria* clones form a number of divergent clusters phylogenetically close to the bacteria from the genus *Cytophaga* (family *Flexibacteriaceae*) described from the marine environment (reviewed in ref. 17). The prevalent *Sphingobacteria* phylotype has closest matches with the uncultured *Cytophaga–Flavobacterium–Bacteroides* group bacterium associated with mucous secretions of the hydrothermal vent polychaete *Paralvinella palmiformis* (20). The clones representing the class *Bacteroidetes* are rare (only two of 229) and they closely cluster with bacteria from the genus *Prevotella* (family *Prevotellaceae*) described from the human oral cavity (21).

***Bacteroidetes* Community Structure.** For the analysis of bacterial composition, we have obtained samples of *S. intermedius* from two additional sea urchin settlements in addition to Zolotoi Cape: Olga Bay and Povorotnyi Cape (see *Materials and Methods*). The three geographical localities extend over more than 700 km of the northern Primorye coastal region. The clones belonging to *Sphingobacteria* and *Flavobacteria* are unequally distributed between the U and G forms in all three localities. The deviation from equal proportion of *Sphingobacteria* and *Flavobacteria* is highly significant for both the U form ($\chi^2 = 65.33$; df = 1; $P < 0.001$) and the G form ($\chi^2 = 22.50$; df = 1; $P < 0.001$). In all three localities the U form is predominantly associated with *Flavobacteria*, whereas the G form is predominantly associated with *Sphingobacteria* (paired *t* test statistic = 9.69; $P < 0.001$; see Fig. 3). However, the bacterial clones from *S. nudus* are evenly distributed along the trees ($\chi^2 = 1.52$; df = 1; $P > 0.05$).

The population genetic structure of each of the two bacterial groups, *Flavobacteria* (upper cluster) and *Sphingobacteria* (lower cluster), is very similar in the three distantly located settlements, but with a statistically significant deviation from neutrality and nonrandom association with the two color forms. The population parameters (Tajima *D* statistic and the Fu and Li test) show an excess of unique polymorphisms ($P < 0.01$ in both tests). The excess of singleton mutations is also highly significant by coalescent simulations ($P < 0.01$). A similar pattern of intraspecific variability and deviation from neutrality has been revealed for many genes of vertically transmitted endosymbionts (eg, refs. 22, 23) and interpreted in light of the near-neutral theory, as indicating that mildly deleterious mutations accumulate in endosymbiotic bacteria as a result of small effective population size. Thus, our data are consistent with the expected effects of genetic drift under the repeated bottlenecking caused by vertical bacterial transmission.

We have used the methods of Martin (24) and Lozupone and Knight (25) to investigate the structure of the *Bacteroidetes* communities associated with the U and G forms of *S. intermedius*, as well as another sea urchin species, *S. nudus*, considering each sea urchin individual, as an "environment" inhabited by a specific array of bacterial symbionts.

We detected significant differences between the *Bacteroidetes* communities associated with the U and G forms of *S. intermedius* from all three sea urchin settlements studied. The phylogenetic *P* test is highly significant in all cases ($P < 0.01$ corrected for multiple comparisons). This result indicates that the sequences are significantly clustered by environment overall, and proves that the microbial communities associated with the *S. intermedius* U and G forms are significantly different. The UniFrac tests are also highly significant for each sample ($P < 0.01$, corrected for multiple comparisons) indicating that the *Sphingobacteria* and *Flavobacteria* sequences obtained from the *S. intermedius* U and G forms represent a significant amount of unique branch length.

Using the raw UniFrac values (data not shown) for all pairs of environments, we obtained scatter plots (Fig. 4) of the first two principal coordinates; the different environments are represented, as described earlier, by the *S. intermedius* forms (U and G) and by *S. nudus*. The principal components produce biologically meaningful groupings. The two principal components, PC1 and PC2, jointly explain more than half of the variation in the data and separate the U-associated bacterial communities from the G-associated com-

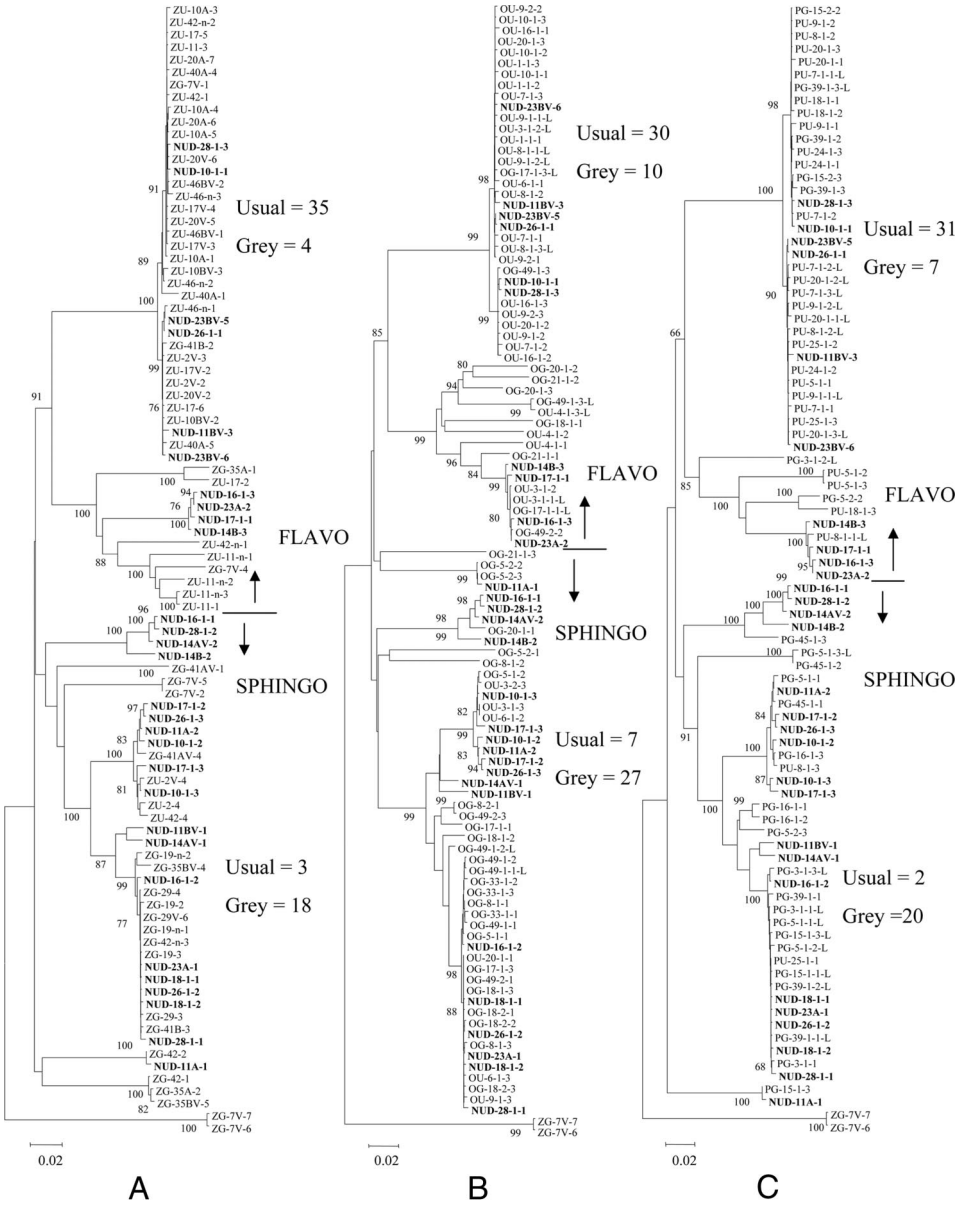


**Fig. 3.** Neighbor-joining tree of the 16S rRNA sequences of *Strongylocentrotus* endosymbionts, based on Kimura 2-parameter distance. The *Bacteroidetes* sequences are from sea urchins inhabiting the northern Primorye coast region, Sea of Japan, Pacific Ocean: Zolotoi Cape (*A*), Olga Bay (*B*), and Povorotnyi Cape (*C*). The sequences are designated by the first letter of the corresponding locality, with second letter referring to the color variant, U or G, following by the clone number. The numbers at the nodes are bootstrap percent probability values based on 10,000 replications. The clones of *S. nudus* are in boldface. The clones ZG-7V-6 and ZG-7V-7 are representatives of the class *Bacteroidetes* (genus *Prevotella*) and used here as common references for all three localities. ''FLAVO'' and ''SPHINGO'' refer to the two bacterial classes *Flavobacteria* and *Sphingobacteria*, respectively. NUD = *S. nudus*.

munities (with four exceptions, however; Fig. 4). The *S. nudus* individuals are dispersed among both the U and G forms, although they were all collected from the same depth as the U form (3 m; see *Materials and Methods*; Fig. 4).

The analysis reveals that the *S. intermedius* morphological forms (U and G), sampled from different depths, harbor significantly different bacterial communities even though they come from the same area of the ocean. The bacterial sequences from *S. nudus* are intermingled among the sequences from *S. intermedius*, independently of the depth at which they were collected, indicating that the depth of the sea urchin settlements by itself does not play a determining role in structuring the bacterial communities.

## Discussion

The sea urchin *Strongylocentrotus intermedius* has been widely used as a model organism for studies of reproductive and developmental biology, as well as toxicology and biotechnology (26, 27). This species is among the sea urchins most intensively studied by marine ecologists and constitutes the majority of the sea urchin fisheries in the Pacific region (3). However, the species-level systematics of *S. intermedius* remains ambiguous. Bazhin (2) examined more than 29,000 Russian museum specimens of *Strongylocentrotus* and noted that *S. intermedius* (along with *S. droebachiensis*) is the species most difficult to identify. The difficulty of reliable identification of *S. intermedius* entails, in turn, that there is no clear picture of the geographical distribution of the species (2). Problems arise from its highly variable morphology and the presence of a variety of forms even within closely located sea urchin settlements. Seeking to clarify the taxonomical status of the morphological forms of *S. intermedius* and to evaluate the possibility that there may be ''cryptic'' species, we first investigated the nucleotide diversity of two genes, one mitochondrial (*COI*) and one nuclear (*BND*), in a sample of *S. intermedius* that includes the most common forms.

There is little genetic differentiation between the two most common forms of *S. intermedius*, U (i.e., usual) and G (i.e., gray). The genetic divergence between the two forms is no greater than within each form; the two forms are not differentiated from each other in either the *COI* or the *BND* phylogenetic tree, as is typical for individuals of a single biological species. The genetic divergence between the two forms is considerably smaller than the differen-

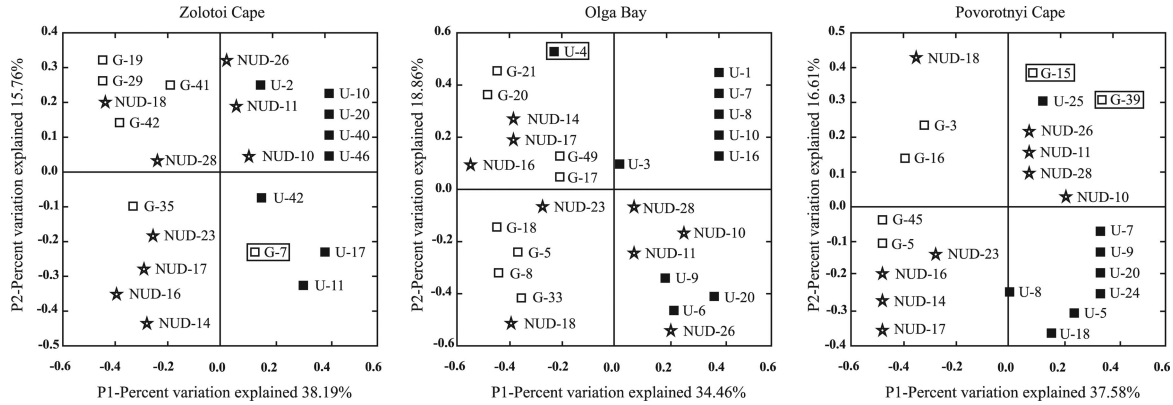


**Fig. 4.** First two principal coordinates from a principal coordinate analysis of the *Bacteroidetes* sequences obtained from three sea urchin settlements: Zolotoi Cape, Olga Bay, and Povorotnyi Cape. *S. intermedius* sea urchins are designated by the letters U and G, followed by numbers that refer to each particular individual. NUD refers to *S. nudus*. The numbers on the axes refer to the percent of the variation explained by each principal component (P1 and P2). Four sea urchin individuals (boxed), one in each of the Zolotoi Cape and Olga Bay localities, and two in the Povorotnyi Cape, are displaced from the expected position (see text).

tiation among different sea urchin species of the genus *Strongylocentrotus.* Intraspecific divergences are rarely greater than 2% and most are less than 1% (28), consistent with the values we have observed for the *COI* (0.74%) and *BND* (0.70%) genes of the *S. intermedius*, whether the U and G forms are considered separately or combined.

The low level of genetic divergence between the morphological forms of *S. intermedius* sharply contrasts with their bacterial content. The U and G forms are infected by symbiont bacteria from the phylum *Bacteroidetes*, but they are preferentially infected by different species belonging to two different classes, *Flavobacteria* and *Sphingobacteria* (Fig. 4). The pattern of bacterial distribution is very similar in three distantly located settlements of *S. intermedius* (Figs. 4 and 5).

Symbiotic bacteria have been shown to play an important role in metazoan evolution (29–32). It has been shown that bacteria of the phylum *Bacteroidetes* are associated with diverse host reproductive manipulations, including cytoplasmic incompatibility, parthenogenesis, and feminization, alterations that may play important roles in the host speciation process (33–35). Symbiont-associated changes in dispersal and mating are likely to play a key role in the initiation of genetic differentiation of populations with different infections, because cytoplasmic incompatibility can have direct consequences on gene flow between populations, making it a potentially important speciation agent (36). The phylum *Bacteroidetes* is one of the most important components of bacterial marine ecosystem (reviewed in ref. 17).

The *Bacteroidetes* symbionts are widespread in *S. intermedius* and, moreover, they have different distribution in the U and G morphological forms. Consequently, symbiont-induced life history changes may have promoted environmental specialization (shallow- and deep-water preferences for the U and G forms, respectively) and might potentially promote speciation in *S. intermedius*. We suggest that the symbiotic bacteria could be an important causative factor leading to morphological and potentially genetical divergence of this sea urchin species. If so, and given the evidence that *Bacteroidetes* symbionts may promote speciation, it might be the case that the U and G forms could be considered incipient species, even though their divergence may have occurred recently. Breeding experiments would not be informative to confirm or disprove this interpretation, because even distantly related sea urchin species, such as *S. intermedius* and *S. nudus*, produce highly viable first-generation hybrids (37); a situation that is not rare in other artificial fertilization experiments with sea urchins (38).

An alternative interpretation of our results is that the difference between the symbionts associated with the U and G forms may be conditioned by the different depths at which the two *S. intermedius* forms settle; that is, it might be the case that different symbionts prevail at different depths. If the settlement depth is the principal factor differentiating the microbial communities, we should expect similar symbiont communities for sea urchins of different species collected from the same (or close) depths and more different communities in hosts collected from different depths. This prediction is not supported by the data obtained for the *Bacteroidetes* symbionts of *S. nudus*: shallow-water *S. nudus* samples have intermingled bacterial distributions belonging to different *Bacteroidetes* lineages, without clear-cut differentiation among the *Bacteroidetes*. That is, the distribution of the symbiotic bacteria in *S. nudus* is not related to the depth of the sea urchin settlements. This observation contradicts the hypothesis that different symbionts associated with the U and G forms of *S. intermedius* are simply determined by the depth at which they are found.

One additional issue is what accounts for the morphological difference between the morphs? It could be the depth of settlement, although this seems *prima facie* unlikely. The morphological differences could also be a result of the different endoparasites, which might interact with nuclear genes or with the cytoplasm in determining the sea urchin phenotypes. This would also seem unlikely. Rather, we consider it likely that there are genetic differences that account for the morphological differentiation as well as the ecological preferences. These genetic differences could be limited to a few genes or to a supergene, whereas the rest of the genome would remain largely undifferentiated between the U and G forms. Our analysis of 62 loci obtained by random amplified polymorphic DNA failed to detect any diagnostic genetic differences between the forms. Future investigations will be necessary to ascertain whether genetic differences exist that would account for the morphological differences and ecological preferences of the U and G forms.

To our knowledge there are no publications concerning the evolutionary genetics of the interactions between symbiotic bacteria and sea urchins. There are multiple examples of morphological variants with uncertain taxonomical status in marine and other species (39–42). It is our conjecture that the morphological differences between forms of this kind, with or without significant genetic differences, may be caused by specific symbiotic associations. The "wrong" position of some individuals in Fig. 5 might imply that there is no absolute association between morphological forms and particular bacterial symbionts. Rather, each form may be associated with a different ensemble of symbionts, so that distinct morphological differences between the hosts would become pronounced when the symbionts' ensembles change significantly.

## Materials and Methods

**Sea Urchin Samples.** The specimens of *S. intermedius* (A. Agassiz, 1863) were obtained from the sea urchin settlement close to Cape Zolotoi (46°15′086′′N, 138°06′646′′E; Sea of Japan, Pacific Ocean). For the analysis of bacterial composition we additionally obtained the specimens of *S. intermedius* from two other localities, Olga Bay (44°15′773′′N, 135°47′630′′E) and Cape Povorotnyi (42°49′530′′N, 133°44′730′′E). The three geographical points cover more than 700 km of the northern Primorye coastal region. The U and G forms were collected at depths of 5 to 10 m and 15 to 20 m, respectively. The specimens of *S. nudus* (A. Agassiz, 1863) were obtained from the Lazurnaya Bay at depth of 3 m (southern Primorye coast region, Sea of Japan).

**DNA Amplification, Cloning, and Sequences.** The procedures for DNA extraction, amplification, and sequencing have been described previously (43). A 1,056-bp *COI* fragment was amplified using the following primers: 5′-ACACTTTATTT-GATTTTTGG-3′ (forward) and 5′-CCCATTGAAAGAACGTAGTGAAAGTG-3′ (reverse) (11). The sequences include the mitochondrial DNA region covering 352 codons of the *COI* gene, corresponding to positions 5854 to 6909 in the complete *S. purpuratus* mitochondrial sequence (44). A 1,809-bp fragment of the *BND* nuclear gene was amplified using the following primers: 5′-TCTGACGATTC-GAAAAGAGGAG-3′ (forward) and 5′-ATTAGCGTCTATATCTAGTTAG-3′ (reverse). The amplified *BND* fragment includes partial exon I (237 bp), intron (951 bp), and exon II (621 bp) and comprises the complete mature BND protein. A 1,360-bp fragment of the 16S rRNA bacterial genes was amplified using the following primers: 5′-CGTAACGCGTATACAATCTGCCTT-3′ (forward) and 5′-AGCCCTAGTTACCAGTTTTACCCT-3′ (reverse). The primers derive from the conserved region of the 16S rRNA gene from *Tenacibaculum* (13), a bacterial genus that has been suggested as an important pathogen of *S. intermedius* (14). The PCR reactions were carried out in final volumes of 25 $\mu$l using TaKaRa Ex Taq in accordance with the manufacturer's description (Takara Biotechnology; see *SI Text*). The *BND*, *COI*, and 16S rRNA sequences have been deposited in GenBank under accession numbers EU003190 through EU003201, EU700092 through EU700094; EU003202 through EU003213, EU700089 through EU700091, EU003214 through EU003229, EU432412 through EU432475, and EU626562 through EU626721.

**DNA Sequence Analysis.** The sequences were assembled using the program SeqMan (Lasergene, DNASTAR). Multiple alignment was carried out manually and using the program CLUSTAL W (45). The computer programs DnaSP, version 4.10.9 (46); and PROSEQ, version 2.9 (47), were used for most intraspecific analyses. Simulations based on the algorithms of the coalescent process with or without recombination (48, 49) were performed with the PROSEQ program to estimate the probabilities of the observed values of neutrality test statistics and the nucleotide diversity values.

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