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Episymbiotic microbes as food and defence for marine isopods: unique symbioses in a hostile environment

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Symbioses profoundly affect the diversity of life, often through novel biochemical services that symbionts provide to their hosts. These biochemical services are typically nutritional enhancements and less commonly defensive, but rarely both simultaneously. On the coral reefs of Papua New Guinea, we discovered unique associations between marine isopod crustaceans (*Santia* spp.) and episymbiotic microbes. Transmission electron microscopy and pigment analyses show that episymbiont biomass is dominated by large (20–30 μm) cyanobacterial cells. The isopods consume these photosymbionts and 'cultivate' them by inhabiting exposed sunlit substrates, a behaviour made possible by symbionts' production of a chemical defence that is repulsive to fishes. Molecular phylogenetic analyses demonstrated that the symbiotic microbial communities are diverse and probably dominated in terms of population size by bacteria and small unicellular *Synechococcus*-type cyanobacteria. Although largely unknown in the oceans, defensive symbioses probably promote marine biodiversity by allowing niche expansions into otherwise hostile environments.

Keywords: coral reefs; chemical defence; cyanobacteria; defensive symbiosis; marine isopods; molecular phylogenetics

1. INTRODUCTION

Symbiotic associations greatly influence the diversity of life by providing expanded ecological and evolutionary opportunities to both host and symbionts (Douglas 1994). The vast majority of described symbioses are associations of macro-organisms with micro-organisms, the latter providing novel biochemical processes that increase host survival and competitiveness in 'extreme' environments in which host fitness would otherwise be low (Douglas 1994). The most commonly recognized symbioses have a nutritional foundation, such as the associations between nitrogen fixing micro-organisms (e.g. *Rhizobium* spp.) and many terrestrial plants.

In the marine environment, nutritionally based symbioses are also common (e.g. reef building corals with *Symbiodium* spp. dinoflagellates (Muller-Parker & D'Elia 1997)) and greatly impact local biodiversity. A clear example of this is deep-sea hydrothermal vents, which, largely due to symbioses between chemosynthetic bacteria and macro-invertebrates, have strikingly different fauna and support significantly more biomass than surrounding deep-sea habitats (Cavanaugh *et al.* 1981).

Symbiont production of defensive chemistry that protects the host against consumers or pathogens is common in terrestrial plants (Saikkonen *et al.* 1998). Many marine invertebrates also harbour microbial symbionts and possess secondary metabolites with structural similarities to known microbial metabolites (Kobayashi & Ishibashi 1993; Piel 2004), yet few studies have progressed sufficiently to even demonstrate symbiont production of these metabolites (Faulkner *et al.* 1999; Piel 2004). Although chemical defence against consumers is a common trait among members of many sessile, soft-bodied marine taxa, including seaweeds, sponges, bryozoans and tunicates (Paul 1992), and particularly on predator-rich coral reefs, there is limited empirical support for defensive symbioses in marine organisms. Gil-Turnes *et al.* (1989) and Gil-Turnes & Fenical (1992) demonstrated that bacterial films on developing embryos of the shrimp, *Palaemon macrodactylus* and the lobster, *Homarus americana*, produce isatin and tyrosol, respectively, both of which prevent infection by the pathogenic marine fungus *Lagenidium callinectes*. While previously supported by circumstantial evidence (Davidson *et al.* 2001), Lopanik *et al.* (2004a,*b*) rigorously demonstrated that *Endobugula sertula*, a bacterial symbiont of the marine bryozoan *Bugula neritina* (Haygood & Davidson 1997), produces bryostatin-class polyketides, which are concentrated on the bryozoan's larvae and protect them from predation by fishes.

At four widely separated regions of Papua New Guinea (PNG), we observed clusters of fluorescent red isopods (*Santia* spp.; Family Munnidae; Suborder Asellota) prominently positioned during daylight hours on fully exposed surfaces of sponges, gorgonians and coral rubble. These non-swimming, slow-moving isopods are up to 5 mm in length and easily seen from a distance of 2–3 m. Aggregations ranging from tens to commonly thousands of individuals were observed from 4 to 45 m depth, and, even at the deeper depths, the isopods had a conspicuous red coloration. Closer examination of the isopods found that their bright coloration is due to a dense carpet of

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unicellular algae covering the isopods' exoskeleton. However, we never observed fishes naturally feeding on these isopods and this observation, combined with their apparent coloration and lack of predator avoidance behaviours, suggested acute unpalatability. The striking coloration and risky behaviour of these isopods stand in sharp contrast to virtually all other small coral-reef crustaceans that typically reduce their risk of predation by being cryptic, refuge dwelling or noctural (Wallerstein & Brusca 1982).

In this paper, we report on the ecological and phylogenetic characterization of this unique complex of previously undescribed symbiotic associations between coral-reef isopods and unicellular microalgae. Specifically, we:

(i) Tested whether these isopods were unpalatable to reef fishes.
(ii) Tested the hypothesis that the isopod's microbial community is the source of the isopod–microbial association's unpalatability.
(iii) Characterized the microbial community through transmission electron microscopy (TEM) and molecular phylogenetic analyses.
(iv) Explored whether symbionts are vertically transmitted or environmentally acquired by examining phylogenetic patterns for concordant regional genetic differentiation in host and symbionts' lineages.

2. MATERIAL AND METHODS

(a) *Description and collection of isopods*

Isopods of the genus *Santia* are generally diminutive and are known from tropical and temperate oceans, primarily in the southern hemisphere (Wolff 1989; Winkler 1993). We collected previously undescribed *Santia* spp. isopods (Brian Kensley, personal communication) bearing microbial episymbionts by scuba-diving on reefs at Kairiru Island, Crown Island, Rabaul and Eastern Fields in PNG (figure 1). Isopods were collected at depths of 4–35 m from sponges and other benthic substrates. In addition to the brightly coloured isopods, a small aggregation of a cryptic *Santia*-like isopod having white eyes rather than the red eyes of the conspicuous red isopods was found on one sponge colony at Eastern Fields. These isopods were covered by non-fluorescent, brown-coloured microalgae.

(b) *Isopod characterization studies*

Isopods for microscopy were preserved in formalin and seawater (1:19) or gluteraldehyde and seawater (1:49). Epifluorescence photomicrographs were recorded using a NIKON Eclipse E800 microscope and NIKON CoolPix 990 digital camera. Isopods for electron microscopy were preserved in 7% formalin. Their preparation for TEM analysis began with three 10-min rinses in 1×phosphate buffered saline (PBS) and fixation overnight in 2% osmium tetraoxide. After two additional rinses in PBS, isopods were dehydrated in an ethanol wash series (30, 50, 70, 95 and 100%) followed by three 30-min rinses in propylene oxide. Samples were incubated overnight in a 1:1-mixture of propylene oxide and Epon 812 and then embedded in Epon 812 at 60 °C. Embedded samples were sectioned with an ultramicrotome and examined using a Zeiss EM 10 electron microscope. Isopods for pigment analysis were preserved in acetone. High performance liquid chromatography (HPLC) analyses of isopod/algal pigments in the acetone extract were conducted according to the procedures described by Paerl and co-workers (Pinckney *et al.* 2001).

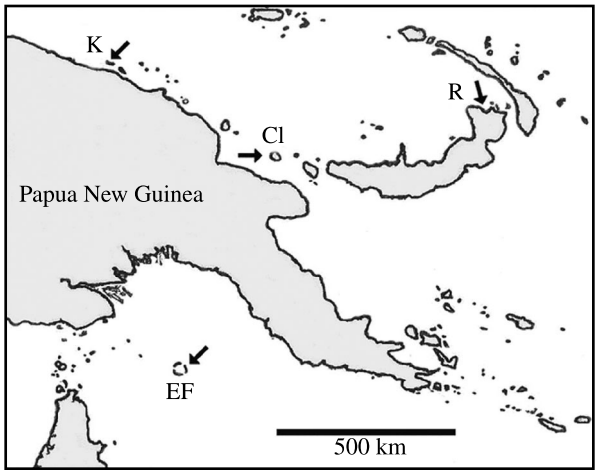

Figure 1. Collection sites of *Santia* spp. isopods in Papua New Guinea. K, Kairiru Island (September 1998); EF, Eastern Fields (December 1999); R, Rabaul (August 2000) and CI, Crown Island (August 2000).

(c) *Feeding assays*

In situ feeding assays were conducted at Kairiru Island and Eastern Fields while scuba-diving from the live-aboard dive boat, the M/V Golden Dawn. To test the palatability of the symbioses to reef fishes, individual isopods ($n=10$ for red isopods at both Kairiru and Eastern Fields and $n=5$ for brown isopods at Eastern Fields) were removed from the substrate using a glass pipette and introduced into nearby schools of reef fishes (mostly *Chromis* and damselfish: typically *Chromis margaritifer*, *Chromis retrofasciata*, *Amblyglyphidodon curacao*, *Dacyllus trimaculata* and *Pomacentrus moluccensis*). Each individual isopod was offered to a separate group of fishes by releasing the isopod 0.5–1 m upstream and slightly above a coral head around which fishes were congregated. The current carried the isopod towards the fishes and their feeding behaviour was observed from *ca.* 1 m away. This pelagic presentation was required to interest fishes in attacking the isopods because fishes ignore the isopods when they are on the substrate despite being fully exposed and conspicuous. Prior to offering an isopod, an isopod-sized pellet (*ca.* 4 mm long×2 mm diameter) of a palatable squid-based control food (see Lindquist & Hay 1995 for the recipe) was released up-current of the schools of fishes—these pellets were always consumed, indicating that close proximity of divers did not deter feeding. Although most fishes ignored the red isopods, some members of the schools would attack (i.e. take the isopod into its mouth) and reject (i.e. spit the isopod out) them. At Eastern Fields, rejected isopods were recaptured to evaluate their 24 h survival rates versus a group of isopods handled in the same manner but not offered to fishes. To test whether isopod unpalatability was a function of their symbionts, red isopods from Eastern Fields were greatly depleted of their episymbionts by keeping them in the dark for 2 days in containers lacking natural substrate and then offered to reef fishes as described above. The level of episymbiont depletion was estimated by visual inspection using a dissecting scope. To test for the presence of defensive chemistry in these isopod–microbial associations, an extract of the red

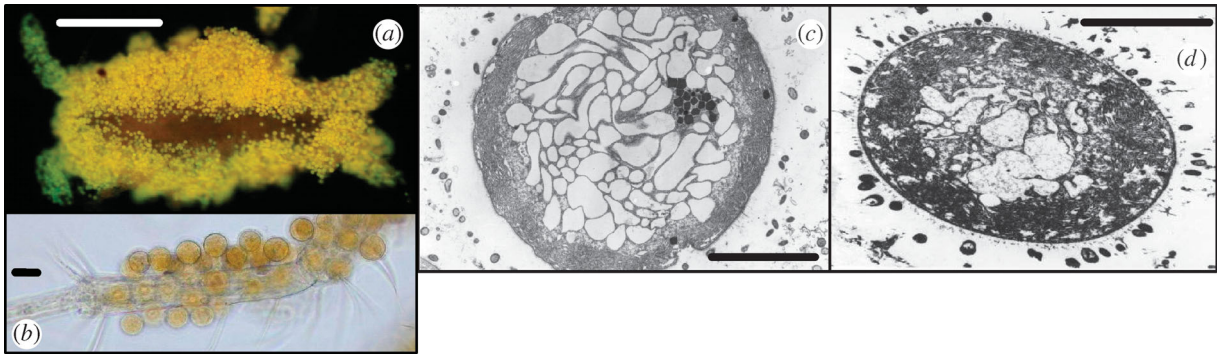

Figure 2. (*a*) Epifluorescence photograph of the *Santia* spp. isopod and its ectosymbiotic cyanobacteria. (*b*) Cyanobacterial cells on an isopod antenna. (*c*) TEM micrograph of a cyanobacterial cell of an unpalatable red isopod and surrounding micro-organisms. (*d*) TEM micrograph of a cyanobacterial cell of a palatable brown isopod and surrounding micro-organisms. Scale bars: (*a*) 1 mm, (*b*) 25 μm, (*c*) 10 μm and (*d*) 5 μm.

isopods from Kairiru was prepared by methanol extraction (3×200 μl) of freshly collected isopods with a packed volume of 100 μl, as measured in a volumetric syringe. After evaporating the methanol with a slow stream of air, the dried extract was thoroughly mixed into 100 μl of the palatable squid-based food and formed into isopod-sized pellets. Paired extract-treated and control pellets (solvent addition only) were offered to schools of small reef fishes on 10 separate coral heads. Because the dark-starvation treatment described above did not entirely eliminate the isopods' episymbiont community, we did not use the extract of episymbiont-depleted isopods as the control. Further, it is highly unlikely that the isopods are the source of the isopod–microbial associations' unpalatability because crustaceans have not been reported to produce defensive natural products (Blunt *et al.* 2003) and appear to be chemically benign without an unpalatable partner (McClintock & Janssen 1990). Fisher's exact test (Zar 1984) was used to statistically compare fishes' consumption of the isopods or the extract-treatment food versus pellets of the palatable control food.

(d) *Genetic analyses*

Samples for genetic analyses were preserved in ethanol. DNA from 28 individual isopods representing four localities (Crown Island $n=3$; Eastern Fields red $n=10$; Eastern Fields brown $n=4$; Kairiru $n=5$; Rabaul $n=6$) was extracted with Chelex (Walsh *et al.* 1991). A portion of each isopod's cytochrome oxidase-1 (CO1) gene was amplified via PCR (Saiki *et al.* 1988) using primers HCO2198 and LCO1490 (Folmer *et al.* 1994) and then sequenced using methods described in Barber *et al.* (2002). For each collection locale, DNA from the microbial symbiont communities attached to individual isopods was extracted using the xanthogenate protocol described by Tillett & Neilan (2000) and then amplified via PCR with primers (SANhop and SAChop; J. Collier, personal communication) for the gamma subunit of the cyanobacterial DNA-dependent RNA polymerase complex (rpoC1). This gene is found in cyanobacteria, but is not present in other eubacteria (Bergsland & Haselkorn 1991). This gene has shown great utility in phylogenetic-based identification of cyanobacteria with greater specificity than 16S (Palenik 1994; Ferris & Palenik 1998) and was targeted for this analysis because, although the TEM studies indicate that episymbiont biomass is dominated by relatively large, unicellular cyanophytes, they are surrounded by a great abundance of small (less than 2 μm) cells. Given this abundance inequity and the general PCR bias favouring enhanced detection of the more abundant microbes within a mixed community (von Wintzingerode *et al.* 1997), we restricted our initial molecular characterization of the symbiont communities to cyanobacteria. PCR products were cloned using the Invitrogen TOPO TA Cloning kit. Positive clones were identified via PCR using primers T7 and M13R, and up to 22 positive clones per individual were sequenced. All sequencing was done on an ABI 377 using BigDye chemistry (Applied Biosystems). Forward and reverse sequences were combined and edited in Sequencher (GeneCodes). Alignments were undertaken via ClustalW (Thompson *et al.* 1994) and adjusted by eye.

All phylogenetic analyses were undertaken using PAUP* 4.0 (Swofford 2002). Isopod CO1 parsimony analysis was performed on DNA sequence data with unordered, equally weighted characters, random stepwise addition and tree-bisection-reconnection (TBR) branch swapping; the robustness of clades was evaluated through 10 000 bootstrap replicates. Outgroup sequences from *Cirolana rugicau*, *Crenoicus buntia* and *Apanthura* sp. were obtained from GenBank.

To investigate the relationship among amplified microbial strains, a neighbour-joining tree was created using mean character distance to identify unique groups of symbiont sequences. Representative DNA sequences from all populations represented in each of these groups were then used in parsimony and bootstrapping (1000 replicates) analyses as described above, with the out-group selected from the results of the amino acid based parsimony analysis described below. Phylogenetic patterns of *Synechococcus* sequences were compared with the isopods through a Shimodaira–Hasegawa test to check for phylogenetic congruence characteristic of co-evolution and vertical transmission (Shimodaira & Hasegawa 1999). This test was performed using likelihood parameters optimized in ModelTest (Posada & Crandall 1998), where regional structures of isopod populations or monophyly of red and brown symbionts were imposed as a topological constraint in symbiont phylogenetic analyses.

To identify cyanobacterial symbionts, phylogenetic analyses on symbiont and comparative rpoC1 sequences from GenBank were performed on amino acid sequence data as translated by MacClade (Maddison & Maddison 1992) with redundant sequences merged. Parsimony search strategies were identical to the above, but gaps were treated as missing data and only 1000 bootstrap replicates were performed.

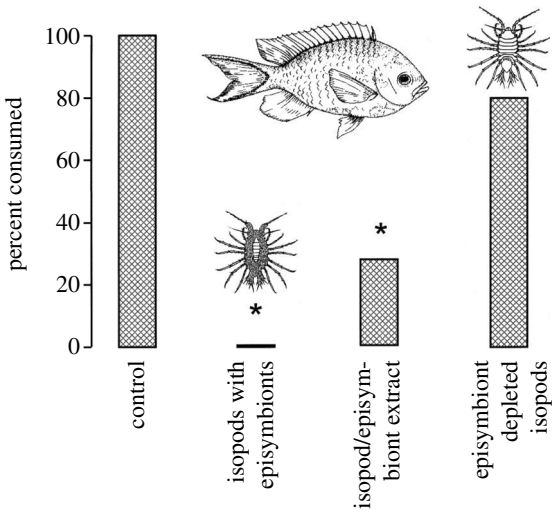

Figure 3. Results of *in situ* feeding assays testing the palatability of: (i) red isopods with their episymbiotic microbial community, (ii) episymbiont-depleted red isopods and (iii) a crude extract of the red isopod/microbial association. A single control bar is shown for comparison with each assay result to simplify the graphical presentation of the data because consumption of control pellets was 100% for all assays, $n=10$ replicates per assay. $p<0.000\,1$ for (i) and $p=0.003\,1$ and 0.47 (Fisher's exact test) for (ii) and (iii), respectively.

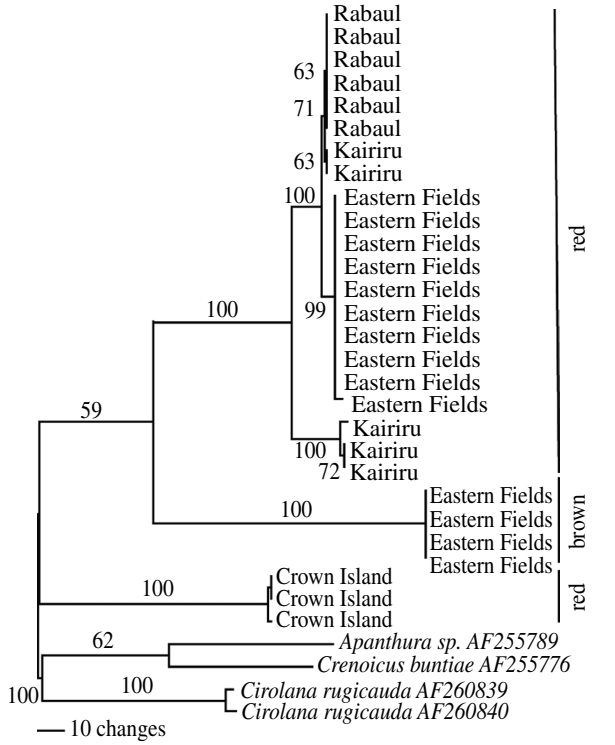

Figure 4. Phylogram representing the most parsimonious tree (522 steps) obtained from 563 bp (246 parsimony informative) of mitochondrial cytochrome oxidase c (subunit 1) data using a heuristic search via simple stepwise addition, characters unweighted and unordered, gaps treated as missing and TBR branch-swapping. Bold numbers represent bootstrap values obtained from 10 000 bootstrap replicates. Sequences were obtained from 28 isopods representing five populations from four localities.

3. RESULTS

Microscopic examination of the red isopods under white and epifluorescent lighting revealed a dense carpet of unicellular microalgae covering the isopods (figure 2*a*,*b*), which produced the isopod–microbial association's fluorescent coloration. HPLC analyses of the association's acetone extract identified chlorophyll a (chlorophyll b, chlorophyll c and divinyl chlorophyll were not found), β,β-carotene, zeaxanthin and astaxanthin as the major pigments. This pigment profile is diagnostic for both prokaryotic cyanophytes (i.e. cyanobacteria) and eukaryotic rhodophytes (i.e. red algae; Jeffrey & Vest 1997), with the exception of astaxanthin which is commonly found in crustaceans (Kleppel *et al.* 1988). TEM micrographs (figure 2*c*,*d*) revealed the presence of thylakoid membranes at the periphery of the large cells, which were up to 30 μm in diameter, and no membrane bound nucleus or organelles were seen, indicating that they are prokaryotes. The absence of divinyl chlorophylls further excludes prochlorophytes as members of the isopods' photosymbionts, thus they must be cyanobacteria. In addition to these large cyanobacteria, TEM micrographs showed high densities of small (less than 2 μm), morphologically diverse cells surrounding the large cyanobacterial cells.

In each of the feeding assays, the number of control food pellets offered matched the number of isopods or extract-treated pellets offered to schools of fishes and these control pellets were always eaten, indicating fishes were actively feeding during trials. When freshly collected, red isopods were offered to schools of reef fishes *in situ* at both Kairiru and Eastern Fields, each isopod was attacked yet all were expelled ($n=10$ at each site, $p<0.000\,1$; figure 3). Only four isopods were recovered after being attacked and rejected because of the difficulty of recapturing rejected isopods as they moved with the currents while sinking towards the substrate—all four of these isopods were alive 24 h after the feeding assay, as were four unattacked isopods that served as the survival controls. The six isopods that drifted into cracks and crevices on the coral heads before they could be recovered appeared to be intact after their rejection by fishes. In contrast to the complete rejection of freshly collected isopods, all of the 10 episymbiont-depleted red isopods offered to fishes at Eastern Fields were attacked and eight were consumed (100% of control pellets consumed, $n=10$, $p=0.47$). Visually, the episymbiont-depleted isopods had only a sparse covering of large algal cells along the margins of their carapace in contrast to the full body coverage observed on freshly collected isopods (figure 2*a*). Addition of an ecologically relevant amount of the red isopod-episymbiont crude extract to the squid-based food reduced fishes feeding by 70% ($n=10$, $p=0.0031$; figure 3). Although time constraints prevented testing the palatability of more than five brown coloured isopods, fishes readily consumed all five brown isopods and control pellets offered.

In total, 28 sequences of isopod CO1 from four localities revealed nine unique 563 bp haplotypes (GenBank accession numbers: AY857824–AY857831). Phylogenetic analysis of 246 parsimony informative characters recovered one most parsimonious tree of 522 steps (figure 4). Red isopods from Crown Island were monophyletic (bootstrap = 100%), as were those from Eastern Fields (bootstrap = 99%) and Rabaul (bootstrap = 63%). Kairiru isopods fell into two

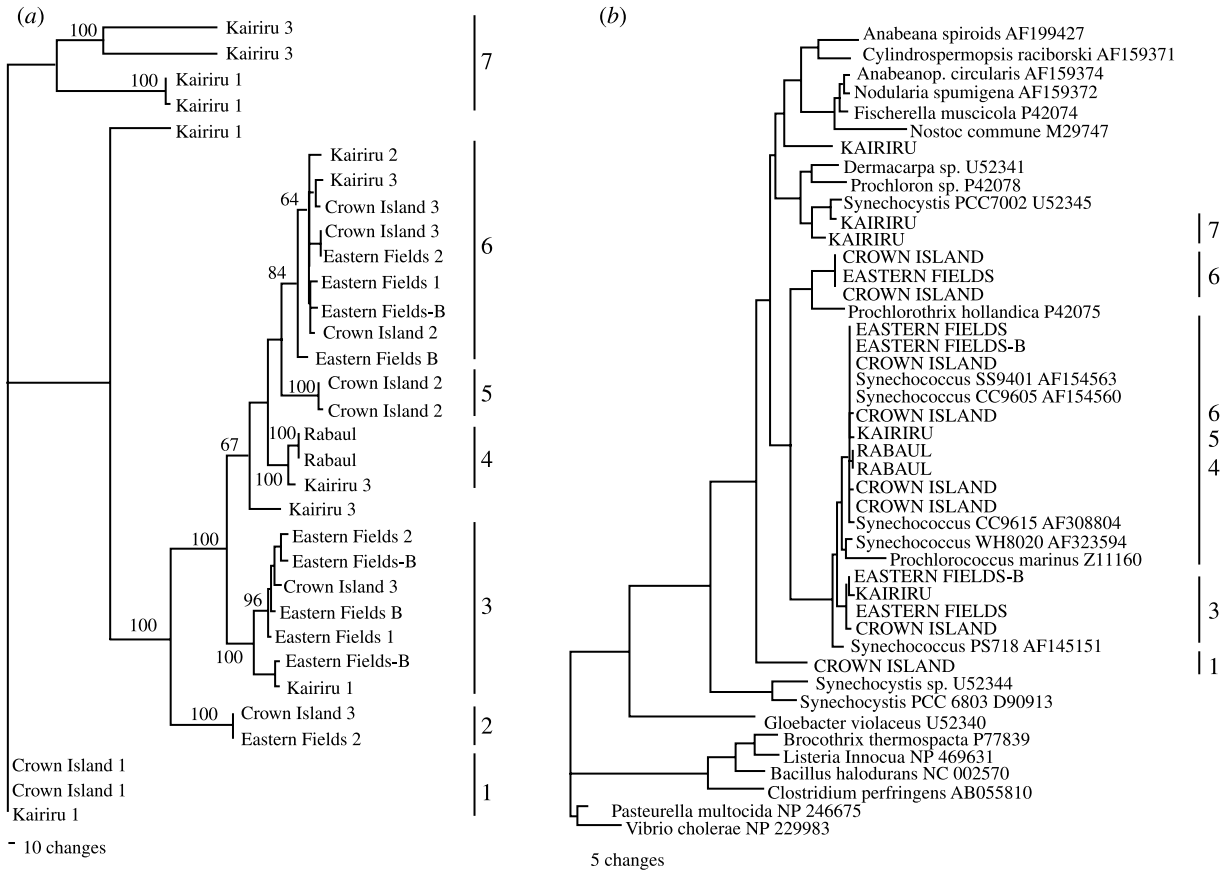

Figure 5. (*a*) One of two most parsimonious trees with 1175 steps obtained from selected 846 bp (380 parsimony informative) rpoC1 clones. (*b*) One of 95 most parsimonious trees with 581 steps obtained from 189 AA (116 parsimony informative) characters from the 846 bp rpoC1 sequence fragment. Numbers at nodes are bootstrap values obtained from 1000 replicates. Outgroup and comparison sequences for AA tree were obtained from GenBank.

monophyletic groups: one (bootstrap = 63%) was sister (bootstrap = 71%) to the Rabaul isopods and a second (bootstrap = 100%) was sister to a clade of Rabaul, Kairiru and Eastern Fields isopods (bootstrap = 100%). Brown isopods from Eastern Fields were monophyletic (bootstrap = 100%) and weakly grouped within the red isopods (bootstrap = 59%).

DNA sequences from rpoC1 (846 bp) from 117 clones, representing 10 individual isopods from four localities (Crown Island $n = 3$, Eastern Fields red $n = 2$, Eastern Fields brown $n = 1$, Kairiru $n = 3$, Rabaul $n = 1$), revealed 97 unique DNA sequences. Neighbour-joining analysis of rpoC1 DNA sequence data revealed seven distinct clades (analysis not shown). To simplify analyses, representative sequences (GenBank accession numbers: AY857832–AY857863) from each population within each of these seven clades were then subjected to parsimony analyses (figure 5*a*) yielding the two most parsimonious trees of 1175 steps from 380 parsimony informative characters. Although Crown Island and Kairiru had unique symbiont sequence variants, the two largest clades (3 and 6) comprised sequences from Crown Island, Eastern Fields and Kairiru (100 and 84% bootstrap support, respectively) indicating no regional differentiation among rpoC1-detected microbes. This result was confirmed by the Shimodaira–Hasegawa test (Shimodaira & Hasegawa 1999) as nine alternative phylogenies with imposed regional structure were significantly less probable than the most parsimonious trees (diff. −log likelihoods = 711.24 to 799.72, $p < 0.000\,1$). Furthermore, sequences from both palatable (brown) and unpalatable (red) isopods from Eastern Fields were included in these two broadly distributed clades and the Shimodaira–Hasegawa test rejected the monophyly of symbionts from unpalatable red and palatable brown isopods (diff. −log likelihoods = 711.23, $p < 0.000\,1$).

Of the 846 bp of rpoC1 DNA sequence data, only 189 AA characters (582 bp sequence data) could be unambiguously aligned with all comparative sequences from GenBank. Parsimony analysis from these 189 AA characters (116 parsimony informative) resulted in 95 of the most parsimonious trees (581 steps). The majority of the microbial sequences grouped strongly (bootstrap = 100%) in a clade containing *Synechococcus* and *Prochlorococcus* (figure 5*b*). Symbionts from both the unpalatable red and the palatable brown isopods were contained within this clade. Microalgal symbionts also grouped strongly with *Synechocystis* (99% bootstrap support) and weakly with *Prochlorothrix hollandica* (63% bootstrap support).

4. DISCUSSION

(a) *Characterization of the symbionts*

Results of the feeding assays demonstrated that the conspicuous red isopods with their associated microbial communities are unpalatable to reef fishes and that the microbial communities confer this unpalatability. Hundreds of structurally diverse, bioactive secondary metabolites are produced by marine bacteria and cyanobacteria

(Blunt *et al.* 2003) and several of these compounds have been shown to deter feeding by fishes (Pennings *et al.* 1997). Although some crustaceans reduce their risk of predation through parasitic, commensual or mutualistic associations with distasteful macro-organisms (Brooks 1988; Hay *et al.* 1989; McClintock & Janssen 1990; Stachowicz & Hay 1999), the isopod–microbial associations described herein are the first examples of symbioses between crustaceans and unpalatable micro-organisms and only the second example from the marine environment of a microbial symbiont producing an antipredator defence for its host. As the first example, Lopanik *et al.* (2004*b*) established bryostatins as a larval defence produced by a bacterial symbiont of the bryozoan *B. neritina*.

Previous studies of sulphur-oxidizing micro-organisms living on taxonomically diverse invertebrate hosts have shown that episymbiont communities may be dominated by one phylotype (Polz *et al.* 2000) or be composed of a great diversity of micro-organisms, some of which are also found in the surrounding environment (Cary *et al.* 1997). TEM data showed that the bulk of the episymbiotic biomass on the red isopods comprised relatively large spherical cyanobacteria. Analysis indicated that 92 of 117 rpoC1 clones grouped strongly with *Synechococcus*. Taken at face value, this result combined with phylogenetic incongruence between host and symbionts, and the inability to differentiate regional strains or strains from red and brown isopods, would suggest that the symbionts are environmentally acquired and that the defensive chemistry is facultative. However, the morphology of the large cells is inconsistent with the identification of *Synechococcus* as these are typically smaller rod-shaped cells (Komarek & Anagnostidis 2000), similar to some smaller abundant cells around the larger cyanobacteria seen in the TEM. Furthermore, *Synechococcus* are not reported to produce unusual secondary metabolites (Blunt & Munro 2003) and therefore are unlikely to be the source of the isopod–microbial associations' chemical defence. Combined, these results suggest that PCR bias may have favoured the amplification of numerically abundant *Synechococcus* that are part of the episymbiotic microbial community, but that the large cyanobacterial cells identified on the TEM are unlikely to be *Synechococcus*. Further evidence against the large episymbionts being *Synechococcus* comes from the comparison of host and symbiont phylogenies. Because isopods have direct development (i.e. their offspring emerge from female brood pouches as miniature versions of the adults), ample opportunity should exist for vertical transmission of symbionts, a characteristic thought to have a stabilizing effect on symbiotic associations (Herre *et al.* 1999). As would be predicted based on their brooding life history, data from CO1 demonstrated that isopod populations were highly geographically differentiated with depths of divergence similar to distinct isopod species (figure 4). However, rpoC1 data failed to differentiate cyanobacterial symbionts among these populations as would be expected if the host and symbionts were tightly coevolving (e.g. Nishiguchi *et al.* 1998). Furthermore, rpoC1 data also failed to distinguish unpalatable red and palatable brown isopods. These results combined suggest that the molecular data are probably skewed towards the numerically dominant small cells within the episymbiotic communities and that these *Synechococcus* (which are abundant in seawater) are environmentally acquired.

Several other cyanophyte sequences were identified that grouped with *Synechocystis* and *Prochlorothrix* or had no obvious affinities. *Synechocystis* tend to be large and spherical (Komarek & Anagnostidis 2000) and some marine *Synechocystis* are reported to produce bioactive secondary metabolites (Nagle & Gerwick 1995) suggesting that the large cyanophyte identified in the TEM may be a *Synechocystis*. Although at present there are no data with which to accurately identify the large cyanobacterial cells, work in progress seeks to link detected cyanobacterial rpoC1 sequences with specific episymbiotic morphotypes through fluorescent *in situ* hybridization (FISH) techniques.

In contrast to the environmentally derived *Synechococcus*, the large cyanobacterial cells are probably transmitted vertically from mother to offspring. This hypothesis is supported by our observations of newly emerged juveniles immediately climbing on to the mother and inoculating themselves with her episymbionts. Further, TEM data show distinct differences in the morphology of the large cyanobacteria on the unpalatable red isopods (spherical cells; figure 2*b*,*c*) and the palatable brown isopods (ovoid cells; figure 2*d*), which is consistent with strain differentiation and vertical transmission. Although the results of the depleted symbiont and methanol extract feeding trials indicate that it is reasonable to assume that the large cyanobacterial cells are the source of the red isopod's unpalatability and that the palatable brown isopods carry a strain that does not produce deterrent secondary metabolites, present data cannot eliminate the possibility that bacteria within the communities produce the isopod–microbial associations' chemical defence (e.g. Lopanik 2004*a*,*b*) or the possibility that the production of the chemical defence may be an induced characteristic of the same microbial community.

(b) *Ecological considerations of the symbiosis*

Theoretically, symbioses arise because the fitness of each partner increases above that experienced as separate, free-living organisms (Douglas 1994). The chemical protection conferred by the microbial symbionts allows the isopods to be active during daylight hours, potentially providing increased opportunities to forage and decreasing interactions with competitors. Additionally, the isopods were observed consuming their symbionts, which they scrape from their backs, antennae and posterior appendages using their legs (note the bare patch on the isopod in figure 2*a*). By positioning themselves on exposed substrates, the isopods maximize their sun exposure and thereby probably optimize symbiont growth. Thus, these isopods appear to farm their symbionts, a relationship with some similarities to leaf-cutter ants (*Atta* spp.) cultivating fungi for food (Mueller *et al.* 1998) and to shrimp, nematods and colonial ciliates that host episymbiotic sulphur-oxidizing bacteria and periodically move between sulphide-rich anoxic and oxic environments to enhance symbiont growth (Polz *et al.* 2000).

Efforts are underway to isolate and structurally characterize the deterrent chemistry of the isopod-microbial symbioses, as well as to determine the number of isopod species and strains of cyanobacteria and bacteria involved in these associations and to characterize the fidelity of the partnerships. Similar isopods have been

photographed in Micronesia (Colin & Arneson 1995) and we have observed similar isopod–episymbiont associations in the Caribbean and throughout Indonesia, suggesting that these or similar associations may be widespread.

In summary, the discovery of these unique episymbioses between marine isopods and microbial communities dominated by unicellular cyanobacteria provide an excellent example of how the merging of two very dissimilar groups of organisms, each with its own unique biosynthetic capabilities or behavioural traits, can provide ecological opportunities for the symbiotic association to radiate into previously uninhabitable niches. For the isopod and cyanobacterial symbioses, the hostile environment created by intense predation pressure is overcome by microbial production of a noxious compound, thus permitting the conspicuous isopods to carry their photosymbionts to sunlit habitats without the threat of being consumed by fishes. Although these isopods represent only the second marine example of microbial symbionts producing a chemical defence against host predators, the risk of predation is high in many marine habitats, suggesting that defensive symbioses may be more common in the marine environment than presently believed.

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