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## Mitochondrial evolution and phylogeography in the hydrozoan *Obelia geniculata* (Cnidaria)

Received: 27 February 2004 / Accepted: 17 June 2004 / Published online: 17 August 2004  
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**Abstract** The distribution and genetic structure of many marine invertebrates in the North Atlantic have been influenced by the Pleistocene glaciation, which caused local extinctions followed by recolonization in warmer periods. Mitochondrial DNA markers are typically used to reconstruct species histories. Here, two mitochondrial markers [16S rDNA and cytochrome *c* oxidase I (COI)] were used to study the evolution of the widely distributed hydrozoan *Obelia geniculata* (Linnaeus, 1758) from the North Atlantic and the Pacific and, more specifically, in the context of North Atlantic phylogeography. Samples were collected from six geographic localities between 1998 and 2002. Hydroids from the North Atlantic, North Pacific (Japan), and South Pacific (New Zealand) are reciprocally monophyletic and may represent cryptic species. Using portions of the 16S rDNA and COI genes and the date of the last trans-Arctic interchange (3.1–4.1 million years ago), the first calibrated rate of nucleotide substitutions in hydrozoans is presented. Whereas extremely low substitution rates have been reported in other cnidarians, mainly based on anthozoans, substitution rates in *O. geniculata* are comparable to other invertebrates. Despite a life history that ostensibly permits substantial dispersal, there is apparently considerable genetic differentiation in *O. geniculata*. Divergence estimates and the presence of unique haplotypes provide evidence for glacial refugia in

Iceland and New Brunswick, Canada. A population in Massachusetts, USA, appears to represent a relatively recent colonization event.

### Introduction

The rate of mitochondrial nucleotide substitution in Cnidaria, particularly in the Anthozoa, is thought to be considerably lower than in other marine invertebrates (Romano and Palumbi 1997; Medina et al. 1999; Van Oppen et al. 1999) and may be 10–20 times lower than in vertebrates (Shearer et al. 2002). Cnidarian mitochondrial evolution has been best studied in the Anthozoa, because there is a fossil record against which sequence divergence can be calibrated. Generalizations from these studies may not be applicable to other cnidarian clades. Analysis of the scyphozoan *Aurelia aurita* COI (cytochrome *c* oxidase I) suggests that the substitution rate may be higher in this group (Dawson and Jacobs 2001). The only previous study on a hydrozoan (Shearer et al. 2002) used a relative rate test on COI amino acid sequences, and found that the hydrozoan (*Limnomedusa*) *Maeotias* sp. was evolving significantly more slowly than echinoderms, molluscs, and arthropods.

A variety of mechanisms, such as selection, a recent bottleneck, introgression, and mismatch repair, could contribute to this unusually slow rate (Shearer et al. 2002). Particularly interesting is the possibility of a mismatch repair system. Mitochondrial gene content is highly conserved among metazoans, but some anthozoans (e.g. octocorals) possess a gene coding for a mismatch repair protein (MSH) that has not been found in any other metazoan mitochondrial genome (Pont-Kingdon et al. 1995, 1998; France and Hoover 2001). However, it is not known if and how this gene functions in mismatch repair in anthozoan mitochondria (Pont-Kingdon et al. 1998; Shearer et al. 2002).

Biogeographic events can sometimes provide firm upper and lower bounds for divergence when a fossil

Communicated by J.P. Grassle, New Brunswick

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record is not available (e.g. Cunningham et al. 1991; Knowlton and Weigt 1998; Wares and Cunningham 2001). Here, assuming migration through the trans-Arctic interchange (Vermeij 1991), we provide the first calibrated substitution rates for a hydrozoan, *Obelia geniculata*. We then use our calibrated substitution rates to interpret the timing of phylogeographic events for *O. geniculata* in the North Atlantic.

The hydrozoan *O. geniculata* (Leptomedusa: Campanulariidae) is very widely distributed (Cornelius 1975). It is found on both sides of the North and South Atlantic and Pacific Oceans, but is apparently absent or rare (or undescribed) in the northern Indian Ocean, the tropical western Atlantic, the Great Barrier Reef region, and the Southern Ocean (Cornelius 1975). The life cycle consists of three stages, in which the adult medusa releases either sperm or eggs, forming relatively short-lived, lecithotrophic planula larvae. The planulae settle on to a substrate and metamorphose into hydroids. Hydroids are colonial and asexually produce medusae, which are released into the plankton, completing the cycle. Additionally, *O. geniculata* hydroids can reproduce asexually by releasing propagules or "capsules" of tissue (Billard 1904; Berrill 1948; Panteleeva 1999). *O. geniculata* hydroids are found from the intertidal down to about 100 m depth, growing on a variety of substrates, especially brown algae and occasionally other invertebrates and fish (Cornelius 1995). This species has the potential for extensive dispersal through planktonic medusae and planulae, or through hydroids rafting on drifting seaweeds (Cornelius 1992).

The goals of this research were to: (1) examine genetic variation and phylogeography in *O. geniculata* using portions of the mitochondrial 16S rDNA and COI genes; (2) determine the substitution rates of 16S rDNA and COI by calibrating nucleotide substitutions to the opening of the Bering Strait, and compare these rates with those in other invertebrates; and (3) investigate phylogenetic patterns and divergence relative to the last glacial maximum in the North Atlantic.

## Materials and methods

### Sample collection and DNA sequencing

Hydroids of *Obelia geniculata* (Linnaeus, 1758) were collected, or obtained from colleagues, from the North Atlantic and Pacific (Table 1) and preserved in 95%

ethanol. Hydroids were identified by A. Govindarajan or P. Schuchert, and vouchers for most samples are available upon request to A. Govindarajan. Genomic DNA was extracted using the DNEasy kit (Qiagen). Portions of the mitochondrial 16S rDNA and COI were amplified under standard PCR (polymerase chain reaction) conditions using the primers of Cunningham and Buss (1993) and Folmer et al. (1994) (LCO1490 and HCO2198, respectively). PCR products were visualized on an agarose gel with ethidium bromide and purified with PCR purification kits (Qiagen). Purified products were cycle-sequenced with either Big Dye 2 or 3 sequencing chemistry (ABI) following the manufacturer's protocol, purified on a Sephadex column, and sequenced in both directions on an ABI 377. Sequences were aligned using Clustal X (Thompson et al. 1994) and confirmed by eye with MacClade (Maddison and Maddison 2000), although there was virtually no length variation in the sequences (only one indel). ModelTest (Posada and Crandall 1998) was used to determine the best-fit model for maximum-likelihood analyses (described below) conducted with PAUP\* 4.0b10 (Swofford 2000). For calculations of substitution rates and divergence estimates, model parameters under the best-fit model were estimated using maximum likelihood in PAUP\*.

### Phylogeny

Because the mitochondrial genome represents a single locus, the 16S rDNA and COI sequences were combined for each individual sequenced and considered a single mitochondrial haplotype. A maximum-likelihood phylogeny was generated from a heuristic search with TBR branch-swapping using the ModelTest parameters (corresponding to an HKY85+I+G model). The starting tree was obtained from the set of most-parsimonious trees found from a heuristic search (starting trees obtained via stepwise addition using ten random addition replicates). Support for the nodes was estimated by conducting a likelihood bootstrap analysis with 300 replicates (identical haplotypes excluded to save computational time).

### Substitution rates

Substitution rates were calibrated using PAUP\* 4.0b10 (Swofford 2000) by calculating the average length of the central internal branch of a likelihood phylogeny using

**Table 1** *Obelia geniculata*. Localities sampled for hydroids

Locality	Sample code	No. of samples	Latitude	Longitude	Sample date
St. Andrews, New Brunswick, Canada	NB	14	45°05'	67°03'	Jul 2002
Woods Hole, Massachusetts, USA	MA	9	41°32'	-70°40'	Oct 2001
Roscoff, France	FR	4	48°43'	-3°59'	Apr 1998
Garour/Sandgerdi, Iceland	IC	8	64°04' to 64°02'	-22°43' to -22°39'	May 2000
Misaki, Sagami Bay, Japan	JP	8	34°19'	135°09'	Sep 2002
Wellington, New Zealand	NZ	8	-41°18'	174°47'	Dec 2001

the best-fit model between Pacific and the North Atlantic (New Brunswick, Icelandic, and French) populations (to get gene, rather than population, divergence, Edwards and Beerli 2000). A likelihood ratio test (Huelsenbeck and Rannala 1997) was used to see if the hydroids were evolving in a clock-like fashion. Likelihood scores were calculated in PAUP\* using the best-fit model with and without the molecular clock constraint, and were used to generate a  $\chi^2$  test statistic with  $n-2$  degrees of freedom ( $n$ =number of taxa). Midpoint rooting was used when the molecular clock was enforced.

For comparison, we similarly calculated the substitution rate for 16S rDNA in another hydrozoan, the genus *Hydractinia* (Hydrozoa, Anthomedusa, Hydractiniidae) using previously published sequences (Cunningham and Buss 1993) and a dated biogeographic event (Cunningham et al. 1991; Young et al. 2002). This calculation had not been done previously.

### Phylogeography and North Atlantic population ages

Evidence for geographic subdivision was obtained by conducting an AMOVA between the three groups (North Atlantic, Japan, and New Zealand) and pairwise  $F_{ST}$  values using Arlequin 2.001 (Schneider et al. 2000). The presence of geographically restricted groups of haplotypes can be used to estimate minimum population ages. This is of interest since several of our populations were in regions thought to have been covered by glaciers during the last glacial maximum.

The age of a clade composed of an ancestral haplotype and its descendants can be estimated using the methods of Saillard et al. (2000). If these form a perfect star phylogeny, the age is easily estimated according to coalescent theory. The more these haplotypes depart from a star phylogeny, the wider the confidence limits.

First, the ancestral haplotype was identified by the method of Castelleo and Templeton (1994), which estimates outgroup weights based on haplotype frequency and connectivity. The haplotype with the highest outgroup weight is most likely the oldest. Following Saillard et al. (2000), the divergence estimate  $\rho$  is the average number of links in terms of observed substitutions between the observed haplotypes from the ancestral haplotype such that:

$$\rho = (n_1 l_1 + n_2 l_2 + \dots + n_m l_m) / n \quad (1)$$

where  $n$  is number of individuals with a given haplotype,  $l$  is number of steps (links) of a given haplotype to the ancestral haplotype, and  $m$  is the number of haplotypes. The variance  $\sigma$  is described by:

$$\sigma^2 = (n_1^2 l_1 + n_2^2 l_2 + \dots + n_m^2 l_m) / n^2 \quad (2)$$

The star index  $\rho/n\sigma^2$  (Torrioni et al. 1998; Saillard et al. 2000), where a value of one equals a perfectly

starlike phylogeny, was calculated. As described above, the more starlike, the smaller the confidence intervals. Since  $\rho$  is expressed in terms of number of observed substitutions, a per-locus rate of substitution is necessary. This is obtained by multiplying the substitution rate times the number of positions in the combined 16S rDNA and COI data.

## Results

### Sample collection and DNA sequencing

A total of 51 *Obelia geniculata* hydroids were collected and sequenced from New Zealand, Japan, and four locations in the North Atlantic (Massachusetts, New Brunswick, Iceland, and France). A total of 440 base pairs (bp) of 16S rDNA and 575 bp of COI were sequenced for all taxa (GenBank accession numbers AY530328–AY530429). The dataset consisted of 1,015 characters, 941 of which were constant, 21 of which were variable but parsimony-uninformative, and 52 of which were parsimony-informative. The alignments were deposited in the EMBL-Align database (accession numbers ALIGN\_000710 and ALIGN\_000711 for 16S and COI, respectively).

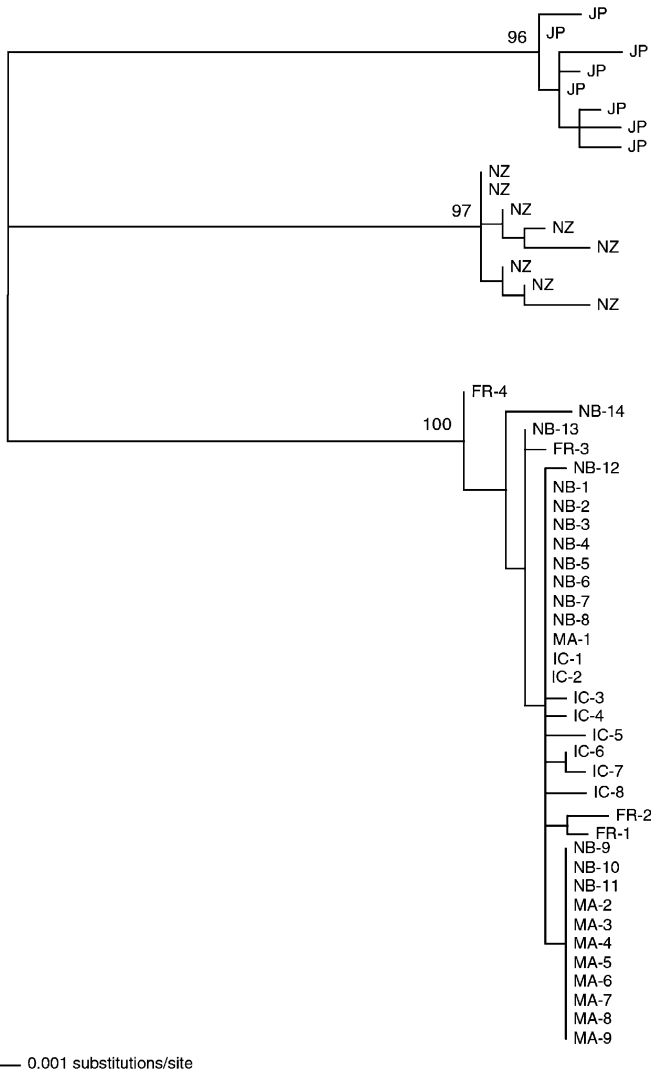
### Phylogeny

A total of 31 mitochondrial haplotypes were found. All Japanese individuals, and all but one New Zealand individual, were unique. The likelihood topology indicated three strongly supported (96–100%), reciprocally monophyletic clades, separated by long branches: one for the North Atlantic, one for Japan, and one for New Zealand (Fig. 1). The arrangement of the three clades with respect to each other is unresolved.

### 16S rDNA and COI substitution rates

Minimum substitution rates were calculated by comparing divergence between North Atlantic and Japanese populations. Three ages for the trans-Arctic interchange were used: the minimum and maximum estimate for the initial opening of the Bering Strait (3.1 and 4.1 million years ago) (Marincovich and Gladenkov 2001) and 3.5 million years ago (Vermeij 1991). Because these dates represent the initial opening, the resulting substitution rate estimate is the minimum possible rate.

To obtain divergence estimates, a maximum-likelihood phylogeny was constructed using the best-fit model. The length of the internal branch between populations estimates the actual age of divergence between populations, and serves to correct for poly-



**Fig. 1** *Obelia geniculata*. Maximum-likelihood topology. Numbers indicate parsimony bootstrap values (300 replicates). Only bootstrap values for the three major clades are shown. Five nodes within these clades had bootstrap values of 50–71, and the rest were < 50. Sample names, see Table 1

morphism in the ancestral population (Edwards and Beerli 2000). The best-fit models for each data partition (16S rDNA, 16S rDNA + COI, COI, COI third codon positions) and their estimated rates are presented in Table 2. The molecular clock assumption was tested, but was not rejected ( $-\ln L_{\text{clock}} = 1,946.40809$ ,  $-\ln L_{\text{no clock}} = 1,913.82546$ ,  $2[-\ln L_{\text{clock}} - (-\ln L_{\text{no clock}})] = 65.16526$ ,  $df = 49$ , critical value at 0.05 significance = 66.339).

Comparison of mitochondrial substitution rates to other invertebrates

The minimum substitution rates for 16S rDNA, COI, and COI third codon positions were higher than those for anthozoans, and within the range of other marine

**Table 2** *Obelia geniculata*. Range of divergence rates based on minimum (3.1 million years ago, mya), maximum (4.1 mya), and commonly used (3.5 mya) estimates for the opening of the Bering Strait

Gene	Substitution rate (site <sup>-1</sup> year <sup>-1</sup> ) ( $\times 10^{-9}$ )			Best-fit model used in rate calculation
	3.1 mya	3.5 mya	4.1 mya	
16S	2.76	2.44	2.08	HKY
16S + COI	6.13	5.43	4.63	HKY + G
COI	7.38	6.54	5.58	HKY + G
COI third codon	22.00	19.48	16.63	HKY

invertebrates (Tables 2, 3). The 16S rDNA rate of *O. geniculata* was comparable to our estimated rate, based on published 16S rDNA sequences, of the hydrozoan *Hydractinia* spp. (Cunningham and Buss 1993). The maximum date of divergence for Atlantic and Gulf of Mexico populations of the hermit crab host of *Hydractinia* spp. is ~4.1 million years (Cunningham et al. 1991; Young et al. 2002). The divergence between Atlantic and Gulf of Mexico *Hydractinia* spp., using an HKY model is 0.01027, which yields a minimum substitution rate of  $1.25 \times 10^{-9}$  substitutions site<sup>-1</sup> year<sup>-1</sup> for *Hydractinia* 16S rDNA. This is lower than the rate for *O. geniculata*, but both rates were considerably higher than published estimates for scleractinian corals (Romano and Palumbi 1997; Table 3).

### Phylogeography

Within the North Atlantic, there appeared to be an ancestral haplotype. As expected (Castelloe and Templeton 1994), the ancestral haplotype was deeply nested and was shared by Massachusetts (MA), New Brunswick (NB), and Iceland (IC) populations (Fig. 2).

The AMOVA indicated that most (92.67%) of the variation corresponded to the three major clades found in the phylogeny (North Atlantic, JP, and NZ); 1.22% of the variation came from between localities within the three major groups (MA, NB, IC, FR, JP, NZ), and 6.11% of the variation came from within locations (Table 4). The pairwise  $F_{ST}$  values indicated significant, although in some cases small, genetic differentiation: only one pair (NB–IC) was not statistically different ( $P > 0.05$ , Table 5).

### Estimates of population age

Both the Icelandic and New Brunswick populations had a number of haplotypes not shared by other populations. In contrast, the Massachusetts sample was entirely composed of haplotypes shared with New Brunswick, consistent with recent colonization from the north (Hewitt 2000).

**Table 3** *Obelia geniculata*. Substitution rates of 16S rDNA and COI (all positions) compared with those from other cnidarians (*O. geniculata* in bold). Substitution rate for cytochrome *b* from an anthozoan is included for comparison, although this gene was not sequenced in *O. geniculata*. *O. geniculata* COI third codon position substitution rate is compared with other invertebrates calculated by Wares and Cunningham (2001) with the F84 model. Even if the

unlikely, earlier (5.5 million years ago, *mya*) opening (which was not accompanied by a large faunal migration) is considered, *O. geniculata* rates would be lower, but still higher than anthozoans (16S rDNA:  $1.55 \times 10^{-9}$  substitutions site<sup>-1</sup> year<sup>-1</sup>; COI:  $4.16 \times 10^{-9}$  substitutions site<sup>-1</sup> year<sup>-1</sup>; COI third codon positions:  $12.40 \times 10^{-9}$  substitutions site<sup>-1</sup> year<sup>-1</sup>)

Gene	Phylum	Species	Substitution rate (site <sup>-1</sup> year <sup>-1</sup> ) ( $\times 10^{-9}$ )	Calibration reference	Source
<b>16S</b>	<b>Cnidaria (Hydrozoa)</b>	<b><i>O. geniculata</i></b>	<b>2.44</b>	<b>Bering Strait (3.5 mya)</b>	<b>Present study</b>
16S	Cnidaria (Hydrozoa)	<i>Hydractiniaspp.</i>	1.25	Florida (4.1 mya)	Cunningham et al. (1991); Young et al. (2002)
16S	Cnidaria (Anthozoa)	Scleractinian corals	0.1–0.6	Fossil record	Romano and Palumbi (1997)
<b>COI</b>	<b>Cnidaria (Hydrozoa)</b>	<b><i>O. geniculata</i></b>	<b>6.54</b>	<b>Bering Strait (3.5 mya)</b>	<b>Present study</b>
COI	Cnidaria (Anthozoa)	<i>Montastraea spp.</i>	0.5	Fossil record	Medina et al. (1999)
Cyt <i>b</i>	Cnidaria (Anthozoa)	<i>Acropora spp.</i>	0.5–0.9	Fossil record	Van Oppen et al. (1999)
<b>COI third codon</b>	<b>Cnidaria (Hydrozoa)</b>	<b><i>O. geniculata</i></b>	<b>19.5</b>	<b>Bering Strait (3.5 mya)</b>	<b>Present study</b>
COI third codon	Arthropoda (Malacostraca)	<i>Alpheus spp.</i>	19	Isthmus of Panama	Knowlton and Weigt (1998)
COI third codon	Arthropoda (Malacostraca)	<i>Sesarma spp.</i>	21	Isthmus of Panama	Schubart et al. (1998)
COI third codon	Mollusca (Gastropoda)	<i>Littorina obtusata</i>	24	Bering Strait (3.5 mya)	Wares and Cunningham (2001)
COI third codon	Arthropoda (Maxillopoda)	<i>Semibalanus balanoides</i>	27.6	Bering Strait (3.5 mya)	Wares and Cunningham (2001)
COI third codon	Arthropoda (Malacostraca)	<i>Idotea balthica</i>	36	Bering Strait (3.5 mya)	Wares and Cunningham (2001)
COI third codon	Arthropoda (Maxillopoda)	<i>Euraphia spp.</i>	38	Isthmus of Panama	Wares (2001)
COI third codon	Mollusca (Gastropoda)	<i>Nucella lapillus</i>	44.3	Bering Strait (3.5 mya)	Wares and Cunningham (2001)
COI third codon	Echinoderma (Asteroidea)	<i>Asterias rubens</i>	48.4	Bering Strait (3.5 mya)	Wares and Cunningham (2001)
COI third codon	Mollusca (Bivalvia)	<i>Mytilus edulis</i>	95.1	Bering Strait (3.5 mya)	Wares and Cunningham (2001)

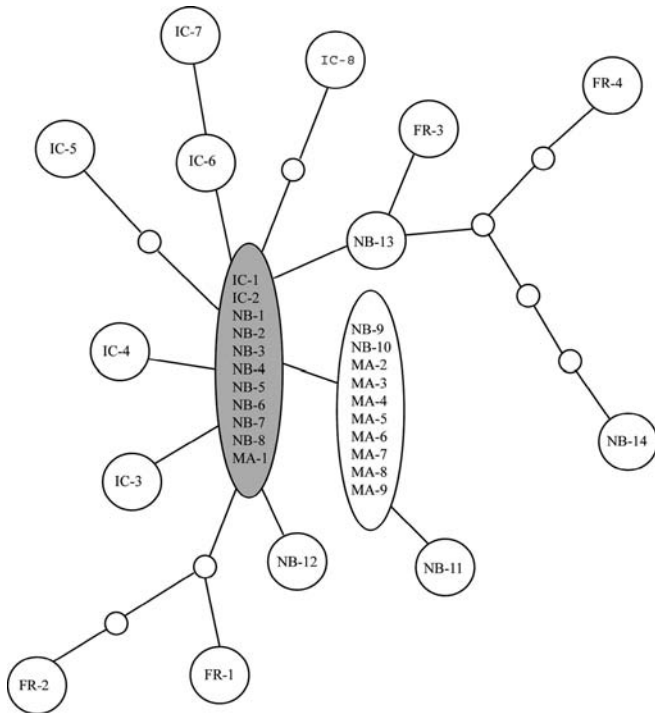
If we consider the ancestral haplotype in the North Atlantic and its descendants restricted only to a particular population, we can estimate the minimum age of that population using the method of Saillard et al. (2000). For New Brunswick the estimated age,  $143 \pm 47$  thousand years ago, predates the last glacial maximum (20 thousand years ago). Including the Massachusetts individuals does not change this result ( $150 \pm 82$  thousand years ago). The Icelandic population appears even older ( $204 \pm 68$  thousand years ago).

To investigate the possibility that the trans-Arctic migration was more recent, thus moving the divergence dates to be more recent than the last glacial maximum, the effects of trans-Arctic migration date on divergence time from the ancestral haplotype and on the corresponding COI third codon position substitution rate were modeled for the New Brunswick samples (Fig. 3). The more recent the date of trans-Arctic migration, the more recent the divergence from the ancestral haplotype and the higher the substitution rate. Trans-Arctic migration dates at or after  $\sim 750,000$  years ago yielded

divergence estimates at or after the last glacial maximum. However, the corresponding substitution rates were exceedingly high. For a trans-Arctic migration date of 750,000 years ago, the COI third codon substitution rate was  $90.93 \times 10^{-9}$  substitutions site<sup>-1</sup> year<sup>-1</sup>, and increased rapidly as trans-Arctic migration dates became more recent.

## Discussion

We found three reciprocally monophyletic clades of *Obelia geniculata* in New Zealand, Japan, and the North Atlantic. The New Zealand and Japanese populations, each collected from a single location, appear to have more haplotype diversity than the four North Atlantic sampling localities combined. This is consistent with a more recent origin for the North Atlantic population, perhaps via an invasion from the Pacific during the trans-Arctic interchange that followed the opening of the Bering Strait 3.1–4.1 million years ago. However,



**Fig. 2** *Obelia geniculata*. Parsimony network for North Atlantic samples. A heuristic search yielded six most parsimonious trees, all with 23 steps; the tree identical to the likelihood topology (Fig. 1) is represented here. The ancestral haplotype is shaded. Sample names, see Table 1. Sample NB-11 is separated from the NB-MA haplotype by one step; because that change was a deletion, it was coded as missing data in the likelihood analyses and not indicated on Fig. 1

additional data are necessary to determine the arrangement of the clades with respect to each other.

Our substitution rate calibrations assume a trans-Arctic migration 3.1–4.1 million years ago, but do not depend on the direction of migration. An earlier opening 5.4–5.5 million years ago has been recently suggested (Marincovich and Gladenkov 1999; Gladenkov et al. 2002), but, unlike the later opening (Durham and MacNeil 1967; Briggs 1970; Vermeij 1991), it does not appear to have been accompanied by a large faunal migration and so was not considered here. Many species, including molluscs, echinoderms, and algae are thought to have been introduced to the North Atlantic from the North Pacific when the Bering Strait opened

**Table 4** *Obelia geniculata*. AMOVA results (groups the three major clades: North Atlantic, Japan, and New Zealand; populations the six populations: Massachusetts, New Brunswick, Iceland, France, Japan, and New Zealand)

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among groups	2	408.972	16.47575	92.67
Among populations within groups	3	8.650	0.21734	1.22
Within populations	45	48.907	1.08682	6.11
Total	50	466.529	17.77990	

**Table 5** *Obelia geniculata*. Pairwise  $F_{ST}$  values and associated  $P$ -values (in parentheses). Sample abbreviations, see Table 1

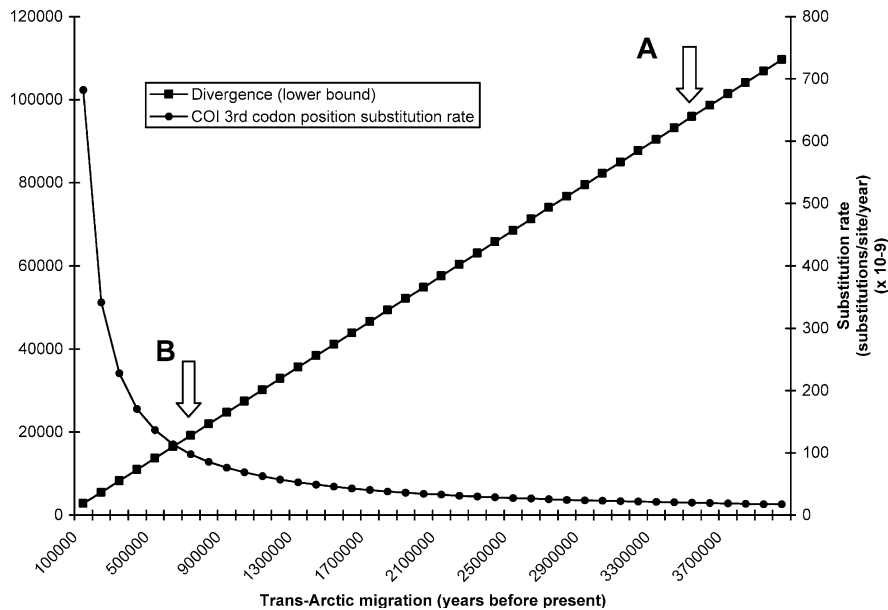
	MA	NB	IC	FR	JP
NB	0.29244 (0.00000)				
IC	0.42176 (0.00000)	0.03547 (0.13514)			
FR	0.49671 (0.00901)	0.19653 (0.01802)	0.14118 (0.03604)		
JP	0.95258 (0.00000)	0.94038 (0.0000)	0.92409 (0.00000)	0.89158 (0.00901)	
NZ	0.95557 (0.00000)	0.94050 (0.0000)	0.92468 (0.00000)	0.89326 (0.00000)	0.89655 (0.00000)

up around 3.5 million years ago (Durham and MacNeil 1967; Van den Hoek and Breeman 1990; Vermeij 1991; Cunningham and Collins 1998). Several species of the kelp *Laminaria*, a common substrate of *O. geniculata*, were among the invaders from the Pacific (Stam et al. 1988).

We used the opening of the Bering Strait to obtain a minimum estimate of the substitution rate for *O. geniculata*. For both the COI and 16S rDNA genes, this rate is about an order of magnitude faster than rates estimated for anthozoans (Table 3). The 16S rDNA rate for *O. geniculata* is also higher than a rate estimated from published sequences for the hydrozoan genus *Hydractinia* (Table 3).

The existence of three well-supported clades separated by long branches suggests that the North Atlantic, Japanese, and New Zealand populations have been separated for a long time. The three clades are reciprocally monophyletic, suggesting they represent different species according to the phylogenetic species concept (Avice 2000). Species distinctions in campanulariid hydroids, particularly in the genus *Obelia*, are controversial. Species-level taxonomy is based in large part on theca (the chitinous covering) morphology, but environmental influences on morphology (e.g. Ralph 1956) make identification difficult. In a revision of the genus *Obelia*, Cornelius (1975) synonymized about 120 species into 3. He later increased that number to 4 (Cornelius 1990). Unlike other *Obelia* species, his treatment of *O. geniculata* was not controversial (Cornelius 1990), and *O. geniculata* is considered easily recognizable by the asymmetrical thickening of the perisarc, or exoskeleton (although the degree of thickening is variable). Nevertheless, our results suggest that diversity may be underestimated in *O. geniculata*. Additional genetic studies will be useful in resolving species boundaries in the genus *Obelia*.

This study presents the first calibrated hydrozoan substitution rates. Because our rates are based on the maximum time of divergence, they represent minimum estimates. *O. geniculata* COI and 16S rDNA genes are evolving at rates similar to those of other invertebrates, countering the hypothesis that all cnidarian mitochondria evolve slowly. Thus, mitochondrial sequences



**Fig. 3** *Obelia geniculata*. Effect of trans-Arctic migration time on divergence of the New Brunswick samples (which diverged more recently than the Icelandic samples) from the ancestral haplotype and on the substitution rate of COI third codon positions (which can be compared to a variety of other invertebrates). To be conservative, the lower bound (mean  $-1$  SD) of the divergence estimate was used [*A* assuming migration at the initial opening, 3.5 million years ago, results in divergence well before the last glacial maximum and a substitution rate as described in the “Results” and Table 3; *B* assuming migration at or after 750,000 years ago results in divergence approximately at or after the last glacial maximum (20,552 years ago) and an exceptionally high substitution rate ( $90.93 \times 10^{-9}$  substitutions site $^{-1}$  year $^{-1}$ )]

should be a useful tool for studying hydrozoan phylogeography.

Anthozoans exhibit very low substitution rates in their mitochondrial sequences, even at COI third codon positions (which are under less selective pressure; Snell et al. 1998; Medina et al. 1999; France and Hoover 2002). In *O. geniculata*, the divergence rate for third codon positions fell within the range for other invertebrates (Wares and Cunningham 2001).

Noting that sponges appear to have a slow rate of mitochondrial evolution, Shearer et al. (2002) (citing R. Watkins, personal communication) suggest that mitochondrial evolution was slow in ancestral metazoans and accelerated near the origin of the Bilateria. They point out that if a higher mitochondrial evolutionary rate were found in cnidarian classes other than the Anthozoa, then that accelerated rate likely evolved at the origin of the Medusozoa, as anthozoans are thought to be the basal cnidarians (Bridge et al. 1992, 1995). Our results are consistent with this interpretation of a second independent origin of rapid mitochondrial evolution.

In addition to the trans-Arctic interchange, the Pleistocene glaciations are thought to have been a major force in shaping the history of the shallow-water flora and fauna in the North Atlantic by causing local extinctions, especially in the west (Ingolfsson 1992;

Cunningham and Collins 1998). Our results suggest that it may have been important for hydroids as well.

Although more comprehensive sampling is required, particularly in France, our divergence estimates, which predate the last glacial maximum, suggest that Icelandic and Canadian populations of *O. geniculata* may have survived in northern, glacial refugia. This conclusion is robust to the calibration of the molecular clock. In some cases, trans-Arctic invaders may have come more recently than after the initial opening of the Bering Strait (Palumbi and Kessing 1991; Van Oppen et al. 1995). If this were the case for *O. geniculata*, the calculated Icelandic and New Brunswick divergence date would be overestimated because the substitution rate estimate would be too low. However, this seems highly unlikely because the minimum substitution rate necessary to push the divergence dates to the last glacial maximum (requiring a maximum trans-Arctic migration date of  $\sim 750,000$  years ago) is considerably higher than the substitution rate in all other invertebrates compared except for *Mytilus edulis* ( $95.1 \times 10^{-9}$  substitutions site $^{-1}$  year $^{-1}$ ; Table 3). *M. edulis* is known to have an exceptionally high mitochondrial evolutionary rate, possibly due to its unusual form of mitochondrial inheritance (Hoeh et al. 1996). Thus, it seems most likely that *O. geniculata* experienced a trans-Arctic migration  $> 750,000$  years ago, supporting our original estimate of pre-glacial ages for *O. geniculata* populations in Iceland and the Canadian Maritimes.

The presence of unique haplotypes in *O. geniculata* from Iceland and New Brunswick also add to the mounting genetic evidence for glacial refugia in these areas (Holder et al. 1999; Dahlgren et al. 2000; Wares et al. 2002; Young et al. 2002). Geological evidence suggests that unglaciated pockets may have existed in northern Norway and Scotland (Dawson 1992; Siegert 2001) and in the Canadian Maritimes and on Georges Bank (Rogerson 1983; Pielou 1991; Holder et al. 1999).

These unglaciated pockets may have provided the refugia indicated by the genetic data.

Our results also suggest a recent southward range expansion by two haplotypes from New Brunswick into Massachusetts [the Woods Hole, Mass., sampling site is located on the southern shore of the biogeographic boundary of Cape Cod (Franz and Merrill 1980a, 1980b)]. As expected for a recent introduction, genetic diversity was lower than, and a subset of, the putative New Brunswick founding population (Hewitt 2000). Similarly, Dahlgren et al. (2000) suggested a southern range expansion for the bivalve *Arctica islandica* due to global climate change. This pattern is also seen in the gastropod *Littorina littorea*, a species believed for a long time to have been introduced into the western Atlantic from Europe, but which is now thought to be a recent southward range expansion from Canada (Wares et al. 2002). Such southward range expansions may be common in other invertebrate taxa as well.

Wares and Cunningham (2001) suggest that species capable of long-distance dispersal may have persisted through glacial periods because they could take advantage of widely scattered, ephemeral refugia (although the role of dispersal ability in colonization is complex, Cunningham and Collins 1998). The presence of a planktonic medusa stage in the life cycle of *O. geniculata* could provide a mechanism for substantial dispersal. Additionally, hydrozoans possessing a medusa stage may have relatively longer-lived planulae (Sommer 1992). In *O. geniculata*, several medusa generations are produced per year, and the medusae may live for several weeks (Kramp 1927). Planulae of *Obelia* spp. may live from 5 days to 3 weeks (Bodo and Bouillon 1968). Dispersal can also occur via the attached hydroid stage by rafting on algae (Cornelius 1992; Ingolfsson 1995). Hydroids living on *Laminaria* spp. live as long as the blades to which they are attached, which may be up to 15 months (Kramp 1927). Finally, dispersal may occur via asexual propagules (Billard 1904; Berrill 1948; Pan-televa 1999).

Nevertheless, there was significant genetic variation despite the dispersal potential of the planktonic medusae, planulae, propagules, and algal rafting. Not surprisingly, because of the large geographic distances between them, there were large differences between the North Atlantic, Japanese, and New Zealand specimens, indicative of cryptic speciation. However, within the North Atlantic, the lack of variation in the Massachusetts samples relative to the New Brunswick samples, and the presence of unique haplotypes in New Brunswick and Iceland, suggest that dispersal may also be restricted at relatively smaller scales. This is counterintuitive given the enormous dispersal potential of *O. geniculata*. Boero and Bouillon (1993) suggested that the presence of a medusa stage does not necessarily imply wide dispersal. In fact, they found that among Mediterranean hydrozoans, nominal species with apparently cosmopolitan distributions were less likely to have a medusa stage. Studies of other cnidarian medusae

also suggest that a planktonic lifestyle does not lead to genetic homogeneity and that there can be considerable genetic differentiation and even cryptic speciation, as in the scyphozoan *Aurelia aurita* (Dawson and Jacobs 2001). Oceanographic or behavioral barriers may be responsible for this restricted planktonic dispersal (Hamner et al. 1994). Additional research is necessary to determine the actual, as opposed to potential, dispersal of the several species presently attributed to *O. geniculata* and its role in surviving the Pleistocene glaciation.

**Acknowledgements** We are grateful to P. Schuchert, L.-A. Henry, Y. Hirano, and B. Grossman for providing specimens, and to F. Boero, L. Madin, J. Pineda, and T. Shank for helpful comments. This work was supported by an NSF PEET grant to C. Cunningham (DEB-9978131) and an Ocean Life Institute fellowship to K.M. Halanych. This is WHOI Contribution number 11181. The experiments comply with the current laws of the USA.

## References

- Avice JC (2000) Phylogeography. Harvard University Press, Cambridge
- Berrill NJ (1948) A new method of reproduction in *Obelia*. Biol Bull (Woods Hole) 95:94–99
- Billard A (1904) Contribution à l'étude des hydroids (multiplication, régénération, greffes, variations). Ann Sc Nat Zool 20:1–251
- Bodo F, Bouillon J (1968) Étude histologique du développement embryonnaire de quelques hydroméduses de Roscoff: *Phialidium hemisphaericum* (L.), *Obelia* sp. Péron et Lesueur, *Sarsia exima* (Allman), *Podocoryne carnea* (Sars), *Gonionemus vertens* Agassiz. Cah Biol Mar 9:69–104
- Boero F, Bouillon J (1993) Zoogeography and life cycle patterns of Mediterranean hydromedusae (Cnidaria). Biol J Linn Soc 48:239–266
- Bridge D, Cunningham CW, Schierwater B, DeSalle R, Buss LW (1992) Class-level relationships in the phylum Cnidaria: evidence from the mitochondrial genome structure. Proc Natl Acad Sci USA 89:8750–8753
- Bridge D, Cunningham CW, DeSalle R, Buss LW (1995) Class-level relationships in the phylum Cnidaria: molecular and morphological evidence. Mol Biol Evol 12:679–689
- Briggs JC (1970) A faunal history of the North Atlantic. Syst Zool 19:19–34
- Castelloe J, Templeton AR (1994) Root probabilities for intra-specific gene trees under neutral coalescent theory. Mol Phylogenet Evol 3:102–113
- Cornelius PFS (1975) The hydroid species of *Obelia* (Coelenterata, Hydrozoa, Campanulariidae), with notes on the medusa stage. Bull Br Mus Nat Hist (Zool Ser) 28:249–293
- Cornelius PFS (1990) European *Obelia* (Cnidaria, Hydrozoa): systematics and identification. J Nat Hist 24:535–578
- Cornelius PFS (1992) Medusa loss in leptolid Hydrozoa (Cnidaria), hydroid rafting, and abbreviated life-cycles among their remote-island faunas: an interim review. In: Bouillon J, Boero F, Cicogna F, Gili JM, Hughes RG (eds) Aspects of hydrozoan biology. Sci Mar 56:245–261
- Cornelius PFS (1995) North-west European thecate hydroids and their medusae, part 2. Sertulariidae to Campanulariidae. Synop Br Fauna New Ser 50:1–386
- Cunningham CW, Buss LW (1993) Molecular evidence for multiple episodes of paedomorphosis in the family Hydractiniidae. Biochem Syst Ecol 21:57–69
- Cunningham CW, Collins TM (1998) Beyond area relationships: extinction and recolonization in molecular marine biogeogra-

- phy. In: Schierwater B, Streit B, Wagner G, DeSalle R (eds) Molecular ecology and evolution: approaches and applications. Birkhauser, Basel, Switzerland, pp 297–321
- Cunningham CW, Buss LW, Anderson C (1991) Molecular and geologic evidence of shared history between hermit crabs and the symbiotic genus *Hydractinia*. *Evolution* 45:1301–1306
- Dahlgren TG, Weinberg JR, Halanych KM (2000) Phylogeography of the ocean quahog (*Arctica islandica*): influences of paleoclimate on genetic diversity and species range. *Mar Biol* 137:487–495
- Dawson AG (1992) Ice age earth: late quaternary geology and climate. Routledge, New York
- Dawson MN, Jacobs DK (2001) Molecular evidence for cryptic species of *Aurelia aurita* (Cnidaria, Scyphozoa). *Biol Bull (Woods Hole)* 200:92–96
- Durham JW, MacNeil FS (1967) Cenozoic migrations of marine invertebrates through the Bering Strait region. In: Hopkins DM (ed) The Bering land bridge. Stanford University Press, Stanford, Calif., USA, pp 326–349
- Edwards SV, Beerli P (2000) Perspective: gene divergence, population divergence, and the variance in coalescence time in phylogeographic studies. *Evolution* 54:1839–1854
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol* 3:294–299
- France SC, Hoover LL (2001) Analysis of variation in mitochondrial DNA sequences (ND3, ND4L, MSH) among Octocorallia (= Alcyonaria) (Cnidaria: Anthozoa). *Bull Biol Soc Wash* 10:110–118
- France SC, Hoover LL (2002) DNA sequences of the mitochondrial *COI* gene have low levels of divergence among deep-sea octocorals (Cnidaria: Anthozoa). *Hydrobiologia* 471:149–155
- Franz DR, Merrill AS (1980a) Molluscan distribution patterns on the continental shelf of the Middle Atlantic Bight (Northwest Atlantic). *Malacologia* 19:209–225
- Franz DR, Merrill AS (1980b) The origins and determinants of distribution of molluscan faunal groups on the shallow continental shelf of the northwest Atlantic. *Malacologia* 19:227–248
- Gladenkov AY, Oleinik AE, Marincovich Jr L, Barinov KB (2002) A refined age for the earliest opening of the Bering Strait. *Palaeogeogr Palaeoclimatol Palaeoecol* 183:321–328
- Hamner WM, Hamner PP, Strand SW (1994) Sun-compass migration by *Aurelia aurita* (Scyphozoa): population retention and reproduction in Saanich Inlet, British Columbia. *Mar Biol* 119:347–356
- Hewitt G (2000) The genetic legacy of the Quaternary ice ages. *Nature* 405:907–913
- Hoeh WR, Stewart DT, Sutherland BW, Zouros E (1996) Cytochrome *c* oxidase sequence comparisons suggest an unusually high rate of mitochondrial DNA evolution in *Mytilus* (Mollusca: Bivalvia). *Mol Biol Evol* 13:418–421
- Holder K, Montgomerie R, Friesen VL (1999) A test of the glacial refugium hypothesis using patterns of mitochondrial and nuclear DNA sequence variation in rock ptarmigan (*Lagopus mutus*). *Evolution* 53:1936–1950
- Huelsenbeck JP, Rannala B (1997) Phylogenetic methods come of age: testing hypotheses in an evolutionary context. *Science* 276:227–232
- Ingolfsson A (1992) The origin of the rocky shore fauna of Iceland and the Canadian Maritimes. *J Biogeogr* 19:705–712
- Ingolfsson A (1995) Floating clumps of seaweed around Iceland: natural microcosms and a means of dispersal for shore fauna. *Mar Biol* 122:13–21
- Knowlton N, Weigt LA (1998) New dates and new rates for divergence across the Isthmus of Panama. *Proc R Soc Lond B Biol Sci* 265:2257–2263
- Kramp PL (1927) The hydromedusae of Danish waters. *K Dan Vidensk Selsk Biol Skr* 8:1–291
- Maddison WP, Maddison DR (2000) MacClade: analysis of phylogeny and character evolution, ver. 4.0. Sinauer, Sunderland, Mass., USA
- Marincovich Jr L, Gladenkov AY (1999) Evidence for an early opening of the Bering Strait. *Nature* 397:149–151
- Marincovich Jr L, Gladenkov AY (2001) New evidence for the age of the Bering Strait. *Quat Sci Rev* 20:329–335
- Medina M, Weil E, Szmant AM (1999) Examination of the *Montastraea annularis* species complex (Cnidaria: Scleractinia) using ITS and COI sequences. *Mar Biotechnol* 1:89–97
- Palumbi SR, Kessing BD (1991) Population biology of the trans-Arctic exchange: mtDNA sequence similarity between Pacific and Atlantic sea urchins. *Evolution* 45:1790–1805
- Panteleeva NN (1999) *Obelia longissima* (Pallas, 1766) and *Obelia geniculata* (L., 1758) (Hydrozoa, Thecaphora, Campanulariidae) in the Barents Sea. Morphology, distribution, ecology and special life history features. *Zoosyst Rossica Suppl* 1:51–65
- Pielou EC (1991) After the ice age. University of Chicago Press, Chicago
- Pont-Kingdon GA, Okada NA, Macfarlane JL, Beagley CT, Wolstenholme DR, Cavalier-Smith T, Clark-Walker GD (1995) A coral mitochondrial *MutS* gene. *Nature* 375:109–111
- Pont-Kingdon G, Okada NA, Macfarlane JL, Beagley CT, Watkins-Sims CD, Cavalier-Smith T, Clark-Walker GD, Wolstenholme DR (1998) Mitochondrial DNA of the coral *Sarcophyton glaucum* contains a gene for a homologue of bacterial *MutS*: a possible case of gene transfer from the nucleus to the mitochondrion. *J Mol Evol* 46:419–431
- Posada D, Crandall KA (1998) ModelTest: testing the model of DNA substitution. *Bioinformatics* 14:817–818
- Ralph PM (1956) Variation in *Obelia geniculata* (Linnaeus, 1758) and *Silicularia bilabiata* (Coughtrey, 1875) (Hydrozoa, f. Campanulariidae). *Trans R Soc NZ* 84:279–296
- Rogerson RJ (1983) Geological evolution. In: South RG (ed) Biogeography and ecology of the island of Newfoundland. Junk, The Hague, The Netherlands
- Romano SL, Palumbi SR (1997) Molecular evolution of a portion of the mitochondrial 16S ribosomal gene region in scleractinian corals. *J Mol Evol* 45:397–411
- Saillard J, Forster P, Lynnerup N, Bandelt H, Norby S (2000) MtDNA variation among Greenland eskimos: the edge of the Beringian expansion. *Am J Hum Genet* 67:718–726
- Schneider S, Roessli D, Excoffier L (2000) Arlequin, ver. 2.000. A software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland
- Schubart CD, Diesel R, Hedges SB (1998) Rapid evolution to terrestrial life in Jamaican crabs. *Nature* 393:363–365
- Shearer TL, Van Oppen MJH, Romano SL, Worheide G (2002) Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). *Mol Ecol* 11:2475–2487
- Siegert MJ (2001) Ice sheets and Late Quaternary environmental change. Wiley, Chichester, England
- Snell TL, Foltz DW, Sammarco PW (1998) Variation in morphology vs conservation of a mitochondrial gene in *Montastraea cavernosa* (Cnidaria, Scleractinia). *Gulf Mexico Sci* 16:188–195
- Sommer C (1992) Larval biology and dispersal of *Eudendrium racemosum* (Hydrozoa, Eudendriidae). In: Bouillon J, Boero F, Cicogna F, Gili JM, Hughes RG (eds) Aspects of hydrozoan biology. *Sci Mar* 56:205–211
- Stam WT, Bot PVM, Boele-Bos SA, van Rooij JM, van den Hoek C (1988) Single-copy DNA–DNA hybridizations among five species of *Laminaria* (Phaeophyceae): phylogenetic and biogeographic implications. *Helgol Meeresunters* 42:251–267
- Swofford DL (2000) PAUP\* (phylogenetic analysis using parsimony). Sinauer, Sunderland, Mass., USA
- Thompson JD, Higgins DG, Gibson TJ (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. *Nucleic Acids Res* 22:4673–4680
- Torrioni A, Bandelt H, D'Urbano L, Lahermo P, Moral P, Sellitto D, Rengo C, Forster P, Savontaus M, Bonne-Tamir B, Scozzari

- R (1998) MtDNA analysis reveals a major late Paleolithic population expansion from southwestern to northeastern Europe. *Am J Hum Genet* 62:1137–1152
- Van den Hoek C, Breeman AM (1990) Seaweed biogeography of the North Atlantic: where are we now? In: Garbary DJ, South GR (eds) *Evolutionary biogeography of marine algae in the North Atlantic*. NATO ASI Ser G22, Springer, New York Berlin Heidelberg, pp 55–87
- Van Oppen MJH, Draisma SGA, Olsen JL, Stam WT (1995) Multiple trans-Arctic passages in the red alga *Phycodrys rubens*: evidence from nuclear rDNA ITS sequences. *Mar Biol* 123:179–188
- Van Oppen MJH, Willis BL, Miller DJ (1999) Atypically low rate of cytochrome *b* evolution in the scleractinian coral genus *Acropora*. *Proc R Soc Lond B Biol Sci* 266:179–183
- Vermeij G (1991) Anatomy of an invasion: the trans-Arctic interchange. *Paleobiology* 17:281–307
- Wares JP (2001) Patterns of speciation inferred from mitochondrial DNA in North American *Chthamalus* (Cirripedia: Balanomorpha: Chthamaloidea). *Mol Phylogenet Evol* 18:104–116
- Wares JP, Cunningham CW (2001) Phylogeography and historical ecology of the North Atlantic intertidal. *Evolution* 55:2455–2469
- Wares JP, Goldwater DS, Kong BY, Cunningham CW (2002) Refuting a controversial case of human-mediated marine species introduction. *Ecol Lett* 5:577–584
- Young AMC, Torres JE, Mack JE, Cunningham CW (2002) Morphological and genetic evidence for vicariance and refugium in the Atlantic and Gulf of Mexico populations of the hermit crab *Pagurus longicarpus*. *Mar Biol* 140:1059–1066