Integrative Biology of *Idas iwaotakii* (Habe, 1958), a ‘Model Species’ Associated with Sunken Organic Substrates

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Abstract

The giant bathymodioline mussels from vents have been studied as models to understand the adaptation of organisms to deep-sea chemosynthetic environments. These mussels are closely related to minute mussels associated to organic remains decaying on the deep-sea floor. Whereas biological data accumulate for the giant mussels, the small mussels remain poorly studied. Despite this lack of data for species living on organic remains it has been hypothesized that during evolution, contrary to their relatives from vents or seeps, they did not acquire highly specialized biological features. We aim at testing this hypothesis by providing new biological data for species associated with organic falls. Within Bathymodiolinea a close phylogenetic relationship was revealed between the *Bathymodiolus sensu stricto* lineage (i.e. “thermophilus” lineage) which includes exclusively vent and seep species, and a diversified lineage of small mussels, attributed to the genus *Idas*, that includes mostly species from organic falls. We selected *Idas iwaotakii* (Habe, 1958) from this latter lineage to analyse population structure and to document biological features. Mitochondrial and nuclear markers reveal a north-south genetic structure at an oceanic scale in the Western Pacific but no structure was revealed at a regional scale or as correlated with the kind of substrate or depth. The morphology of larval shells suggests substantial dispersal abilities. Nutritional features were assessed by examining bacterial diversity coupled by a microscopic analysis of the digestive tract. Molecular data demonstrated the presence of sulphur-oxidizing bacteria resembling those identified in other Bathymodiolinea. In contrast with most *Bathymodiolus s.s.* species the digestive tract of *I. iwaotakii* is not reduced. Combining data from literature with the present data shows that most of the important biological features are shared between *Bathymodiolus s.s.* species and its sister-lineage. However *Bathymodiolus s.s.* species are ecologically more restricted and also display a lower species richness than *Idas* species.

Introduction

Despite the number and variety of studies on organisms from deep-sea chemosynthetic environments, their evolutionary origins and the biological processes explaining their diversity remain poorly resolved [1]. Among these organisms, bathymodioline mussels are studied as a model to elucidate the biological processes in vent and seep environments often designated as ‘extreme’ [e.g. [2,3]]. A comparative approach with the most closely related organisms from different environments is, however, required to understand the evolutionary significance of the organisms’ features in such ‘extreme’ environments. Distel et al. (2000) [4] were the first to show that bathymodioline mussels inhabiting vents and seeps form a monophyletic lineage with the modioline mussels associated with sunken organic substrates. This pioneering phylogenetic result implied the inclusion of the small and poorly-known deep-sea mussels from organic falls in the Bathymodiolinea. Recently, an evolutionary scenario was proposed in which sunken-wood is considered as an “early stage” towards adaptation to more extreme chemosynthetic environments in the deep-sea [5,6]. However, a recent molecular phylogenetic study, including more than 20 potentially new species associated with organic remains, suggested a more complex evolutionary scenario in which the history of hydrothermal vent and cold seep species is entangled with that of the small mussels associated with organic remains (Fig. 1) [7]. Lorion et al. (2010) [7] confirmed that the vent and seep mussels are divided into several distinct lineages, among which the “thermophilus” group (i.e. *Bathymodiolus sensu stricto* since *thermophilus* is the type-species of the genus) is the most thoroughly-studied. This clade is closely related to a much more diversified lineage of small mussels, here provisionally attributed to the genus *Idas*, with most of the species associated with organic remains but also with a few from vents or seeps (Fig. 1). Unfortunately, biological data are scarce for most species within this lineage [7] and thus the evolutionary significance of biological features observed in vent and seep species is difficult to establish.
Figure 1. Dendrogram obtained from Bayesian analyses of the combined dataset of COI mtDNA and the 28S rRNA. Asterisks correspond to nodes with posterior probabilities (PP) obtained from BA analysis and bootstrap values obtained from ML analysis that are higher than 0.90 and 50%, respectively. (modified from [7]).
doi:10.1371/journal.pone.0069680.g001
One of the most striking features discovered with these ‘extreme’ environments is symbiotic interactions with chemosynthetic bacteria [8], interpreted as a key adaptation of bathymodioline mussels to the particular environmental conditions of deep-sea vents and seeps. In Bathymodiolus s.s., chemosynthetic bacteria use reduced compounds such as hydrogen sulphide (H₂S) and/or methane (CH₄) to gain energy and for carbon fixation from dissolved CO₂ or methane providing a source of carbon to their host [8]. Most species within Bathymodiolus s.s. have both sulphur- and methane-oxidising symbionts, (e.g. [9]). Hydrogen was also recently shown to be an additional energy source for the sulphur-oxidising symbionts of Bathymodiolus (s.s.) puteoserpentis [10]. In all species, bacteria are located within the gill epithelial cells suggesting a tight relationship between metazoan and bacterial partners. Moreover for several Bathymodiolus s.s. species, the digestive tract is reduced [11–15] and it has been hypothesized that filter-feeding plays only a minor role in the diet compared to the food supply offered by their symbionts, especially in deeper-dwelling species.

By contrast, for the small species within the sister lineage of Bathymodiolus s.s., data about symbiosis are only available for two cold seep species (Idas modioliformis: [16,17]; I. macdonaldi: [9]) and for three species associated with organic remains (Idas washingtonius on whale bone: [18–20]; Idas sp. D on wood falls: [9] and Idas sp. C on both wood and bone: [21]). Located on gills, symbiotic bacteria are intracellular in I. washingtonius and I. macdonaldi but extracellular for I. sp. D and I. sp. C. Up to six different bacteria were observed in the gills of the seep species I. modioliformis [16,17], meanwhile other species seem to harbour only sulphur-oxidisers. No data are available concerning the anatomy of the digestive tracts of Idas species.

Larval dispersal is a key issue for understanding the evolutionary success of specialised species, because hydrothermal vents and cold seeps are patchily distributed over large distances [22]. In bivalves, larval dispersal capability can be inferred, to a certain extent, from larval shell morphology. Indeed since the size of the prodissococonch I of the oocyte are correlated, the size of the prodissococonch II relative to that of the prodissococonch I is indicative of the time spent in plankton by larvae [23]. Most bathymodioline species are considered planktotrophic because of their small egg size and prodissococonch morphology. Moreover, evidence for long-distance dispersal is available for several Bathymodiolus s.s. species [24–27].

### Table 1. Geographical localities, substrates, depths and sequences of specimens of I. iwaotakii used in this study.

| Locality             | Latitude     | Longitude  | Substrate | Depth (m) | COI  | 28S  | 16S bac |
|----------------------|--------------|------------|-----------|-----------|------|------|--------|
| Japan unknown        |              |            | wood      | 490       | (3)  | (1)  | (2)*   |
| Japan unknown        |              |            | wood      | 490       | (3)  | (1)  | (2)*   |
| Philippines 9° 24.3’ N | 124° 10.7’ E | wood      | 1764      | (1)      | (1)  | (2)  |
| Philippines 9° 25.6’0’ N | 124° 2.1’ E  | wood      | 1750-1767 | (1)*     | (1)* | (7)* |
| Philippines 9° 20.9’0’ N | 124° 8.7’ E  | wood      | 1764      | (3)*     | (3)* | (10)* |
| Philippines 8° 51.0’ N | 123° 10.0’ E | wood      | 982-989   | (1)*     | (1)* |       |
| Philippines 8° 51.3’0’ N | 122° 58.9’ E  | wood      | 2303–2307 | (1)*     | (1)* |       |
| Philippines unknown  |              |            | unknown   |           | (1)* |       |        |
| Papua-New Guinea 6° 55.8’5’ S | 147° 08.2’2’ E | wood   | 700–740   | (2)*     | (1)* |       |
| Papua-New Guinea 6° 46.9’8’ S | 147° 12.5’3’ E | wood   | 592–660   | (2)*     |       |       |
| Papua-New Guinea 2° 14.0’5’ E | 150° 15.1’0’ E | wood   | 490–505   | (1)*     | (1)* |       |
| Papua-New Guinea 5° 04.2’4’ S | 152° 0.2’1’ E | wood   | 752–998   | (3)*     | (2)* |       |
| Papua-New Guinea 5° 04.0’9’ S | 152° 0.2’7’ E | wood   | 782–1085  | (1)*     | (1)* |       |
| Solomon Islands 6° 37.6’0’ S | 156° 13.1’5’ E | wood   | 490–520   | (1)*     |       |       |
| Solomon Islands 6° 38.2’7’ S | 156° 13.2’0’ E | wood   | 508–522   | (2)*     |       |       |
| Solomon Islands 7° 44.5’0’ S | 156° 27.9’0’ E | wood   | 518–527   | (1)*     | (1)* |       |
| Solomon Islands 7° 42.8’3’ S | 156° 24.4’5’ E | wood   | 686–690   | (1)*     | (1)* |       |
| Solomon Islands 7° 45.2’1’ S | 156° 25.6’1’ E | wood   | 650–673   | (2)*     | (2)* |       |
| Solomon Islands 7° 57.5’0’ S | 156° 51.3’5’ E | wood   | 460–487   | (3)*     |       |       |
| Solomon Islands 7° 54.3’5’ S | 156° 50.8’6’ E | wood   | 515–520   | (2)*     |       |       |
| Vanuatu 15° 42.5’0’ S | 167° 03.0’0’ E | wood   | 441       | (24)     | (13) | (3)* | (9)*   |
| Vanuatu 15° 3.0’8’ S | 166° 56.1’0’ E | wood   | 548–560   | (1)*     |       |       |
| Vanuatu 15° 1.1’4’ S | 166° 53.7’6’ E | wood   | 630–670   | (11)*    | (4)* |       |
| Vanuatu 15° 1.4’2’ S | 166° 53.7’6’ E | wood   | 630–705   | (2)*     | (2)* |       |
| Vanuatu 16° 27.4’8’ S | 167° 54.7’8’ E | wood   | 562–580   | (2)*     | (1)* |       |
| Vanuatu 16° 2.6’7’ S | 167° 30.3’8’ E | wood   | 802–900   | (3)*     | (1)* |       |
| Vanuatu 16° 37.4’8’ S | 167° 57.5’3’ E | wood   | 641–677   | (4)*     | (1)* |       |
| Vanuatu 16° 27.6’2’ S | 167° 53.6’6’ E | wood   | 568–591   | (1)*     |       |       |
| New Caledonia 22° 33.2’5’ S | 166° 24.7’0’ E | bone   | 800       | (22)     | (4)* | (15)* | (6)* |

The number of sequences is indicated in brackets. The superscripts refer to the authors of sequences. The stars indicate the sequences obtained during this study. doi:10.1371/journal.pone.0069680.t001
The distribution of organic falls is patchy, but accumulations of vegetal remains are predictably found in the vicinity of tropical islands [28]. Several species associated with wood falls can also prosper on animal remains [7]. Thus, animal remains (notably the carcasses of large marine vertebrates), although not predictably located, are spread throughout the sea floor [29] and may constitute steps for dispersal among distant locations.

No study has yet documented population connectivity patterns among mussel species associated with organic remains. We selected the species *I. iwaotakii* to initiate such studies. This species was frequently sampled during the cruises of the Tropical Deep-sea Benthos program [28]. It has been found mainly on wood substrates, but also occurs on turtle bones [7]. It is distributed in the Western Pacific (sampled in Japan, Vanuatu and New Caledonia).

To document connectivity patterns in *I. iwaotakii*, we describe larval developmental morphology and prodissoconch I and II, infer the genetic structure of populations at different geographic scales considering two environmental factors (depth and organic substrate type). We describe the bacteria associated with the gill tissue and describe the structure of the digestive tract to infer the nature of its diet. We also re-evaluate the evolutionary significance of features often presented as adaptation to extreme environments by comparing the biological features revealed here with those of features often presented as adaptation to extreme environments.

**Materials and Methods**

**Sampling**

Specimens were collected during recent cruises of the Tropical Deep-sea Benthos program [31] off the Solomon Islands (2004), Vanuatu (2005, 2006), the Philippines (2005, 2007), New Caledonia (2008) and Papua New Guinea (2010) [28]. Part of the material has already been used as described in Lorion et al. (2009, 2010) [7,30]. The studied organisms are not protected and do not require specific export permits. Research permits were obtained for the EEZ of each country. In the Solomon Islands and Vanuatu, officers from Fisheries departments collaborated on the cruises. In the Philippines and Papua New Guinea, cruises were organised under a Memorandum of Understanding with Bureau of Fisheries and Aquatic Resources and the University of Papua New Guinea respectively. Collecting in New Caledonia was done within the EEZ of each country. In the Solomon Islands and Vanuatu, officers from Fisheries departments collaborated on the cruises. In the Philippines and Papua New Guinea, cruises were organised under a Memorandum of Understanding with the Bureau of Fisheries and Aquatic Resources. In the Philippines, cruises were organised under a Memorandum of Understanding with the University of Papua New Guinea respectively. Collecting in New Caledonia was done within the EEZ of each country. In the Solomon Islands and Vanuatu, officers from Fisheries departments collaborated on the cruises.

**PCR Amplification, Cloning and Sequencing**

DNA was extracted either from the gills of large individuals or from entire specimens using the QiAmp® DNA Micro Kit (Qiagen). For all specimens, a 379 bp fragment of the Cytochrome Oxidase I (COI) mitochondrial gene was amplified using primers LCO1490 [34] and H901 5’-GTRTTAAARTGRCGAT-CAAAAT-3’ [30]. For selected individuals representing distinct COI haplotypes, a 999 bp fragment of the 28S rRNA nuclear gene corresponding to the domains D1, D2 and D3 was amplified using the primers C1 5’-ACCCGGTGAATTTAAGGAT-3’ and C4 5’-TCGGAGGAAAGCAGTGACTA-3’. PCR reactions were performed in a final volume of 25 μL containing approximately 5 ng of template DNA, 1.5 mM of MgCl₂, 0.26 mM of each dNTP, 0.75 μM of each primer, 5% DMSO, and 0.75 unit of Taq polymerase (Qbiogene). Amplicons were generated by an initial denaturation step of 4 min at 94°C followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 50°C for COI and 52°C for 28S for 50 sec, and extension at 72°C for 1 min; and a final elongation at 72°C for 10 min.

The bacterial gene encoding 16S rRNA, used for the characterisation of bacterial communities, was amplified as described in Duperron et al. (2005) [33]. Symbiont diversity was evaluated for six specimens: two from the Philippines, two from Vanuatu (sunken wood) and two from New Caledonia (turtle bone). For each specimen, five PCR products were pooled and purified (Montage PCR kit, Millipore) prior to cloning using the QIAGEN PCR Cloning Kit. Inserts from a total of fifty positive clones were amplified using the plasmid-specific primers M13F and M13R.

PCR products were purified with AMPure XP beads, cycle-sequenced using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (1/32 reactions), and purified using an EtOH/sodium acetate precipitation. These products were then electrophoresed on an ABI PRISM (R) 3730xl Genetic Analyzer.

**Microscopy**

**Larval shell observation.** The prodissoconch I and II of 15 specimens (sample ID: MNHN-IM-2009–22278 to MNHN-IM-2009–22292) from New Caledonia were coated with gold-palladium and examined under a JEOL JSM-840A scanning electron microscope (SEM) operating at 80 kV. Shells were subsequently measured and described.

**Symbiotic bacteria.** The specimen used to study the localisation of symbiotic bacteria was collected off Vanuatu in association with coconut fibers. Gills were pre-fixed at sea in cacodylate-buffered 2% glutaraldehyde for two hours at room temperature (RT) and briefly rinsed in a fresh volume of the same buffer. Gills were stored at RT in that buffer until they were brought to the laboratory a few weeks later. Samples were then fixed for 45 minutes at RT in 1% osmium tetroxide in the same buffer, rinsed in distilled water and post-fixed with 2% aqueous uranyl acetate for one hour before embedding in Epon-Araldite by the method of Mollenhauer described by Glauert (1975) [32].

**Digestive system.** Two specimens (sample ID: MNHN-IM-2009–22293 and MNHN-IM-2009–22294, preservation in 80% ethanol), were chosen for the analysis of the digestive tract structure, both living on turtle bone off New Caledonia. Digestive tracts of specimens (n = 2) were observed in longitudinal sections, and cross sections.

**Materials and Methods**

**Sampling**

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Data Analysis

Genetic structure of mussel populations. Sequences were edited using Sequencher 4.1.4 and aligned using the Clustal W module implemented in Mega 4.0 [36]. Sequences were deposited both in BOLD and GenBank (COI: KC861674-KC861725; 28S: KC861726-KC861749 and KC904226-KC904234).

For the COI dataset, for each locality, the number of haplotypes (Hap), the number of segregating sites (Seg), haplotype (Hd) and nucleotide (Pi) diversity were determined using Arlequin 3.1.1.1 [37]. Phylogenetic relationships among haplotypes were constructed using the Median-Joining algorithm [38] and maximum parsimony post-processing calculation [39] implemented in Network 4.5.1.6 (http://www.fluxus-engineering.com).

FST was estimated among sampling locations using the formula of Weir and Cockerham (1984) [40] to assess population genetic structure. These calculations were based both on haplotype frequencies and on their pairwise nucleotide differences. A distribution of FST values under the null hypothesis of spatial genetic homogeneity was drawn from 10,000 datasets simulated by permutations of specimens between south-western localities. Genetic structure was also analysed with an exact test of differentiation using a Markov chain process with a burnin of 1000 steps. An Analysis of Molecular Variance (AMOVA; [41]) was performed among populations of the south-western part of the sampling region (Papua New Guinea, the Solomon Islands, Vanuatu and New Caledonia). An AMOVA was also assessed in order to evaluate hypothesised patterns of bathymetric genetic structure by using two depth categories (sampling, and >600 m). All tests were performed in Arlequin 3.1.1.1 [37].

Tajima’s D [42] and Fu’s FS [43] statistics were calculated to trace demographic events. Their statistical significance at alpha = 5% was estimated with a permutation test (10,000 replicates). Tests were performed for south-western localities, and for groups of localities among which no structure was detected. The parameters of a demographic expansion model, including the expansion factor (t), the mutation parameter (θ = 2 μN, where μ is the mutation rate and N is the effective population size) initial (θ0) and actual (θ1) of the south-western populations were estimated using Arlequin. These estimators were used to plot the distribution of the number of pairwise differences between mitochondrial sequences (‘mismatch distributions’) based on Slatkin and Hudson (1991) [44] for south-western Pacific populations. Under the coalescent model modified from Hudson (1990) [45] 1,000 replicates were carried out to calculate parameters, to define 99% confidence intervals, and to calculate the sum of squared deviations (SSD) of these parameters between the observed and estimated mismatch distribution.

Phylogenetic relationships. Phylogenetic relationships at the intra-specific level were inferred from COI sequences. A

Table 2. Geographical location, number of sequences (N), number of haplotypes (h), haplotype diversity (Hd) (with standard deviation), nucleotide diversity (π) and the number of segregating sites (S) of *I. iwaotakii* populations.

| Localities       | N  | h  | Hd (SD)      | π     | S  |
|------------------|----|----|--------------|-------|----|
| Japan            | 6  | 4  | 0.867 (0.129)| 4.467 | 9  |
| Philippines      | 8  | 4  | 0.643 (0.184)| 2.429 | 9  |
| PNG              | 9  | 7  | 0.9444 (0.070)| 2.500 | 9  |
| Solomon Islands  | 12 | 6  | 0.682 (0.149)| 1.47  | 8  |
| PNG+Solomon Islands | 21 | 10 | 0.8429 (0.069)| 1.962 | 14 |
| Big Bay          | 14 | 9  | 0.8791 (0.079)| 1.38  | 8  |
| Malo             | 24 | 12 | 0.7572 (0.094)| 1.275 | 11 |
| Malekula         | 10 | 5  | 0.7556 (0.130)| 0.956 | 4  |
| Vanuatu total    | 48 | 19 | 0.8023 (0.058)| 1.268 | 18 |
| New Caledonia    | 22 | 11 | 0.797 (0.087) | 1.221 | 9  |

PNG = Papua New Guinea.

doi:10.1371/journal.pone.0069680.t002

Figure 2. South Western Pacific map showing the sampling localities of mussels of *Idas iwaotakii*. Specimens from Malo and New Caledonia have already been studied by [7,30].
doi:10.1371/journal.pone.0069680.g002
Figure 3. Population structure of *I. iwaotakii*. Left part: K2P neighbour-joining tree of unique COI haplotypes of *I. iwaotakii*. The localities at which each haplotype have been sampled is figured by a coloured circle (see legend for correspondence between localities and colours), the number of individuals sharing the same COI haplotype at each locality is given in brackets. The grey rectangles underline the haplotypes shared by the two studied regions. The scale bar represents 0.02% estimated base substitutions. The mitochondrial haplotypes of *I. iwaotakii* are divided in two clades, A' and A", represented by one panel with large dots and the other one with small dots, respectively. Right part: Median-joining networks (MJN) from COI mtDNA data drawn independently for each of the two studied regions (North Western and South Western haplotypes). Size of haplotype circles is proportional to the number of specimens. Colours used to figure the localities within each region are the same of that used for the NJ tree. A small black circle represents a hypothetical haplotype. The haplotypes shared by the two studied regions are underlined using grey circles over haplotype labels.

Table 3. AMOVA results comparing genetic variation in *I. iwaotakii* populations at two hierarchical levels.

| Source of variation | Degrees of freedom | Variance components | FST    | P values |
|---------------------|--------------------|---------------------|--------|----------|
| Geography           |                    |                     |        |          |
| Among populations   | 2                  | -0.00376            | -0.00540 | 0.66020  |
| Within populations  | 86                 | 0.70109             |        |          |
| Bathymetry          |                    |                     |        |          |
| Among populations   | 1                  | -0.00292            | -0.00398 | 0.61762  |
| Within populations  | 87                 | 0.73635             |        |          |

Populations are clustered according to geographical locations (among populations from Papua New Guinea and the Solomon Islands grouped together, Vanuatu and New Caledonia) and depth ranging, below and above 600 m, from samples collected in the South-West of the Pacific Ocean.

doi:10.1371/journal.pone.0069680.g003
sequence of the species *I. macdonaldi* (AY649804), which belongs to the same clade as *I. iwaotakii*, was added to the dataset as an outgroup. A phylogenetic tree based on the neighbour-joining (NJ) method of Kimura two-parameter (K2P) distances was built using MEGA version 4.0 [36]. The bootstrap analysis was based on 1000 replicates.

**Symbiont diversity.** The sequences obtained for the bacterial 16S fragment were aligned using the Clustal W module implemented by Mega 4.0 [36]. Sequences with ≥99% identity were considered as a single phylotype and a single representative clone from each phylotype was chosen for phylogenetic analysis. Sequences were deposited on GenBank (KC904235-KC904238). Sequences were compared with the NCBI (http://www.ncbi.nlm.nih.gov/) database using BLAST [46]. The most similar BLAST sequences were included in phylogenetic analysis. Phylogenetic reconstructions were performed using maximum likelihood (ML) and Bayesian (BA) analyses.

The most appropriate evolutionary model was estimated using jModelTest 0.1.1. The GTR+I+C model was chosen based on AIC. The ML analysis was performed using the software Treefinder [47] and node support was assessed with 1,000 bootstrap replicates [48]. BA was performed using MrBayes v3.1.2 [49]. Two independent analyses were run in parallel. Each analysis comprised four Markov chains and each chain was run over 8 million generations with a sampling frequency of one tree every hundred generations and a burnin period of 40,000 generations. Convergence between the two analyses was assessed using likelihood curves, standard deviation of split frequencies and potential scale reduction factor [50].

**Results**

**Genetic Diversity and Phylogenetic Analyses**

A total of 50 COI mtDNA sequences of 579 bp were obtained. Moreover, 53 sequences available on Genbank were added to this new dataset for the analyses. Because the three sequences available

![South-East localities (Papua-New Guinea, Solomon Islands, Vanuatu, New Caledonia)](image)

**Figure 4. Mismatch distributions of COI haplotypes.** COI haplotypes observed, simulated under a constant population size and a model of population expansion for the South-west Pacific populations of *A. iwaotakii*. Along x-axis are indicated the numbers of nucleotide differences between all pairs of sequences and on y-axis the frequencies of pairs. The table summarizes the parameters of demographic analyses under constant population size and demographic expansion. The confidence intervals at 99% of the parameters under a demographic expansion model are given in square brackets.

doi:10.1371/journal.pone.0069680.g004
Figure 5. Scanning electron micrographs of the larval shell of a specimen of Idas iwaotakii. Pictures: (A) Dorsal view showing prodissoconch I (Pl) and prodissoconch II (Pll); the white arrow indicates the boundary between the dissoconch and prodissoconch. (B) Detail of Pl; the grey arrow indicated of the boundary between Pl and Pll.

Connectivity Patterns

Two separate median-joining networks were computed for the north-western and south-western regions, as these locations harboured distinct phylogenetic lineages and the sampling effort was very unbalanced between the two regions. In agreement with the NJ analysis, median-joining networks revealed the co-occurrence of both lineages in the Northwest (Fig. 3).
most likely represent environmental bacteria, as they were >97% similar to Proteobacteria (epsilon- and gamma-Proteobacteria), Planctomycetes, and Bacteroidetes (Cytophaga Flavobacter Bacteroides, or CFB) that are also commonly found at/near vent communities.

Only thiotrophic symbiont sequences (corresponding to phylogenotypes 1 to 4) were included in the phylogenetic analyses. Trees were rooted on the methanotrophic symbiont of *B. brooksi* [30,52]. The topologies of the ML and BA reconstructions were identical, and confirmed that phylogenotypes 1 through 4 belonged to a single large clade which included all known thiotrophic symbionts of the Bathymodiolinae and other mussels from sunken organic substrates (Fig. 6). Moreover, these four phylogenotypes are resolved within a group of symbionts of organic falls mussels. The four phylogenotypes were closely related to symbionts of tiny mussels associated with sunken wood from the Philippines (AM503926, AM51093) [21] and to the symbionts of *G. crypta* and *I. sp. C* from Vanuatu [30].

**Localisation of Associated Bacteria**

TEM observations of sections of gill filaments indicated the presence of extracellular, gram negative bacteria located between the microvilli of the host cells, throughout the lateral zone of each gill filament (Figs 7A–B).

**Digestive Tract Structure**

LM observations of semi-thin sections of the digestive system revealed the presence of typical features of bivalve digestive tract consisting of an oesophagus, a stomach, an intestine and a well-differentiated digestive gland that surrounds the stomach and a part of the intestine. In all observed specimens: the unilammar

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*Figure 6. Bayesian tree displaying bacterial symbionts based on the analysis of the 16S rRNA.* Phylogenotypes associated with *I. iwaotakii* are shown in bold. Posterior probabilities (PP) and bootstrap values obtained from ML analysis are given above and below branches respectively. PP and bootstrap values lower than 0.90 and 50%, respectively, are not shown. The scale bar represents 0.5% estimated base substitution.

doi:10.1371/journal.pone.0069680.g006
prismatic epithelium of the oesophagus had ciliated cells (Fig. 8B); the stomach epithelium had cubic cells (Fig. 8D) and a chitinous area corresponding to the gastric shield; the prismatic epithelium of the intestine (Fig. 8C) displayed a high density of microvilli. The digestive tract was curved and formed at least one loop (Fig. 8A). Both LM and TEM observations revealed the presence of digestive contents in the stomach and the intestine. The alimentary bolus was nevertheless difficult to identify. One rigid structure, about 296 μm wide and little damaged by digestion, could be identified as a foraminiferan test (Fig. 8E).

Discussion
Genetic Divergence, Population Connectivity, and Demographic History

Our study confirms that *Idas iwaotakii* populations are divided into two distinct mitochondrial lineages, A’ and A”, and confirmed their coexistence in Japan. But, although we confirm that specimens from the lineage A” from the Philippines have a distinct 28S rRNA haplotype, we show that in Japan, specimens from both A’ and A” lineages share the 28S rRNA haplotype typically associated with A’ in the south-western area. The combination of nuclear and mitochondrial data on a larger number of mussels thus suggests that the two mitochondrial lineages are not, as it has been hypothesised before [7], reproductively isolated.

At the regional scale (SW Pacific) the sampling of *I. iwaotakii* initiated by Lorion et al. (2009, 2010) [7,30] is improved here. Although the localities are separated by 500 km (New Caledonia/ Vanuatu) to 2400 km (New Caledonia/Papua New Guinea), no genetic structure was detected. Moreover, our sampling effort covers a wide depth range, and the recruitment experiments deployed off New Caledonia allowed us to collect *I. iwaotakii* on bone, a substrate much more difficult to locate than sunken wood. Indeed, trees and other plant substrates can reliably be found in the vicinity of islands and continents skirted with well-developed coastal forests, whereas the distribution of skeletal remains has an unpredictable, idiosyncratic distribution. The New Caledonian specimens sampled from turtle bones show no genetic difference from other samples collected from sunken-wood at other localities. Similarly, no genetic structure is detected over a large bathymetric range (i.e. 460 m –2,307 m). Thus, *I. iwaotakii* is a widespread species that may colonise, over a wide depth range, organic substrates such as sunken-wood and bone [7]. These results are congruent with those available for other species associated to organic remains (i.e. *I.* sp. C and *G. crypta* [30]).

The absence of genetic structure between south-western sampling localities suggests either high larval dispersal ability and an absence of barriers to dispersal or historical connectivity still detectable with COI and 28S. These results are consistent with the long larval stage here inferred from the size of prodissoconch II, and documented for close relatives from hydrothermal vent and cold seep environments. Indeed, larvae from *B. azoricus* could remain in the water column for 5 to 6 months [53,54]. Evidence of long-distance dispersal has also been found for *I. modiolaeformis*, *I. argenteus* and other Bathymodiolinae [14,17,23,55–58]. Notably, Lutz et al. (1980) [23] revealed that *B. thermophilus* produces large numbers of small eggs that develop into planktotrophic larvae and potentially disperse over long distances. The assumption of large-scale dispersal ability is supported by the high gene flow documented among *B. thermophilus* populations from 13°N to 11°S latitude on the East Pacific Rise (~2,500 km) [24,26]. Vents, seeps and organic remains, are patchy and often ephemeral habitats. In such highly fragmented habitats, establishment and maintenance of populations probably relies on the long, free-swimming, larval stage that in turn might also explain the wide geographical distribution of most species.

Based on our genetic data, we hypothesise that a small number of migrants from the northwest area have recently founded new successful populations in the south-western area. The association

Figure 7. Gill filaments of *Idas iwaotakii*. (A) TEM view of gill filament of the lateral zone from a specimen of *I. iwaotakii* collected on wood off Vanuatu. Bacteria (black arrows) are located extracellularly in contact with microvilli. BL: blood lacuna; Lm: basal lamina. (B) Electron micrograph of the extracellular symbionts. Symbiotic bacteria possess a double membrane (white arrow) typical of Gram negative bacteria. doi:10.1371/journal.pone.0069680.g007
of a demographic expansion signal and a reduction of the genetic diversity in the southern populations support this hypothesis. However, this pattern is also consistent with allopatric divergence followed by secondary contact. The complex history of the Western Pacific, marked by tectonic plate movement (e.g., [59,60]), volcanism and periodic sea level changes (e.g., [61]), might have impeded larval dispersal, during some periods, and thus favoured the emergence of new lineages through founder events but also the secondary contacts among isolated populations.

Geographic breaks between pairs of closely related species have been repeatedly documented in both *Bathymodiolus s.s* and *Idas* lineages. In *Idas*, the cold seep species *I. modiolaeformis* and *I. macdonaldii* (3.3% divergent at COI), were proposed as a potential amphi-Atlantic species-complex [17]. In the *Bathymodiolus* lineage, the same was proposed for the seep complex of species *B. boomerang* and *B. aff boomerang* (2% divergent at COI, [62,63]). Two pairs of vent species have a geographic structure along a mid-oceanic ridge system. The two sister-species *B. thermophilus* and *B. antarcticus* are respectively distributed in the Galapagos rift to the North and not to the South of the East Pacific Rise [26,27,64]. Similarly the sister-species *B. azoricus* and *B. puteoserpentis* are respectively distributed in the North and in the South part of the Mid Atlantic Ridge [65,66]. However, in the Indo-West Pacific, the populations attributed to *B. septemdierum*, *B. brevis* and *B. marisindicus*, are not reproductively isolated and display no genetic structure over a very wide geographical area (see [5]).

**Symbiotic Association and Insights into Nutritional Features of *I. iwaotakii***

In previous studies [21,30,67], thioautotrophic symbionts have predictably been found in each newly examined deep-sea mussel associated with organic remains. This new dataset supports this predictability and moreover shows that, within a species, the same dominant bacterial phylotype is found, independent of geographic location (the Philippines, Vanuatu and New Caledonia), substrate type (wood versus bone) or depth (441 m to 1767 m). This dominant phylotype is closely related to a symbiont detected in another species within the *Idas* lineage (*I. sp. C*) and also in another species from a distinct lineage (*G. crypta*) [30]. These two species were also collected both on wood and bone. Phylogenetic relationships among sulphur-oxidizing symbionts are however poorly resolved. This lack of resolution is probably due to the relatively slow evolution rate of the 16S rRNA. More variable genetic markers have to be developed to better resolve symbiont phylogeny.

**Figure 8.** Semi-thin sections of the digestive tract of *Idas iwaotakii*. The specimen was collected on turtle bone off New Caledonia. (A) Overall view of the digestive tract (longitudinal sections). The black arrow indicates the location of microvilli in the intestine. (B) Cross section of the oesophagus lined by a stratified epithelium. (C) Cross section of the intestine. (D) Cross section of the stomach lined by a cuboidal epithelium. The white arrow indicates the gastric shield. (E) Longitudinal section of a detail of intestinal contents.

**Figure 9.** Comparison of data available for the “thermophilus” lineage and its sister lineage. The “thermophilus” lineage is represented by the dark grey rectangle and its sister lineage, corresponding to the genus *Idas*, by the light grey rectangle. Phylogenetic relationships correspond to a part of the combined COI mtDNA and 28S RNA phylogenetic tree from [7]. T: thiotrophic symbiont; M: methanotrophic symbiont. In geographical patterns section, the ovals correspond to pairs of sister-species with an allopatric distribution and location maps illustrate the allopatric distribution of these sister pairs. References: [7–9], [11], [14], [16], [24], [26–27], [30], [52], [63–64], [66], [70], [72], [76–83].

doi:10.1371/journal.pone.0069680.g008
Electron microscopic analyses confirm the presence and abundance of bacteria in association with gill filaments. The bacteria are extracellular and are thus directly exposed to the reduced compounds from degrading organic remains. The extra- cellular localisation of symbionts has already been documented for other mussels associated with wood falls, notably for the *Idas* lineage in *I. sp. C* and *I. sp. D* [21,30,67,68]. By contrast, the symbionts of bathymodioline mussels from hydrothermal vents and cold seeps are mostly intracellular. Likewise, *I. washingtonius*, which is associated with whale bone, possesses intracellular bacteria located within gill bacteriocytes [18,30]. Thus, by contrast with the species included in *Bathymodiolus* s.s. lineage, the species included in the *Idas* lineage harbour either intracellular symbionts (*I. macdonaldii* or *I. washingtonius*) [18] or extracellular symbionts (*I. iwaoi*, *I. sp. C* and *I. sp. D*) [8,30]. As *I. washingtonius* is sampled on organic remains, we suggest that the extra- or intra-cellular location of the bacteria is not strictly related to the environments as was previously suggested [5,6].

Bathymodioline mussels possess a functional but considerably reduced digestive tract [11–14]. Some *Bathymodiolus* species (*B. boomerang, B. heckerae, B. brooks, B. thermophilus* and *B. aff. thermophilus*) have a simple digestive tract with a small stomach and a straight midgut without any coiling or loop [14,69,70]. Others such as *B. azoricus* and *B. putosserpentis* have a coiled intestine and well-developed lobial palps [71,72]. Intestines with recurrent loops have been observed in seeps specimens of *I. macdonaldii* [14]. Based on stable isotope measurements, Deming et al. [18] suggested that *I. washingtonius*, may have a mixotrophic nutritional strategy involving both filter feeding and supply from symbionts. *I. iwaoi* has a coiled intestine. Digestive contents were relatively abundant and varied. For example a rigid structure found in the intestine could be a foraminiferan test resembling *Sprylopectammina taiwania* (Chang, 1956) [73]; see figures 29 A and B). Le Pemec and Prieur (1984) [11] also identified the tests of benthic Foraminifera and diatom frustules in the stomach contents of vent mytilids.

Overall, sulphur-oxidizing symbionts closely related to those previously identified in Bathymodiolinidae are present in the gills of *I. iwaoi* and the digestive tract is non-reduced and functional. These mussels associated with organic falls are thus potentially able to obtain carbon and other nutrients either from their autotrophic symbionts and/or by filter feeding. A longer intestine with recurrent loops suggests a significant contribution of filter feeding to the mussels’ diet, likely supplemented by nutrition derived from symbiont chemoautotrophy (e.g. [70]). The relative contribution of each pathway remains to be quantified. The concentration of hydrogen sulphide (*H*₂*S*) available in the environment determines the ability of bacterial symbionts to provide food supply. Concentrations of reduced compounds probably vary depending on quantity, nature and state of degradation of the available organic substrates (e.g. [74]). A mixed diet might allow individuals to adapt to high variability in the availability of reduced compounds.

**Evolutionary Significance of Biological Features Related to Environments**

Although living on organic remains, *Idas iwaoi* shares many biological similarities with the large mussels of *Bathymodiolus s.s.* living in either vents or seeps: (i) association with chemosymbiotic symbionts, (ii) high larval dispersal and (iii) lack of geographical genetic structure. However, *I. iwaoi* apparently accommodates to a wider range of ecological conditions than *Bathymodiolus s.s.* species (Fig. 9). Indeed, *I. iwaoi* displays a very wide geographical distribution and a large bathymetrical range, has probably a mixed diet and is able to colonise various organic substrates (i.e. bone and vegetal remains). By contrast, *Bathymodiolus s.s.* species are restricted either to vents or seeps but are never found in both habitats nor on organic remains. This contrasts with giant mussels belonging to the “childressi” group that, like *Bathymodiolus* (sensus lato) *platyons* or *B. (s.l.) japonicus* are found both at vent and seep sites [75]. The straight intestine evidenced for most *Bathymodiolus s.s.* species suggests a greater dependency on the food supply provided by their autotrophic symbionts. This dependency may explain why they are restricted to habitat in which the reduced compounds remain available regularly, even if at variable concentrations.

**Acknowledgments**

New material was collected on board of M/V DA-BFAR (PANGL AO 2005, Co-PI P. Bouchez and L. Labe and Aurora 2007, Co-PI M. Manuel, and P. Bouchez) and on board of R/V Alis (BOA1, Co-PI B. Richer de Forges and S. Samadi; SANT02006, Co-PI P. Bouchez, O. Pascal and H. Le Guayder; Salomon2, Co-PI B. Richer de Forges and P. Bouchez, Biopapa, Co-PI S. Samadi and L. Corbari). Sincere thanks to J. Lorion for his keen insight and his support, C. Djediat for his assistance with microscopic experiments and analysis, B. Buge for curation of specimens and the staff of the “Service de Systématique Moléculaire” (UMS2700 CNRS-MNHN). It is part of the agreement no.2005/67 between the Genoscope and the Museum National d’Histoire Naturelle on the project “Macrophylogeny of life” directed by G. Lecointre. Special thanks to E. Pante for constructive comments and English improvement of the manuscript.

**Author Contributions**

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3. Bettencourt R, Costa V, Laranjo M, Pires L, et al. (2011) Out of the 'Macrophylogeny of life' directed by G. Lecointre. Special thanks to E. Forges and S. Samadi; SANT02006, Co-PI P. Bouchet, O. Pascal and H. Le Guayder; Salomon2, Co-PI B. Richer de Forges and P. Bouchez, Biopapa, Co-PI S. Samadi and L. Corbari). Sincere thanks to J. Lorion for his keen insight and his support, C. Djediat for his assistance with microscopic experiments and analysis, B. Buge for curation of specimens and the staff of the “Service de Systématique Moléculaire” (UMS2700 CNRS-MNHN). It is part of the agreement no.2005/67 between the Genoscope and the Museum National d’Histoire Naturelle on the project “Macrophylogeny of life” directed by G. Lecointre. Special thanks to E. Pante for constructive comments and English improvement of the manuscript.

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