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Cryptic species, cryptic endosymbionts, and geographical variation in chemical defences in the bryozoan *Bugula neritina*

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Abstract

Molecular markers often offer the only means to discriminate between species and to elucidate the specificity of many community interactions, both of which are key to the understanding of ecological patterns. Western Atlantic populations of the bryozoan *Bugula neritina* vary in the palatability of their larvae to predators: individuals south of Cape Hatteras produce chemical deterrents to fish predators that are absent in more northern individuals. We use mitochondrial cytochrome oxidase *c* subunit I (COI) sequences to show that the differences in palatability between populations correlate with the geographical distributions of two cryptic species within *B. neritina*. Furthermore, these cryptic species differ in their associations with bacteria that may confer chemical resistance to predation. Small subunit rRNA primers specific to a subset of γ-proteobacteria amplified only the bacterium *Endobugula sertula* from the southern cryptic species. *Endobugula sertula* produces a family of chemical compounds (bryostatins) that may deter predators of its animal host. In contrast, the same primers amplified an array of γ-proteobacteria from the unprotected northern cryptic bryozoan species, but never *E. sertula*. In combination, these findings suggest that the geographical variation in palatability observed in the larvae of *B. neritina* is not the result of local adaptation of a single species to regions of differing predation pressure, but rather results from the comparison of cryptic species that differ in the presence or absence of a bacterium that may provide protection against predators. The ability to identify the cryptic *Bugula* species and their differing relationships with bacterial associates provides an example of the important role molecular techniques may play in addressing ecological questions.

Keywords: chemical defence, cryptic species, endosymbiotic bacteria, geographical variation, γ-proteobacteria, symbiosis

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Introduction

A prerequisite for studies of population and community ecology is the ability to identify species. For many organisms, this was problematic prior to the advent of molecular tools. Microbial ecology, for example, has been revolutionized by molecular markers that permit ecologists to discriminate between species that are morphologically indistinguishable. This has not only enabled species tallies that greatly boost estimates of community diversity (Giovannoni *et al*. 1990), but has also allowed inquiries into conventional ecological patterns, such as niche partitioning (Ferris & Palenik 1998) that were previously possible only in macroscopic species.

Molecular identification of species can also illuminate studies of macroscopic organisms in at least two ways. First, many nominal species turn out to be collections of morphologically indistinguishable, but reproductively isolated, cryptic species (Knowlton 1993; Perkins 2000; Shaw 2001). These cryptic species may interact with other species

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and with their environments in different ways (Haylor *et al*. 1984; Knowlton *et al*. 1992; Mokady & Brickner 2001), and by definition have independent population dynamics. Ecological differences that previously might have been attributed to local adaptation or intraspecific variation can be correctly identified as interspecific differences once cryptic species are recognized. Second, recent work has suggested that the response of many organisms to their biotic (Clay *et al*. 1985) and abiotic (Rowan *et al*. 1997; Schützendübel & Poole 2002) environment often hinges on variation in their endosymbionts. The detection and identification of these endosymbionts may be difficult by any other than genetic means. Characterization of the symbiotic associations between macroscopic organisms and micro-organisms may thus shed light on previously unexplained variations in how organisms respond to their environments.

The strength of predation in marine communities varies latitudinally, peaking in equatorial waters (Vermeij 1978). When the geographical range of a nominal species spans regions of differing predation pressure, selection should favour divergent defensive capabilities in different populations (Harvell *et al*. 1993). Along the Atlantic coast of North America, the Cape Hatteras region separates southern waters, where predation pressure is high, from comparatively low predation to the north (Vermeij 1978; Bertness *et al*. 1981; Lankford *et al*. 2001). Chemical defences in several marine animals are known to vary to either side of Cape Hatteras. Stachowicz & Hay (2000) found that the crab *Libinia dubia* decorated itself with seaweeds that chemically deter feeding by omnivorous fishes in southern portions of its range, but that individuals from northern populations, where omnivory is less intense, applied living camouflage solely based on its relative abundance. Sotka & Hay (2002) showed an analogous pattern of geographical variation in behaviourally mediated response to predation risk: southern populations of an amphipod species exhibited greater feeding preference for chemically defended seaweeds and tolerance for their chemical metabolites than did northern populations.

The bryozoan *Bugula neritina* is likewise geographically variable in its palatability to predators. Larvae (Lindquist & Hay 1996) and adults (Stachowicz & Hay 1999) of *B. neritina* from North Carolina are chemically defended and unpalatable to fish and crab predators, respectively. In contrast, the larvae of morphologically indistinguishable bryozoans from Delaware, also identified as *B. neritina*, are palatable to the same fish that avoid larvae from North Carolina (N. Lopanik and N. Lindquist, unpublished data). The larvae of *B. neritina* are large, conspicuous and nutrient rich (Wendt 2000). They are released during the day (Lindquist & Hay 1996), swim slowly (Wendt 2000), and spend little time in the plankton (Keough & Chernoff 1987; Wendt 2000). Lindquist & Hay (1996) argued that such larvae may face especially strong selective pressure for defensive adaptations because of their association with the benthos where predation is great. Unlike the crustaceans mentioned above, larvae, particularly large nonfeeding larvae of taxa such as *B. neritina*, tend to defend themselves chemically via the internal production of secondary compounds (Lindquist *et al*. 1992; Lindquist & Hay 1996). Bryozoans have proven rich sources of novel organic compounds, several with potential pharmaceutical values (Pettit 1991), as have many other sessile marine invertebrates (including sponges, gorgonians and tunicates; Faulkner 2002). Interestingly, these compounds often appear to be produced not by the animal itself, but rather by bacterial endosymbionts (Lee *et al*. 2001). The larvae of *B. neritina* harbour such bacterial symbionts (Woollacott 1981; Haygood & Davidson 1997).

The secondary compounds of *B. neritina* that have received greatest attention are a family of pyran ring structures, termed bryostatins. Bryostatins appear to be clinically useful both in fighting some cancers and in promoting lymphocyte survival during therapy via action on the protein kinase C signal transduction pathway (see references in Davidson *et al*. 2001). Davidson *et al*. (2001) have confirmed previous suspicions (Anthoni *et al*. 1990) that bryostatins are produced by an endosymbiotic γ-proteobacteria, which they have identified genetically as *Endobugula sertula* (Davidson & Haygood 1999).

The array of bryostatins produced by *B. neritina* varies among populations (Pettit 1991). Davidson & Haygood (1999) surveyed genetic variation within *B. neritina* along its Pacific range in California. They found two cryptic species of *B. neritina* that harboured different strains of *E. sertula*. The different strains of symbionts produced different bryostatins. Davidson & Haygood (1999) suggested (but did not demonstrate) that the suite of bryostatins produced by each endosymbiont may determine the predators to which its animal host is susceptible. If so, or if endosymbionts produce defensive compounds other than bryostatins, then bryozoan hosts and their bacterial endosymbionts may co-evolve in response to geographical variation in predation pressure.

In this study, we examine genetic differentiation among populations of both *B. neritina* and its bacterial associates from its Atlantic range between the Gulf of Mexico and southern New England. Specifically, we ask (i) whether populations of *B. neritina* from north and south of Cape Hatteras, which differ in their palatability, belong to a single genetically mixed species or constitute genetically distinct cryptic species, and (ii) whether differently defended bryozoan populations harbour genetically distinct endosymbionts.

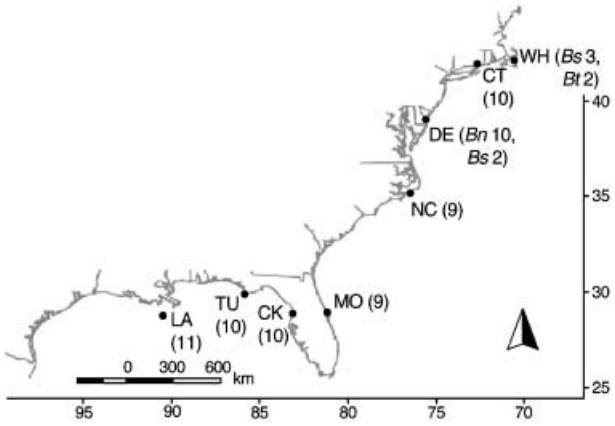

Fig. 1 Location of sampled populations with the number of sequenced colonies in parentheses. LA, South Terrebonne platform ST67H, LA (28°48′ N, 90°25′ W); TU, Turkey Point, FL (29°54′ N, 84°29′ W); CK, Cedar Key, FL (29°08′ N, 83°02′ W); MO, Mosquito Lagoon, FL (28°45′ N, 80°45′ W); NC, Beaufort, NC (32°25′ N, 80°40′ W); DE, Indian River, DE (38°35′ N, 75°17′ W); CT, Waterford, CT (41°20′ N, 72°08′ W); WH, Woods Hole Oceanographic Institute, MA (41°31′ N, 72°08′ W).

Materials and methods

Population sampling

Figure 1 shows the location of sampled populations and the number of individuals sampled from each. We sampled 9–11 colonies of *Bugula neritina* from five populations south of Cape Hatteras ('Southern' populations), including the population from Beaufort, North Carolina that was shown to be unpalatable to fish predators (N. Lopanik and N. Lindquist, unpublished data). We also sampled 10 individuals from each of two 'Northern' populations, including the Delaware site from which palatable animals were collected (N. Lopanik and N. Lindquist, unpublished data). In addition, we sampled two or three colonies of *B. stolonifera* from the same Delaware site where *B. neritina* was collected and from Woods Hole, Massachusetts (Marine Biological Laboratory Supply Center), and two colonies of *B. turrita* from Woods Hole. All samples were preserved in 95% ethanol and stored at −80 °C until processed.

Genetic variation within B. neritina

We extracted DNA (QIAGEN QIAamp extraction kit) from 20 to 30 healthy, unfouled zooids from the tips of each colony. We sampled only colonies for which the holdfast was still present to avoid multiple samplings of the same genetic individual. 1–2 μL of extracted DNA served as template in the 50 μL polymerase chain reactions (PCR) used to amplify a portion of cytochrome oxidase *c* subunit I (COI) with primers LCO1490 and HCO2198 (Folmer *et al.* 1994). Standard PCR profiles were used, with annealing temperatures of 46 °C (Connecticut and Delaware and outgroups) or 50 °C (all other populations). We pooled two to three amplifications per individual, purified the amplicon (Stratagene PCR purification kit), and cycle-sequenced using Big-Dye Terminators (ABI). The resulting products were sequenced on an ABI 377 Automated DNA Sequencer.

The 624 base pairs (bp) of the resulting COI sequence were aligned by eye; there were no indels or stop codons. COI sequences of both deep- and shallow-water forms of *B. neritina* (Davidson & Haygood 1999; GenBank accession numbers AF061422, AF061424, AF061425, AF061432) were included in the alignment. *Bugula stolonifera* and *B. turrita* were used as outgroups. Phylogenetic trees were constructed using the neighbour joining method in PAUP* 4.0b10 (Swofford 2001) with Kimura two-parameter distances (to account for the high transition bias in animal mitochondrial DNA). Maximum parsimony trees were generated using both equal and 8 : 1 weightings of transversions (tv) and transitions (ts). We used MODELTEST 3.06 (Posada & Crandall 1998) to determine that a TVM + Γ model was the most appropriate for maximum likelihood analysis of our COI data. Bayesian posterior probabilities were then generated using MRBAYES (Huelsenbeck & Ronquist 2001). These analyses were initiated with random trees and then run for 500 000 generations. Sampling (every 100 generations) began at generation 20 000 (having previously determined that parameters reached stationarity well before this point), so posterior probabilities were based on 4800 trees.

Genetic variation of bacteria associated with B. neritina

To identify the bacterial associates of the different bryozoans, we amplified and sequenced > 1000 bp of the bacterial gene encoding the small ribosomal subunit (SSU rRNA). SSU amplification was a two-step process. First, general bacterial primers 10f and 1390r (Table 1; Lane 1991) were used at an annealing temperature of 55 °C. The resulting amplicons from two or three reactions were pooled, purified, and then diluted to serve as a template in subsequent nested PCR. The next step employed *Endobugula sertula*-specific primers 240f and 1253r (Table 1; Haygood & Davidson 1997) and an annealing temperature of 60 °C. We then pooled and cleaned two or three amplification products per individual bryozoan colony. For some individuals, we were able to sequence this purified amplicon directly using the amplification primers and additional internal sequencing primers (Table 1). Amplicons that would not yield unambiguous direct sequences were cloned using the Zero Blunt TOPO PCR kit (Invitrogen). We obtained sequence from a minimum of three clones per individual bryozoan colony using 240f and 1253r, the cloning vector primers M13 forward and M13 reverse, and a combination of internal sequencing primers (Table 1).

Table 1 Primers used in this study

Primer	Sequence (5′–3′)
Bugula COI primers	
LCO1 (f)	GGT CAA CAA ATC ATA AAG ATA TTG G
HCO1 (r)*	TAA ACT TCA GGG TGA CCA AAA AAT CA
Bacterial SSU rRNA primers	
General	
10f†	GAG TTT GAT CCT GGC TCA G
1390r†	GGT GTG TRC AAG GCC C
Endobugula sertula	
240f‡	TGC TAT TTG ATG AGC CCG CGT T
1253r‡	CAT CGC TGC TTC GCA ACC C
Internal	
92f	ACT CCT ACG GGA GGC AGC AGT G
688f	GCA CAA GCG GTG GAG CAT G
927f	ACA AAC CGG AGG AAG GTG GG
126r	TGC AAT ATT CCC CAC TGC TGC CTC
514r	TCA GCG TCA GTA TCA GTC CAG
975r	CGT AAG GGC YAT GAT GAC TTG ACG

*Folmer *et al*. 1994.
†Lane 1991.
‡Haygood & Davidson 1997.

In the SSU alignment, we included all of the sequences we obtained, as well as the *E. sertula* sequences obtained by Haygood & Davidson (1997; GenBank accession numbers AF006606–AF006608) and the four most similar bacterial sequences identified using BLAST searches: *Oceanopirillum maris* (AB006763, Satomi *et al*. 1998), *O. multiglobuliferum* (AB006764; Satomi *et al*. 1998) and two uncultured γ-proteobacteria (AB015541; Li *et al*. 1999 and AF228694). The program ARB (http://www.arb-home.de) was used to position the indels; most sequences could be aligned unambiguously by eye. The two most distant of the sequences obtained from our BLAST search (the two *Oceanopirillum* spp.) were used as outgroups. Equally weighted unrooted neighbour-joining and parsimony trees were generated using PAUP*. We also generated a maximum likelihood tree using a TrN + I + Γ model and settings that were determined by using MODELTEST 3.06. Posterior probabilities were calculated as before using MRBAYES, except that tree sampling was begun at generation 200 000 (so that 3000 trees were used to compute consensus and posterior probabilities) because more generations were required to achieve stationarity.

Results

Cryptic species within Bugula neritina

COI sequences from *Bugula neritina* sampled from the Gulf of Mexico and from the Atlantic coast south of Cape Hatteras were identical (Figs 2A, 3A, GenBank accession number AY173425). These sequences were also identical to those of Davidson & Haygood (1999) from Beaufort, NC over the 618 bp where they aligned, thus falling within their Shallow clade of Californian *B. neritina*. There was a deep divergence (11.5%), however, between these Southern/Shallow form sequences and those obtained from *B. neritina* populations in Delaware and Connecticut. As with the Southern form, these Northern populations were both fixed for the same haplotype (GenBank accession number AY173426). This Northern Atlantic form of *B. neritina* differs not only from the Shallow/Southern form, but also from two other species of *Bugula* found north of Cape Hatteras, *B. stolonifera* (AY173427) and *B. turrita* (AY173428), and from the Pacific deep water form of *B. neritina* identified by Davidson & Haygood (1999).

Qualitatively similar trees were obtained using the parsimony (both ts : tv ratios) and neighbour-joining methods (Fig. 2A). These trees placed the Southern/Shallow and deep forms as sister taxa, with the Northern clade falling basal within a monophyletic *B. neritina*. Maximum likelihood trees differed topologically, placing the Northern form as the sister to the deep-water clade (Fig. 3A).

Variation in associated bacteria between the Northern and Southern forms of B. neritina

For the bacterial SSU rRNA sequences, all tree building methods produced trees that were topologically similar with regard to relationships among variants of *Endobugula sertula* and between this symbiont and other γ-proteobacteria (Figs 2B, 3B). All bacterial sequences obtained from the Southern populations of *B. neritina* are identical (GenBank accession numbers AY173429–AY173431). They differ by only 2 bp (0.2%) and 4–5 bp (0.5%) from SSU rRNA sequences reported for Haygood & Davidson's (1997) shallow- and deep-water *E. sertula*, respectively (Fig. 2B). In contrast, the bacterial sequences obtained from *Bugula* found north of Cape Hatteras differ by from 5% to 11% from the *Endobugula* sequences from the Southern populations and by 0% to 10% from each other (GenBank accession numbers: AY173432–AY173454). The bacterial sequences from the Northern *Bugula* show no apparent correlation with either geography or host phylogeny; bacteria from the three species and the six collecting sites (Woods Hole, Connecticut and Delaware) are completely interspersed (Fig. 2B). Whereas all Southern bryozoan colonies were associated with identical bacterial sequences, only two sequences associated with the Northern *B. neritina* and the other *Bugula* species were found more than once. Two of three clones from CT7 were found to have identical sequences, but this was the only case in which bacterial clones isolated from the same northern *Bugula* colony had identical sequences. The other instance in

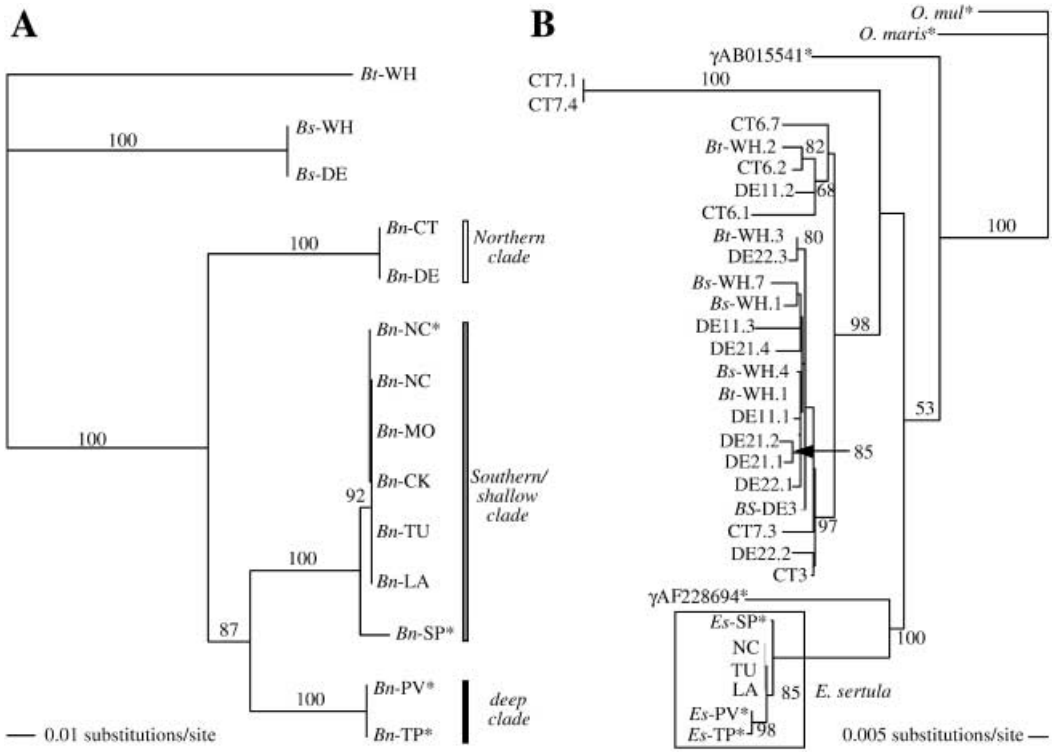

Fig. 2 Neighbour-joining trees for (A) *Bugula* species and (B) their associated bacteria. See Fig. 1 legend for locality abbreviations. Bootstrap values shown are based on 1000 replicates. In (A), the Northern, Southern/shallow, and deep clades of *B. neritina* (*Bn*) are indicated. In (B), individuals from which multiple clones were obtained are indicated, with the population and individual identification followed by the clone number after the periods. Only clones obtained from *B. stolonifera* (*Bs*) and *B. turrita* (*Bt*) are labelled with their species tags; all others were isolated from *B. neritina* colonies. Asterisks indicate sequences obtained from GenBank.

which we found identical sequences came from two different *Bugula* species (*B. stolonifera* and *B. turrita*) collected from two different sites (Delaware and Woods Hole). BLAST searches (13 August 2002) with the bacterial sequences from the northern *Bugula* bacterial isolates produced no matches closer than 94%.

Discussion

The reciprocal monophyly and high level of genetic divergence between the Southern and Northern populations of *Bugula neritina* (Fig. 2A) indicate that this nominal species is most probably a complex of cryptic species along the east coast of the United States, just as Davidson & Haygood (1999) found for *B. neritina* living along the west coast. We suggest that *B. neritina* as currently recognized in US waters comprises three cryptic species: the deep water Pacific form delineated by Davidson & Haygood (1999), a Shallow/Southern form present both in warmer waters along the Pacific coast and along the Atlantic coast south of Cape Hatteras, and a third form present in the Atlantic north of the Cape Hatteras region (our 'Northern' form), identified here for the first time (Fig. 2A).

Cape Hatteras has long been considered a biogeographical boundary (Briggs 1974; Burke *et al*. 2000), although its effectiveness varies among taxa (Engle & Summers 1999; Jones & Quattro 1999). Phylogeographic breaks also occur north of Cape Hatteras (reviewed in Wares 2002), including the region around Delaware that marks our southernmost sampling of the Northern form of *B. neritina*. Whatever the precise location of the break between the two Atlantic forms of *B. neritina*, the identification of two allopatric cryptic species means that differences in larval palatability between Northern and Southern *B. neritina* (N. Lopanik and N. Lindquist, unpublished data) do not result from intraspecific responses to geographical variation in predation pressure, as suggested for other species (Sotka *et al*. 1999; Stachowicz & Hay 2000; Sotka & Hay 2002.). Instead, our results indicate that the geographical differences in larval (and perhaps adult) palatability within *B. neritina* result from the presence of two cryptic and allopatric species, each with different defensive capabilities. Other instances of apparent intraspecific variation in morphology and behaviour have likewise been found to be the result of grouping together morphologically similar cryptic species (Knowlton *et al*. 1992; Rowan *et al*. 1997; reviewed in Shaw 2001). We cannot eliminate

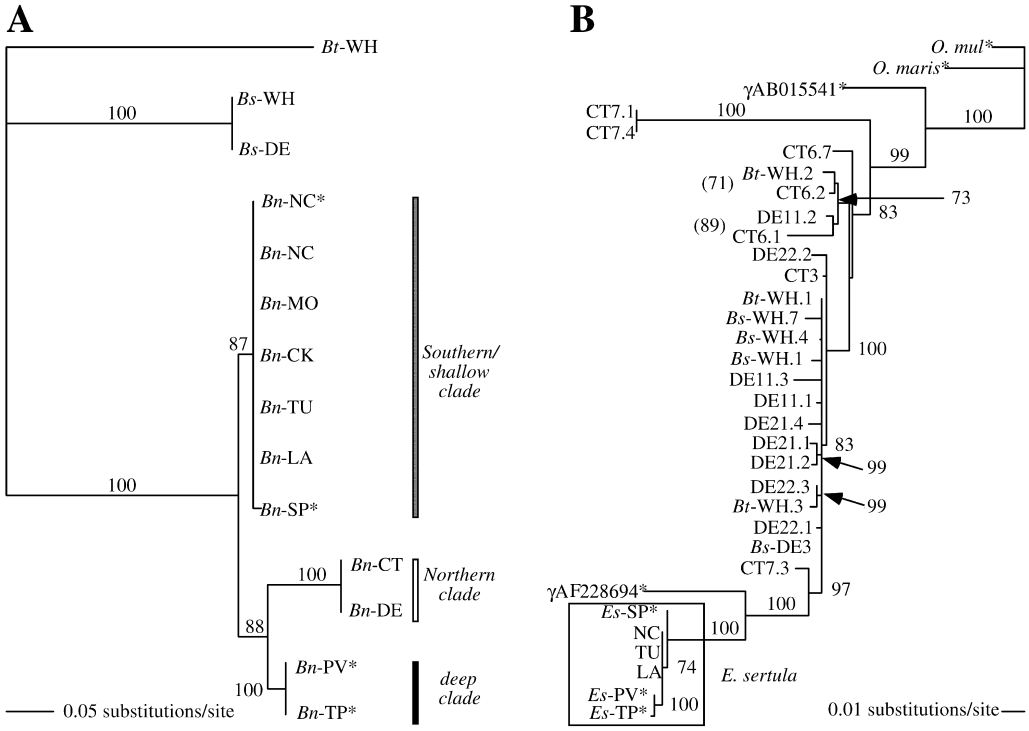

Fig. 3 Maximum likelihood trees with Bayesian posterior probabilities for (A) *Bugula* species and (B) their associated bacteria. In (B), individuals from which multiple clones were obtained are indicated with the population and individual identification followed by the clone number after the periods. Only clones obtained from *B. stolonifera* (*Bs*) and *B. turrita* (*Bt*) are labelled with their species tags; all others were isolated from *B. neritina* (*Bn*) colonies. Asterisks indicate sequences obtained from GenBank.

the possibility that past adaptation to differing predation pressures initiated the differentiation of the two cryptic species, but at present the evidence suggests that these are fully isolated species, and current local adaptation need not be invoked.

The Shallow/Southern species of *B. neritina* has a widespread distribution despite the low dispersal potential of larvae (Keough & Chernoff 1987; Wendt 2000), probably reflecting both anthropogenic transport (Carlton & Geller 1993) and this species' success as a fouling organism. The absence of intraspecific variation seen here is consistent with the possibility that both the Southern/Shallow and Northern *Bugula* species may have been introduced to the ranges studied here. In the face of this demonstrated colonization ability, the failure of the Southern/Shallow species to establish populations north of the Cape Hatteras area, and the failure of the Northern species to establish populations further south, suggests that ongoing ecological forces may act to restrict range expansion of both cryptic species. These factors may include different abiotic tolerances for the two bryozoan species, perhaps for temperature (Davidson & Haygood 1999). However, differences between the two species in their associations with bacteria may provide a better-supported explanation.

Our results demonstrate that the Northern and Southern species of *B. neritina* differ in their associations with γ-proteobacteria (Fig. 2B). The Southern *B. neritina* populations appear to have an endosymbiotic relationship with a single species of γ-proteobacteria: *Endobugula sertula*. Direct sequences of PCR amplifications were unambiguous, despite the ability of the primers used to amplify a wide range of γ-proteobacteria (in the first stage of the nested PCR), suggesting *E. sertula* was the only γ-proteobacteria within Southern *B. neritina* tissues. While differing by only 2 bp from sequences reported by Davidson & Haygood (1999) for the shallow (Type S) form of *E. sertula*, it is also closely related to the deep (Type D) form (notably, the two nucleotides at which sequences from Southern populations differ from the Type S *E. sertula* are identical to the Type D sequences). Chemical characterization of the suite of bryostatins present in Southern populations may shed more light on the phylogenetic affinity of their symbiont. In contrast, in the Northern populations there was no evidence of a close (or indeed any) symbiotic relationship between *B. neritina* and *E. sertula* or any other γ-proteobacteria. Northern populations were sampled within their reproductive time season (N. Lopanik, personal communication). Thus, even if only larval *Bugula* carry the endosymbiont, as perhaps suggested by the finding that larvae but not adults were distasteful to fish predators [Lindquist & Hay 1996; although note that Stachowicz & Hay (1999) found extracts from adult Southern *B. neritina* deterred

feeding by crabs], we should have been able to detect *E. sertula* in adults from these populations. Instead, we found no *E. sertula* sequences in any northern *Bugula*, despite performing PCR amplifications that employed *E. sertula*-specific primers (in the second stage of the nested PCR) and thus should have biased our efforts in favour of detecting this species.

The suite of bryostatins produced by *E. sertula* may determine the predators to which its bryozoan host is susceptible (Davidson & Haygood 1999; Davidson *et al*. 2001). Because the Northern form of *B. neritina* lacks *E. sertula*, their larvae should also lack both bryostatins and any defensive capabilities these compounds confer, capabilities that should be present in larvae of the Southern form. Consistent with this reasoning, the larvae of the Northern form of *B. neritina* are palatable to fish predators whereas the Southern form is unpalatable (N. Lopanik and N. Lindquist, unpublished data). Bryostatins were not specifically isolated and tested for their deterrent properties (N. Lopanik, personal communication), but even if the bryostatins are not the source of deterrence, the correlation of deterrence with the presence of *E. sertula* suggests a role for the *Bugula–Endobugula* symbiosis in the response to predators.

The diversity of bacterial sequences isolated from the two *Bugula* outgroups and the Northern form of *B. neritina*, both within populations and from individual colonies, suggests that the bacterial sequences are probably environmental samples rather than host-specific endosymbionts. The lack of any relationship with *E. sertula* or a closely related γ-proteobacteria in these three northern *Bugula* species may reflect a lack of access to suitable endosymbionts. Alternatively, the association between *B. neritina* and *Endobugula* may have arisen after the divergence of the Northern and Southern forms. The neighbour-joining tree (Fig. 2A) suggests that the Southern/shallow and deep forms of *B. neritina* are monophyletic. It may be that the common ancestor of these bryozoans developed an endosymbiotic relationship with the common ancestor of *E. sertula* (also monophyletic; Figs 2B, 3B) and that this relationship has co-evolved. The topology of the maximum likelihood (Fig. 3A), however, suggests that the two symbiont-bearing forms are paraphyletic. A greater sampling of both *Bugula* and bacterial genes may help resolve the branching pattern in both host and symbiont to address questions of co-evolution.

There may also be active selection maintaining the association between the Southern form of *B. neritina* and *E. sertula* that is not occurring in the northern species. *Bugula* in regions of high predation risk may derive great benefit from the chemicals produced by bacterial endosymbionts. In areas of low predation, there may be little selective pressure on bryozoans to enter into such a symbiotic association, despite the presence of γ-proteobacteria, which are found in symbiotic relationships with a variety of taxa (Douglas 1997; Krueger & Cavanaugh 1997; Spaulding & von Dohlen 1998; Rohwer *et al*. 2001; Von Dohlen *et al*. 2001). A wider sampling of *Bugula* species and the bacteria associated with them could shed further light on this issue.

The presence or absence of a symbiotic relationship between *Bugula* and *Endobugula* may mediate the geographical distributions of the cryptic bryozoan species studied here. Tolerance limits of the symbiont rather than the bryozoan host could prohibit the establishment of populations of the Southern species north of the Cape Hatteras region, particularly if the symbiotic relationship were obligate for *Bugula*. The southward expansion of the symbiont-free northern species of *Bugula* may be limited because its larvae are chemically undefended and more vulnerable to predators (N. Lopanik and N. Lindquist, unpublished data) that are more prevalent in the south (Vermeij 1978). Ecologists have become increasingly aware that geographical variation in species interactions may determine species distributions (Travis 1996; Rowan *et al*. 1997; Bertness *et al*. 1981), and the three-way interaction between predators, bryozoan larvae and bacterial symbionts may play a role in shaping the distribution of *B. neritina*.

In conclusion, genetic markers have allowed us to investigate the source of geographical variation in predator-resistance within Atlantic populations of the bryozoan *B. neritina* (N. Lopanik and N. Lindquist, unpublished data). Using mitochondrial COI sequences, we found that this geographical variation in chemical defence does not result from the local adaptation of a single widespread species to different predation regimes. Rather, the populations surveyed by Lopanik and Lindquist (unpublished data) belong to two genetically differentiated cryptic species: one northern, one southern. Using bacterial small ribosomal subunit sequences, we found that the southern bryozoan species is engaged in a symbiotic relationship with a bacterium (*Endobugula sertula*) known to produce compounds (bryostatins) that may serve in the host's chemical defence. The northern species showed no evidence of such an association. The ability to identify genetically both cryptic species of *Bugula neritina* and their bacterial associates has allowed us to reject a hypothesis of intraspecific geographical variation in chemical defences. Instead, the pattern of chemical defences, and possibly the geographical range of the undefended species, may be determined by species-specific patterns of endosymbiosis.

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