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Cryptic speciation along a bathymetric gradient

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The deep ocean supports a highly diverse and mostly endemic fauna, yet little is known about how or where new species form in this remote ecosystem. How speciation occurs is especially intriguing in the deep sea because few obvious barriers exist that would disrupt gene flow. Geographic and bathymetric patterns of genetic variation can provide key insights into how and where new species form. We quantified the population genetic structure of a protobranch bivalve, *Neilonella salicensis*, along a depth gradient (2200–3800 m) in the western North Atlantic using both nuclear (28S and calmodulin intron) and mitochondrial (cytochrome *c* oxidase subunit I) loci. A sharp genetic break occurred for each locus between populations above 2800 m and below 3200 m, defining two distinct clades with no nuclear or mitochondrial haplotypes shared between depth regimes. Bayesian phylogenetic analyses provided strong support for two clades, separated by depth, within *N. salicensis*. Although no morphological divergence was apparent, we suggest that the depth-related population genetic and phylogenetic divergence is indicative of a cryptic species. The frequent occurrence of various stages of divergence associated with species formation along bathymetric gradients suggests that depth, and the environmental gradients that attend changes in depth, probably play a fundamental role in the diversification of marine organisms, especially in deep water. *Biological Journal of the Linnean Society*, 2014, ••, ••–••.

ADDITIONAL KEYWORDS: cryptic species – deep sea – gene flow – phylogeography – population genetics – protobranch – speciation.

INTRODUCTION

The evolutionary processes that gave rise to the remarkably diverse fauna inhabiting the deep ocean are not well understood. Species formation requires the isolation of gene pools, but few obvious barriers exist in the deep sea that would impede gene flow and allow new species to form. In fact, many taxa appear to have broad bathymetric and geographic distributions, sometimes spanning entire oceans or even multiple oceans (e.g. France & Kocher, 1996; Allen & Sanders, 1996a; Herrera, Shank & Sánchez, 2012). Such enormous ranges suggest that populations are well connected via dispersal and that barriers to gene flow are rare. The high diversity, lack of obvious isolating barriers, and broad-scale distribution of many taxa raise intriguing questions about how and where new species form in this vast, remote, and complex ecosystem.

Molecular genetic analyses of deep-water taxa have begun to document geographic and bathymetric patterns of divergence that implicate several mechanisms potentially limiting gene flow and allowing populations to diverge. Population divergence has been associated with distance (France, 1994; Knutsen *et al*., 2012), depth (France & Kocher, 1996; Chase *et al*., 1998; Etter *et al*., 2005), topography (Iguchi, 2007; Etter *et al*., 2011), hydrographic features (Stepien, Dillon & Patterson, 2000; Roques, Sevigny & Bernatchez, 2002; Le Goff-Vitry, Pybus & Rogers, 2004), environmental heterogeneity (Etter *et al*., 2005), and vicariance (Kojima *et al*., 2001; Aboim *et al*., 2005; Stefani & Knutsen, 2007), and is often large enough to arguably reflect cryptic species. Cryptic species are quite common in marine environments (Knowlton, 1993), but may be more common in the deep sea where much less is known about the biology and natural history of the fauna and where morphology may be highly conserved. The presence of cryptic species suggests that geographic distributions may be greatly overestimated and biodiversity underestimated, which will have important implications for identifying the ecological forces that shape

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local and regional levels of diversity, understanding the evolutionary processes that promote diversification, and protecting the ecosystem properties essential for managing and preserving the deep-water fauna. This final concern is especially important now because the deep sea is experiencing increasing stresses from a wide variety of anthropogenic activities, including fisheries, energy extraction, and mineral mining (Ramirez-Llodra *et al*., 2011; Levin & Sibuet, 2012; Mengerink *et al*., 2014).

Although many species are thought to have broad bathymetric ranges, often exceeding 2500 m (e.g. bivalvia: *Cuspidaria atlantica* and *Poromya tornata* (Olabarria, 2005); echinoidea: *Paragonaster subtilis* and *Porcellanaster ceruleus* (Howell, Billett & Tyler, 2002); and polychaeta: *Nephtys sphaerocirrata* and *Ophelia profuna* (Cosson-Sarradin *et al*., 1998)), they are typically defined morphologically. The strong environmental changes that occur across such large depth ranges, however, are likely to engender population differentiation and possibly lead to the formation of new species. A number of environmental gradients attend changes in depth, including pressure, temperature, oxygen, nutrient flux, topographic complexity, environmental heterogeneity, and sediment characteristics (reviewed in Gage & Tyler, 1991). Each of these gradients, singly or in combination, has been invoked as a key force in regulating bathymetric distributions (Carney, 2005), altering ecological processes (Levin *et al*., 2001), shaping macroecological patterns (reviewed in Rex & Etter, 2010), fostering adaptation (e.g. Somero, 1992; Levin, 2003; Brown & Thatje, 2011), and promoting diversification (Etter *et al*., 2005). If these strong environmental gradients promote diversification, and deep-water taxa are limited in the range of biotic and abiotic conditions they can successfully tolerate, then many species that are defined morphologically and thought to have broad bathymetric ranges (e.g. > 2500 m) might instead be composed of complexes of cryptic species that have adapted to specific depth regimes. Quantifying bathymetric patterns of genetic variation and identifying cryptic species are critical for providing a better understanding of the abundance, distribution, and diversity of the deep-water fauna and the ecological and evolutionary processes that shape local and regional patterns of biodiversity.

Here we quantify bathymetric patterns of genetic variation for a common protobranch bivalve, *Neilonella salicensis* (Seguenza, 1877), in the western North Atlantic. This species is abundant throughout the Atlantic, ranging in depth from 508 m to 3800 m, but may occur deeper in the West European Basin (Allen & Sanders, 1996a, b). Protobranchs are the most basal bivalve group (Kocot *et al*., 2011; Smith *et al*., 2011; Sharma *et al*., 2012), are infaunal deposit feeders, and reach their greatest success in the deep sea (reviewed in Zardus, 2002). Most protobranchs, including *N. salicensis*, have a long, complicated taxonomic history of species synonymizations, splitting, and renaming, primarily based on morphological characters of the shell (e.g. height/length ratios, umbo position, and presence and shape of hinge teeth) and internal structures (e.g. size and shape of adductor muscles, gill plates, and positioning and shape of the gut) (reviewed in Warén, 1989; Allen & Sanders, 1996b). Although morphology forms the basis for most species-level identifications, recent genetic analyses of other deep-sea protobranch bivalves have frequently identified significant genetic divergence within putative morphological species, suggestive of cryptic species (Chase *et al*., 1998; Etter *et al*., 1999, 2005; Zardus *et al*., 2006; Jennings *et al*., 2013). In addition, the first comprehensive molecular phylogenetic analysis of the protobranch bivalves discovered considerable inconsistencies in the phylogenetic relationships within and among genera (Sharma *et al*., 2013), suggesting that even at these higher taxonomic levels, delineating evolutionary affinities may be challenging with traditional morphological features. Population genetic and phylogenetic analyses of *N. salicensis* within the North American Basin revealed strong genetic divergence among populations at different depths, likely indicative of a cryptic species and suggesting that the environmental gradients which attend changes in depth play an important role in population differentiation and speciation in the deep sea.

MATERIAL AND METHODS

Samples

Epibenthic sled samples were collected in 2008 from 1000 to 5200 m depth in the western North Atlantic along the Gayhead–Bermuda transect from south of Massachusetts to Bermuda (Fig. 1). Samples were sorted at 2 °C on board or were stored in chilled 95% ethanol to be sorted in the laboratory. Protobranch bivalves sorted on board were either flash-frozen at –80 °C or placed in 95% ethanol and stored at –20 °C to maintain the integrity of the DNA. *Neilonella* individuals were identified morphologically as *N. salicensis* or *Neilonella whoii* (Allen & Sanders, 1996b) based on characters described in Allen & Sanders (1996b) and were found in samples from 2200 to 3800 m.

Extraction, PCR Amplification and Sequence Processing

Genomic DNA was extracted from 50 whole individuals using the QiaAMP Mini Tissue kit (Qiagen, Valencia, CA, USA) and the standard protocol for tissues, with two elutions of 100 μL. PCR amplifica-

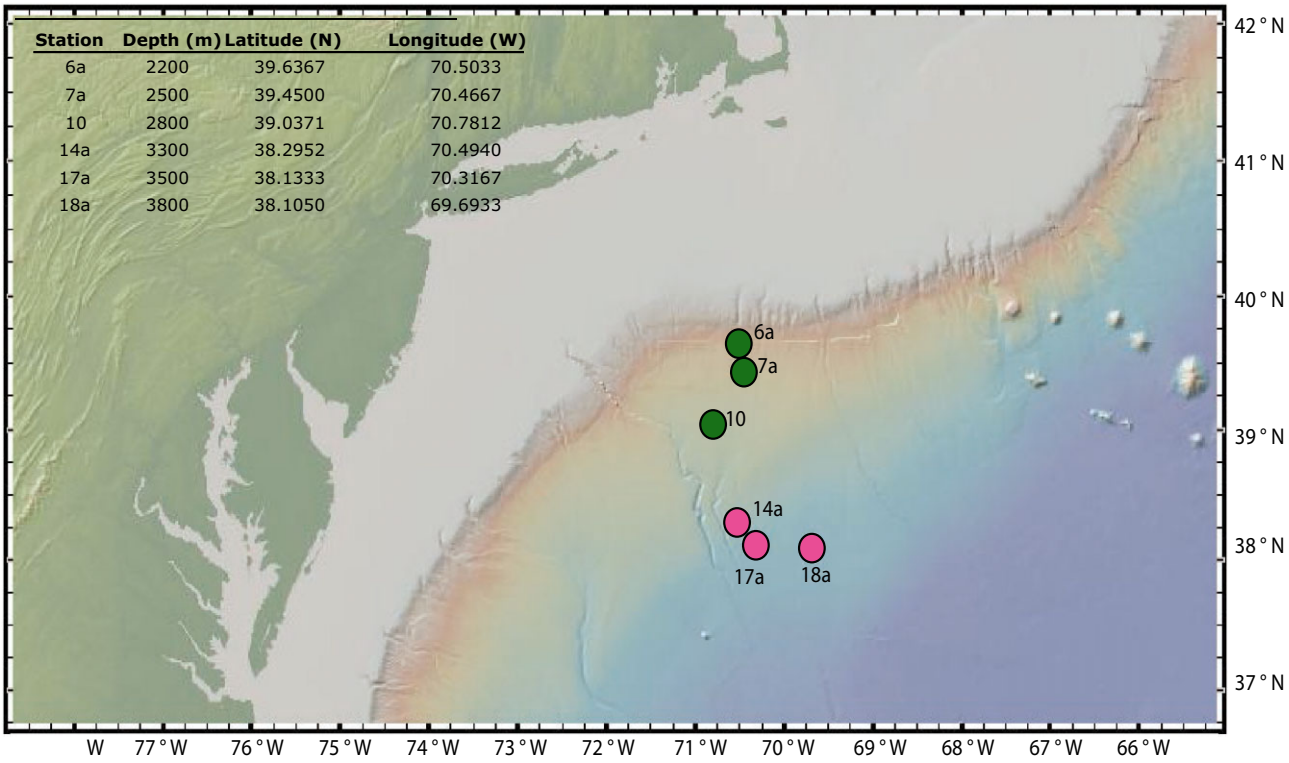

Figure 1. Map of station locations. Geographic location and depth of sampled stations. Bathymetry is coloured to represent depth. Stations are coloured by the clade identified in phylogenetic analyses: grey corresponds to shallower than 2800 m and white to deeper than 3200 m.

tion reactions (50 μL) consisting of 1× GoTaq flexi buffer (Promega, Madison, WI, USA), 1.2 pmol of each primer, 2 pmol of deoxyribonucleotide triphosphates (dNTPs), 1.25 mM bovine serum albumin (BSA), 2.5 mM MgCl, 1 U of Gotaq Flexi polymerase (Promega), and 2 μL of genomic DNA template were carried out for all loci. The mitochondrial cytochrome *c* oxidase subunit I gene (COI), nuclear calmodulin intron (CAL), and nuclear 28S rRNA gene were amplified and sequenced. The PCR profiles consisted of an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 45 s at the annealing temperature specific for the locus, and 72 °C for the extension time specific for the locus, with a final hold at 4 °C. Primers, annealing temperatures, and extension times are listed in Table 1. Amplification of COI consisted of two rounds, the preliminary round with the primers LCO1490 and HCO2198 and the secondary round with a nested forward primer, NSCOIF2, and HCO2198 as a result of poor initial amplification. Negative controls from the original round were also included to test for contamination. Twenty individuals were sequenced at the 28S rRNA gene, 10 from each of the two *N. salicensis* clades. Sequencing of COI and CAL were attempted for all *N. salicensis* individuals, but success varied. Two *N. whoii* individuals collected from station 18a at a depth of 3800 m were sequenced for 28S, COI and CAL and were used as an outgroup. All three loci in two individuals of the more distantly related protobranch, *Malletia johnsoni* Clarke 1961, were sequenced for use in phylogenetic analyses. These three loci were selected to span a range of evolutionary rates.

The PCR products were checked for the presence of single bands by gel electrophoresis and were outsourced to Agencourt (a Beckman-Coulter company, Beverly, MA, USA) for bidirectional sequencing. Raw chromatograms were provided to us. The forward and reverse sequences were edited and aligned using Sequencher v 5.0.1 (Gene Corp. Ann Arbor, MI, USA) and checked by eye to ensure correct base calling. Individuals heterozygous for CAL were detected with clear double peaks in the chromatogram. Alleles containing indels were resolved using the online program Indelligent (Dmitriev & Rakitov, 2008). Heterozygotes were phased using the Parent-Independent-Mutation (PIM) model and a threshold of 0.65 in PHASE v 2.1.1 (Stephens, Smith & Donnelly, 2001; Stephens & Donnelly, 2003). Each direction was resolved separately before realigning. Alignments of multiple

Table 1. Primer sequences and annealing temperatures

Locus	Primer	Sequence	Annealing Temperature (°C)	Elongation Time	Reference
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	48	1 min 30 s	Folmer *et al.* 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	48	1 min 15 s	Folmer *et al.* 1994
	NSCOIF2	GTCATCTTYTCDCTGTTGGCCT	45	1 min 30 s	This work
28S	28dd	GTCTTGAAACACGGACCAAGGAGTCT	60	2 min	Hillis and Dixon 1991
	28 mm	GAGCCAATCCTTWTCCCGAAGTTACGGATC	60	2 min	Hillis and Dixon 1991
Calmodulin	Cal1	GCCGAGCTGCARGAYATGATCAA	50.5	2 min	Duda and Palumbi 1999
	NESCALiR	GGACCAGCCCTGCAGTAGTC	50.5	2 min	This work

Primers, primer sequence, and corresponding annealing temperature for the loci analyzed are given. PCR profiles and reactions are described in the text.

individuals were created using ClustalX (Larkin *et al.*, 2007) in BioEdit and were visually checked in MacClade (Maddison & Maddison, 2005) to ensure accuracy of alignment.

Genetic Analyses

Arlequin v 3.5 (Excoffier & Lischer, 2010) was used to calculate basic diversity indices and to test for neutrality. The number of haplotypes, gene diversity, and nucleotide diversity were calculated for each locus. Neutrality was tested using both Tajima's D (tested at $P < 0.05$) and Fu's Fs (tested at $P < 0.02$). Indels in CAL were excluded from these analyses.

Phylogenetic relationships were inferred using BEAST v 1.7.4 (Drummond *et al.*, 2012) for each locus (COI, CAL, and 28S), individually and for all loci combined. Tracer was used to ensure sufficient burn-in and run time based on Effective Sample Size (ESS) estimations of at least 100. The COI tree was inferred using the SRD06 mutation model. The CAL tree and the combined three-locus tree were inferred using an HKY mutation model based on the AIC and BIC models selected in jMODELTEST (Guindon & Gascuel, 2003; Darriba *et al.*, 2012). A Yule speciation prior was enforced with all trees, and analyses were carried out with an uncorrelated lognormal clock and an MCMC chain of 3×10^7 steps, logging every 1000 trees and a starting UPGMA tree. Individuals were collapsed into haplotypes and alleles for the COI and CAL analyses, respectively. The tree with all three loci contained only the individuals for which all three loci were successfully sequenced. Tree models were linked to create one combined tree. Two *N. whoii* individuals were used as an outgroup in each tree analysis. These analyses were repeated using *M. johnsoni* as an outgroup to determine if topologies were robust.

A haplotype network was inferred for each locus using statistical parsimony in TCS v 1.21 (Clement, Posada & Crandall, 2000), treating gaps as a fifth state. The connection limit was increased until all haplotypes for each locus were incorporated into a single network for each of the two *N. salicensis* clades defined from our phylogenetic analyses.

Species Delimitation

Two species-delimitation methods were used to evaluate the probability that the node between the two *N. salicensis* clades was indicative of different species: a discovery method and a validation method (Carstens *et al.*, 2013). The heuristic search tool implemented in Brownie (O'Meara, 2010) was used in the discovery phase. This method takes ultrametric gene trees as input to estimate species assignments and relationships. Three gene trees were used as

input, a tree each for 28S, COI, and CAL. These trees contained only the individuals for which all loci were sequenced and were estimated using BEAST v 1.7.4 with parameters as described in the preceding section. The heuristic search was carried out in triplicate, with default settings changing only the minimum number of individuals per species to 2.

The validation approach used was BPP v 2.2 (Rannala & Yang, 2003; Yang & Rannala, 2010). BPP uses the multispecies coalescent with a guide tree in a Bayesian framework to estimate the posterior probability of trees with differing numbers of lineages (potential species delimitations). The analysis included all three loci and a guide tree that specified whether individuals were from the shallow or deep clades of *N. salicensis*, or from *N. whoii*. Only individuals sequenced for all three loci were included in the analysis. To assess convergence, three replicate runs were conducted with 250 000 steps and a burn-in of 25 000. This ensured ESSs of ≥ 1000 for all parameters. The validity of the delimitation between shallow and deep clades of *N. salicensis* was further tested by mixing shallow and deep individuals in the guide tree and running two variations (different individuals mixed among clades) three replicate times. To determine how robust the results were to the selection of priors, the fine-tune parameter of the species-delimitation algorithm 1 (2 and 20), and the gamma (α, β) distribution of theta and tau (mean = 0.0001, 0.025, and 0.01) were altered. Each parameter variation was run with both the correctly mapped individuals and the mixed model.

RESULTS

Identification and Amplification

A 405-base pair (bp) segment of COI and a 583-bp segment of CAL was successfully amplified from 41 and 29 individuals, respectively, identified as *N. salicensis*. A 672-bp segment of 28S was successfully amplified for 18 of the 20 *N. salicensis* individuals attempted. Amplification failures showed no clear pattern based on depth or station. All loci were successfully amplified for two *N. whoii* and two *M. johnsoni* individuals to be used as outgroups. Sequences were deposited in GenBank under accession numbers KM102340-KM102448.

Genetic Diversity

Overall gene diversity of COI was high, with diversity greater than 0.9 at all stations, except for station 17a, where only three individuals were sequenced. Nucleotide diversity was low, ranging from 0.03 at station 6a to 0.14 at station 14a. No haplotypes were shared between stations shallower than 2800 m and deeper than 3200 m. Gene diversity was similar between the two depth groups, but nucleotide diversity was greater in the deeper group. (Table 2)

The overall gene diversity of CAL was also high. The greatest diversity was at station 17a, and the lowest diversity was at station 7a. Nucleotide diversity was low, ranging from 0.00 at station 7a to 0.02 at station 14a. Again, no haplotypes were shared between individuals found at shallow (< 2800 m) and deep (> 3200 m) stations. Both the haplotypic and nucleotide diversity of CAL was greater in the deeper group than in the shallower group (Table 2). More heterozygous individuals were also detected in the deep group: 12, compared with one in the shallow group. Five indels were resolved, ranging in length from 1 to 9 bp.

The ten individuals sequenced for 28S from the stations above 2800 m shared a single haplotype, whereas individuals from the stations below 3200 m had two haplotypes. Diversity indices were greater for the deeper group, and no haplotypes were shared between shallow and deep groups. Pooled results are reported for the shallow and deep groups because of the small sample size for each station (Table 2).

Tests of Neutrality

Tests of neutrality for both COI and CAL were nonsignificant at all stations, except at station 18a, for which Tajima's D was significant for CAL. When samples were pooled into shallow and deep groups, both Tajima's D and Fu's Fs for CAL were significant for the deep group whereas both were nonsignificant for the shallow group (Table 2).

Phylogenetic Analysis

Bayesian phylogenetic analyses of the COI locus resulted in two distinct clades, one for *N. whoii* and another for *N. salicensis*, with posterior probabilities of 1.00 for both branches (Fig. 2). Within the *N. salicensis* group, there was a secondary split of two clades, with branch supports of 1.00 and 0.61, individuals shallower than 2800 m forming one clade and those deeper than 3200 m forming another. Within both clades there were two distinct subgroups supported by posteriors of 1.00 and 0.61 in the shallow clade and 0.59 and 1.00 in the deep clade.

Phylogenetic analysis of CAL supported a similar branching pattern with strong divergence between a shallow and deep clade within *N. salicensis* (Fig. 3). Branches between *N. whoii* and *N. salicensis* and between the two clades within *N. salicensis* were supported by posterior probabilities of 1.00 on each branch. The two clades within the shallow and deep clades were again well resolved, the shallow clades supported with a posterior of 1.00 and the deep clades

Table 2. Diversity indices and tests of neutrality

Locus: length	Station	Sequenced	Haplotypes	H	π	Tajima's D	Fu's Fs
COI: 405	6a	6	5	0.93	0.03	7.95	1.38
	7a	5	4	0.9	0.04	14.62	2.53
	10	9	8	0.97	0.04	6.18	–0.24
	Shallow group	20	13	0.95	0.04	6.64	0.59
	14a	8	6	0.93	0.14	7.63	4.85
	17a	3	2	0.67	0.12	3.50E+08	7.08
	18a	10	7	0.93	0.08	6.54	4.12
	Deep group	21	9	0.9	0.11	6.64	13.2
	Total	41	22	0.96	0.15	6.34	8.87
CAL: 583	6a	6	2	0.53	0.0034	2.76	2.9
	7a	4	1	0	0	0	NA
	10	14	5	0.79	0.007	0.63	1.96
	Shallow group	24	6	0.65	0.0053	–0.55	1.32
	14a	12	11	0.98	0.02	–0.67	–2.64
	17a	6	6	1	0.019	1.77	–0.81
	18a	16	10	0.9	0.0095	**–1.25**	–1.51
	Deep group	34	24	0.94	0.015	**–1.53**	**–8.47**
	Total	58	30	0.92	0.09	3.61	6.35
28S: 672	Shallow group	10	1	0	0	0	NA
	Deep group	8	2	0.54	0	4.91	2.91
	Total	18	3	0.62	0.02	6.79	13.5
	NW	4	1	0	0	NA	NA

Genetic diversity indices and tests of neutrality were calculated in Arlequin v 3.5. The total number of individuals sequenced for COI and 28S, and the total number of alleles sequenced for CAL is reported for each station. Neutrality indices given in bold were statistically significant. Station names correspond to stations sampled on the Endeavor 2008 cruise in the western North Atlantic; shallow and deep groups correspond to clades delimited by phylogenetic analyses in which stations above 2800 m and below 3200 m grouped together respectively. Total rows correspond to indices calculated for all N. salicensis individuals taken together. NW corresponds to Neilonella whoii.

supported with a posterior of 0.81. Phylogenetic analysis of the 12 individuals for which all three loci were sequenced produced a very similar topology, with posteriors of 1.00 on each branch between the outgroup *N. whoii* and *N. salicensis,* as well as on the branches of the shallow and deep clades within *N. salicensis* and of 1.00 and 0.54 for clades within the deep group. The split within the shallow clade was not well supported (Fig. 4). All phylogenetic topologies remained unchanged when *M. johnsoni* or both *M. johnsoni* and *N. whoii* (not shown) were used as the outgroup. A 28S tree was not reported because of the lack of polymorphisms within each group.

The 28S rRNA network depicts a clear split, with 12 substitutions separating the shallow and deep samples and over 100 substitutions separating each from *N. whoii* (Fig. 5). Haplotype networks for COI and CAL are reported for each group individually because shallow and deep clades were separated by a large number of substitutions, and phylogenetic divisions were distinct and consistent. Shallow and deep clades were separated by 58 substitutions for COI and by 65 substitutions for CAL. The COI network for the shallow group has a somewhat stellate appearance, but the deeper group does not. Both groups have a stellate haplotype network for CAL but the samples below 3200 m exhibit a more complex network, with longer branches and more alleles (Fig. 5).

Species delimitation

Four species were resolved using O'Meara's (2010) heuristic search. These corresponded to *N. whoii*, a shallow *N. salicensis* clade, and two deep *N. salicensis* clades. The two deep *N. salicensis* clades correspond to the two well-supported clades on the phylogeny of all three loci and the two divergent haplotypes at 28S within individuals deeper than 3200 m (Fig. 6). These individuals do not consistently group together in the single-locus phylogenies, and thus the split appears to be driven by the 28S divergence and might represent two clades that are in the process of diverging but

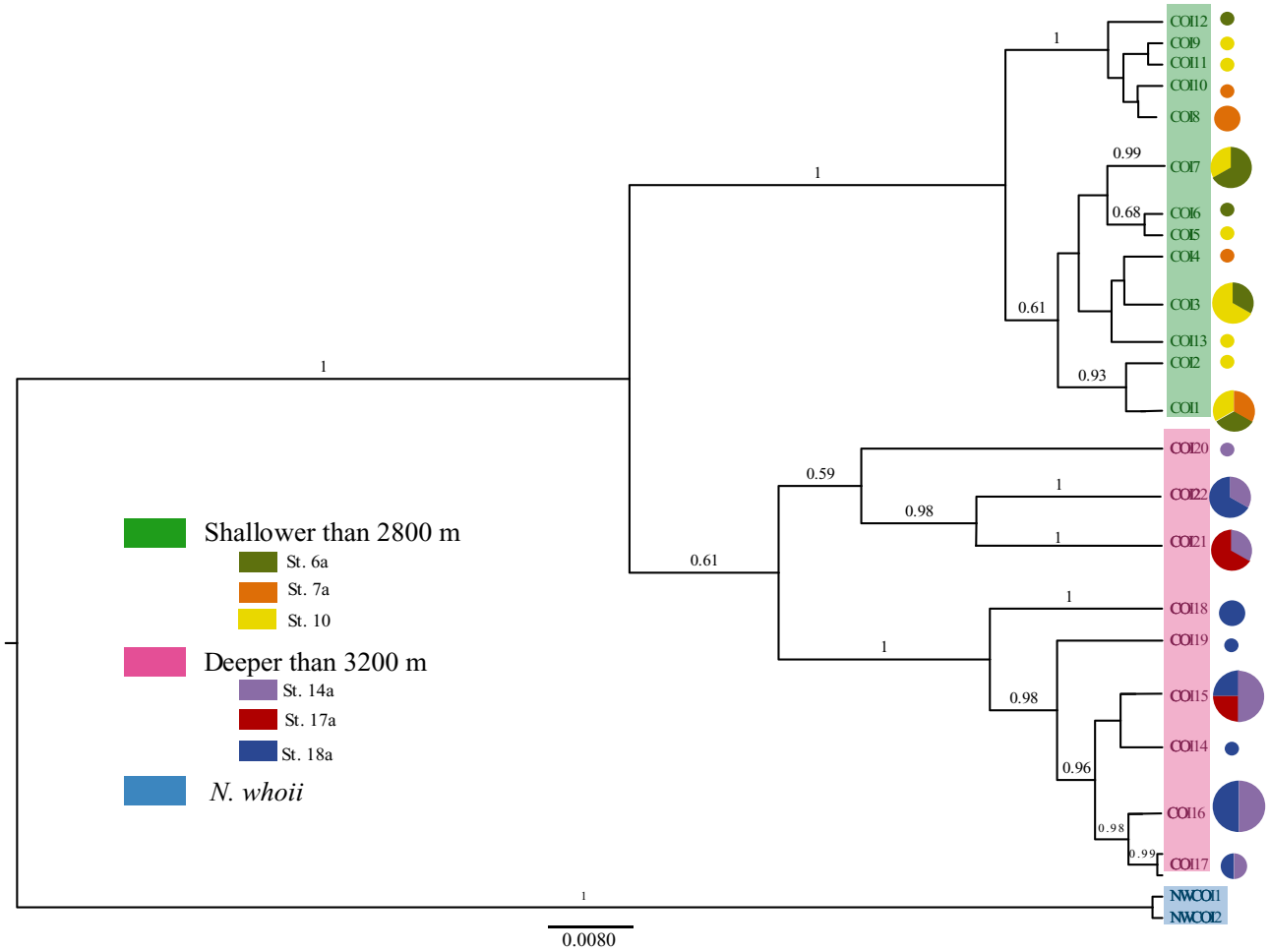

Figure 2. *Neilonella* COI. Phylogeny estimated with COI sequences. Two well-supported clades consist of *Neilonella whoii* and *Neilonella salicensis*. Two clades are also apparent within *N. salicensis*. Pie chart size represents the number of individuals sharing the haplotype, the smallest corresponding to a single individual, and colour corresponds to the stations at which they were found. Posterior probabilities greater than 0.5 are reported on the branches. Branch lengths are proportional to the number of substitutions per site.

are not as far down this path as the shallow and deep clades. These results were consistent over all trials.

BPP analyses using a guide tree with individuals correctly mapped to shallow and deep clades of *N. salicensis* reported tree frequencies of 1.00 for the 11 tree, with posteriors of 1.00 for the node between shallow and deep, as well as for the node between *N. salicensis* and *N. whoii*. In contrast, all model runs using a guide tree with individuals mixed between shallow and deep clades of *N. salicensis* resulted in tree frequencies of ≥ 0.9 for the 10 tree, with the node between the two mixed populations having a posterior of ≤ 0.1 and the node between *N. salicensis* and *N. whoii* having a posterior of 1.00. The BPP analyses provide strong support that the multilocus divergence between the shallow and deep lineages is indicative of different species.

DISCUSSION

CRYPTIC SPECIES?

Neilonella salicensis (Seguenza, 1877) was originally described as a single species based on conchology and internal anatomy (Warén, 1989; Allen & Sanders, 1996b), but molecular genetic analyses suggest that it is composed of at least two genetically distinct groups separated bathymetrically that likely represent cryptic species. The two highly supported clades shared no haplotypes at nuclear loci (28S and CAL) or the mitochondrial locus, COI. Although validation of genetic divergences between putative cryptic species with morphological analysis has resulted in diagnostic characters in other species (Piggott, Chao & Beheregaray, 2011; Barata *et al.*, 2012; Takeuchi *et al.*, 2012), a close examination of individuals from

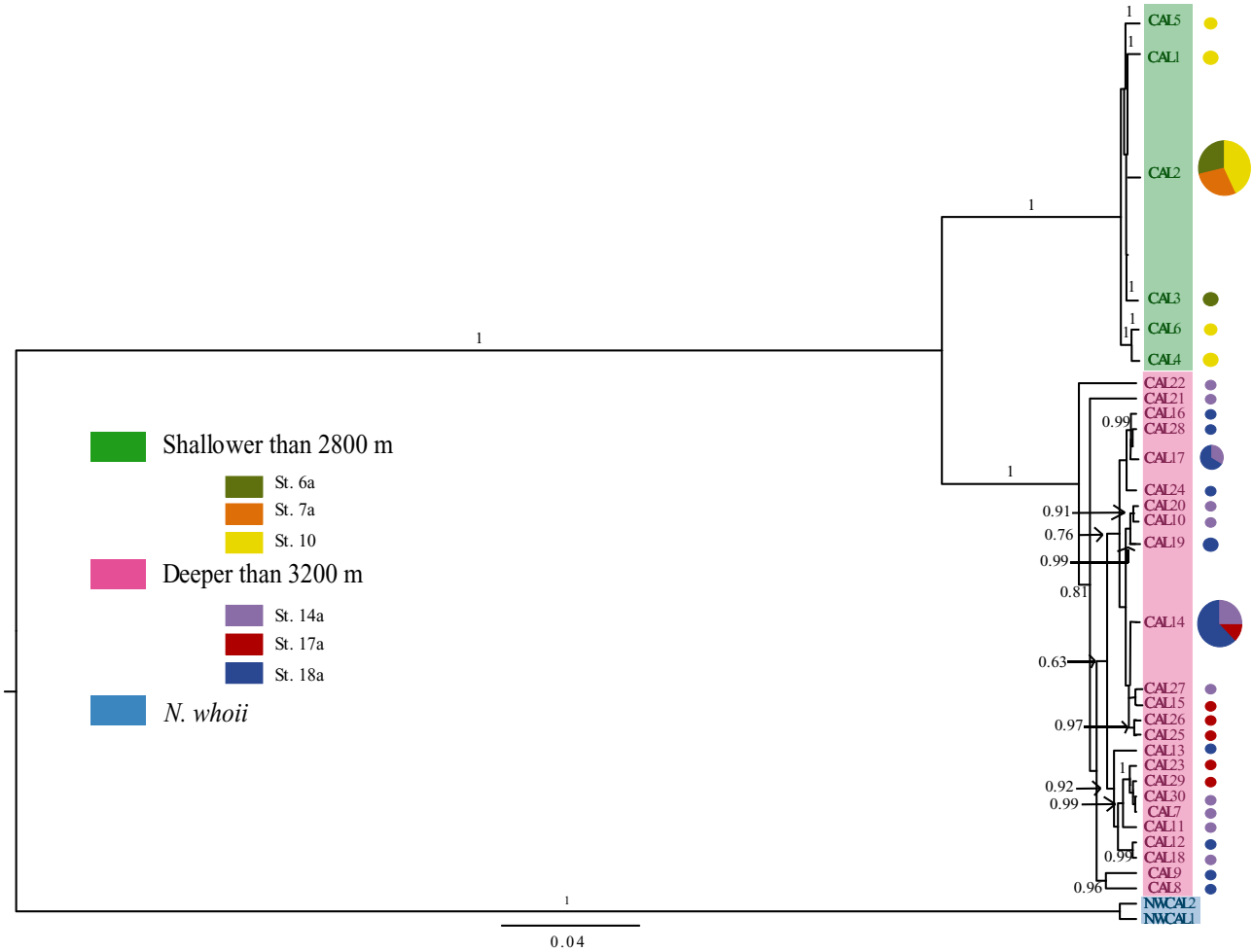

Figure 3. *Neilonella* calmodulin intron. Phylogeny estimated from the calmodulin intron sequences. Two well-supported clades are apparent within *Neilonella. salicensis*. Pie chart size represents the number of allele copies, and colour corresponds to the stations at which they were found. Posterior probabilities greater than 0.5 are reported on the branches. Branch lengths are proportional to the number of substitutions per site.

the two different depth regimes revealed no clear morphological differences, suggesting either that they have not diverged phenotypically or that more detailed multivariate analyses will be needed.

Identification of cryptic species has become increasingly prevalent in a variety of environments (e.g. Jackson & Austin, 2012; Marin *et al*., 2013; Millar & Byrne, 2013; Rheindt, Cuervo & Brumfield, 2013; Hammer *et al*., 2014). The criteria and data required to identify morphologically cryptic species genetically remains controversial, however (Sites & Marshall 2003; DeSalle, Egan & Siddell, 2005; de Queiroz, 2007; Whelan, 2011; Carstens *et al*., 2013; Kvist, 2013), and often vary among taxa and markers. Many species are inferred solely based on the 'barcoding' COI gene (e.g. Hebert *et al*., 2004; Brix, Riehl & Leese, 2011; Knox *et al*., 2012; Pfeiler *et al*., 2013), yet considerable debate exists on how best to use COI to delineate putative species and whether a single locus is sufficient (DeSalle *et al*., 2005; DeSalle, 2007; Waugh, 2007; Birky, 2013). More compelling arguments for delimiting species involve recently developed computational approaches that utilize statistical analyses of multilocus data sets to infer species-level divergences (e.g. O'Meara, 2010; Yang & Rannala, 2010; Ence & Carstens, 2011; Carstens *et al*., 2013; Rannala & Yang, 2013). Controversy and discordance remain with these methods (e.g. Leaché & Rannala, 2011; Carstens *et al*., 2013; Carstens & Satler, 2013; Miralles & Vences, 2013; Parmakelis *et al*., 2013; Satler, Carstens & Heinrich, 2013), but consistent results across multiple methods provide well-supported evidence for independent evolutionary lineages.

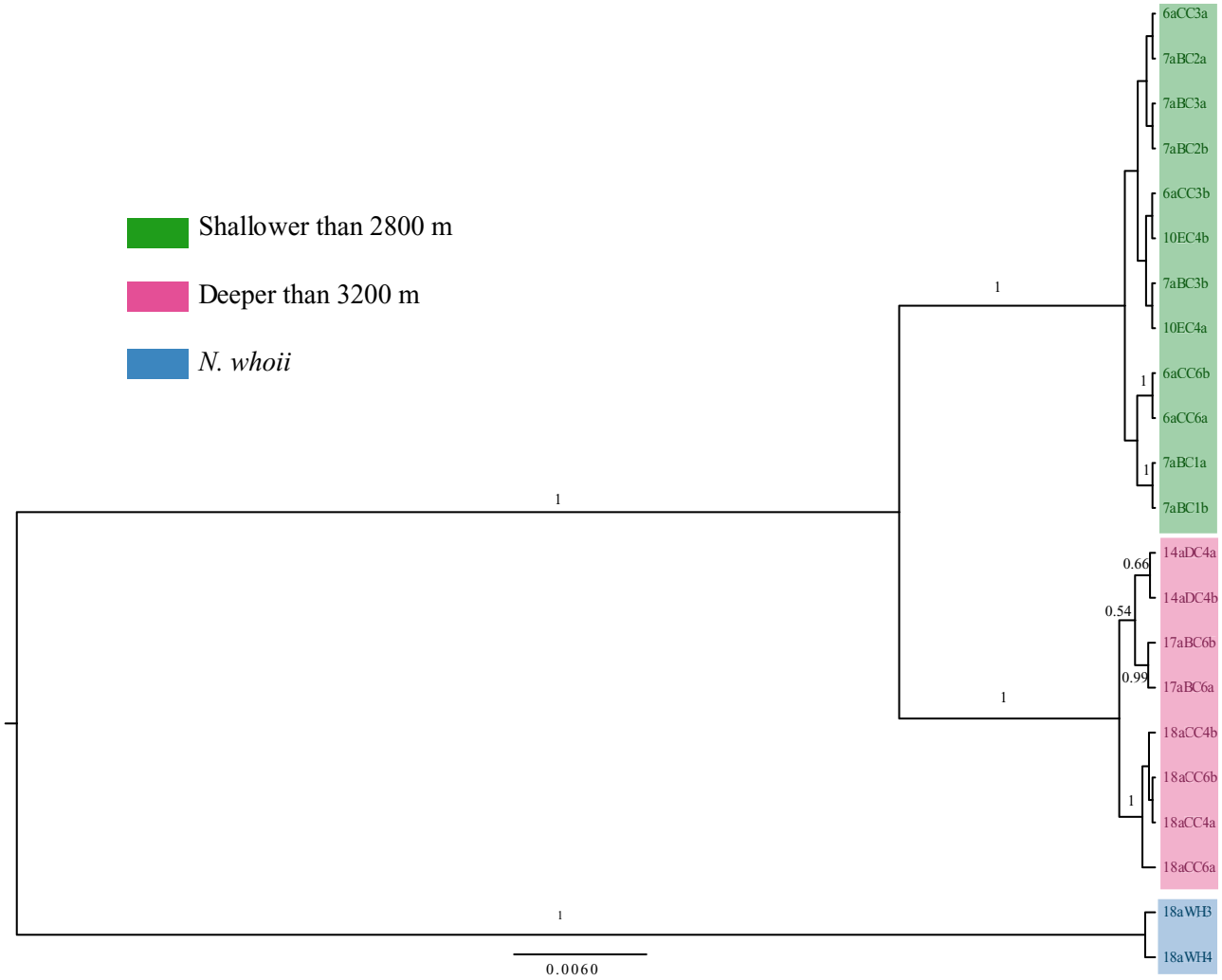

Figure 4. Three *Neilonella* loci. Phylogeny was estimated from 28S rRNA, CAL, and COI sequences. Individuals for which all loci were sequenced are represented. Two well-supported clades are apparent within *Neilonella salicensis*, corresponding to clades above 2800 m and below 3200 m. Posterior probabilities greater than 0.5 are reported on the branches. Branch lengths are proportional to the number of substitutions per site.

Our work suggests that three congeners of *Neilonella* exist within the western North Atlantic and that they have partitioned the deep sea bathymetrically, with little overlap among their depth ranges. The traditional *N. salicensis* is found at bathyal depths and is probably composed of two morphologically cryptic species that have separated into upper and lower bathyal depth regimes. Even if they have not yet met species-level status, they are sufficiently divergent to be independent evolutionary lineages. At abyssal depths, *N. salicensis* is replaced with *N. whoii,* which is widely distributed throughout the Atlantic and is genetically and morphologically quite distinct. Sporadic records of *N. salicensis* at abyssal depths probably reflect misidentifications, a view shared by Allen & Sanders (1996b). Two other congeners occur within the deep Atlantic, but these are quite rare, have not been found in the western North Atlantic, and are easily distinguished from *N. salicensis* based on morphology (Warén, 1989; Allen & Sanders, 1996a, b; Allen, 2008).

Species Formation

Morphologically identical, yet genetically divergent, populations appear to be common in the deep sea (Etter *et al.*, 1999; Zardus *et al.*, 2006; Brandão, Sauer & Schön, 2010; Baird, Miller & Stark, 2011; Knox *et al.*, 2012), especially across bathymetric gradients, but the forces that foster population differentiation and speciation are not well understood. Divergence has been associated with a wide variety of potential

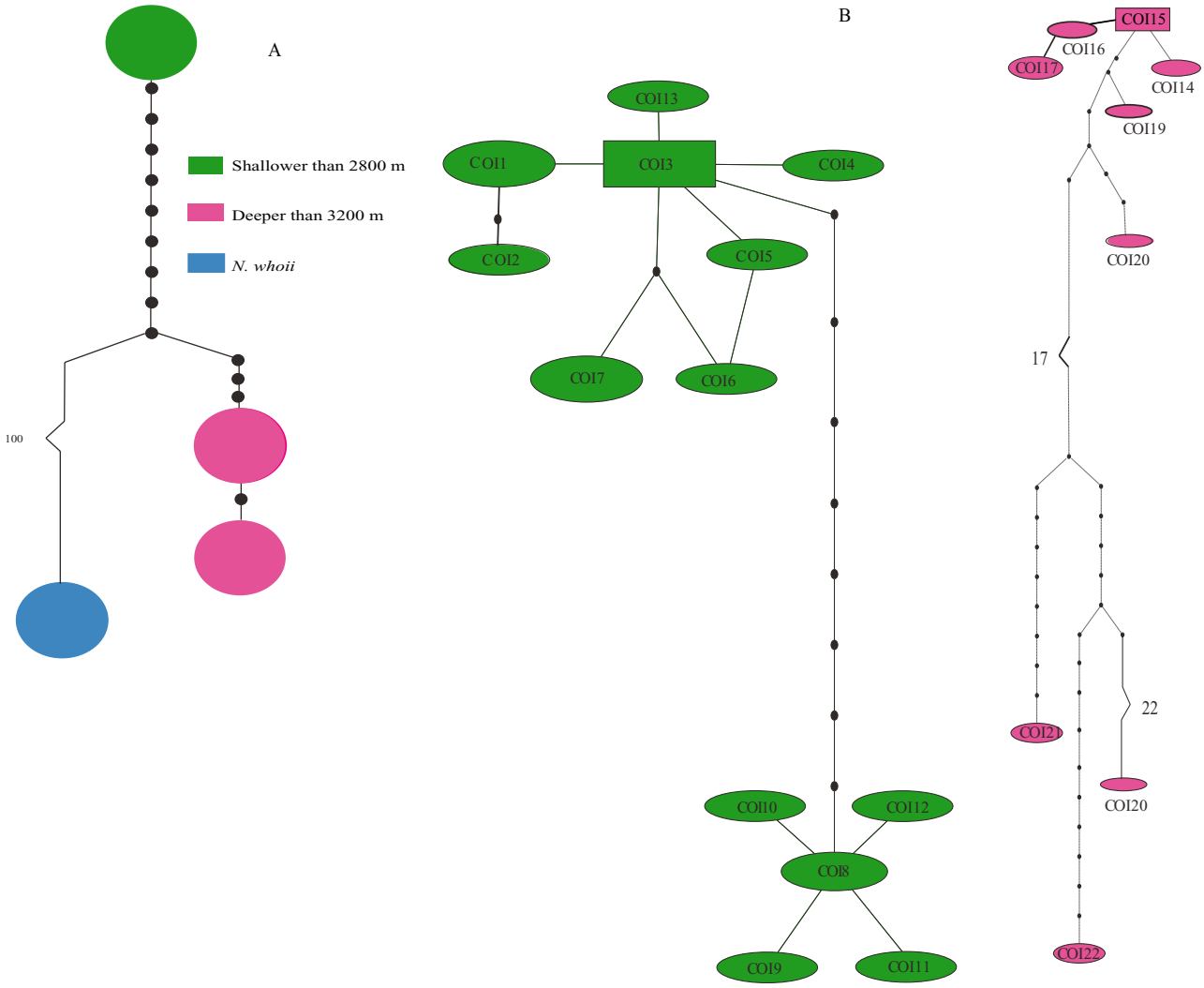

Figure 5. Haplotype networks. Haplotype networks were created in TCS v 1.21 from (A) 28S rRNA, (B) COI, and (C) CAL. Each block represents a different haplotype, and each line between represents a base-pair difference. Rectangles represent expected ancestral haplotypes.

mechanisms, including both selective and nonselective processes (e.g. distance, depth, hydrography, vicariance, and selection along environmental gradients). Although we cannot identify specific mechanisms with the present data, we highlight a few that seem to be the most plausible.

Divergence and the Deep Western Boundary Current

The genetic break between upper and lower bathyal clades occurs where the Deep Western Boundary Current (DWBC) flows south-west along the slope (Bower, Lozier & Gary, 2011; Toole *et al*., 2011), which might be sufficiently powerful to entrain essentially passively dispersing larvae and prevent gene flow between depth regimes. However, both empirical and simulated trajectories indicated considerable mixing with a high potential of movement between depth regimes, especially where the DWBC interacts with the Gulf Stream (Bower *et al*., 2011, 2013; Lozier, Gary & Bower, 2012), suggesting that the present DWBC is unlikely to impede larval exchange among upper and lower bathyal populations. Of course, the nature and the scale of dispersal will be influenced by the length of time that larvae disperse and whether they are passive. Little is known about how protobranch larvae disperse in the deep ocean, whether they are passive, or even how long they spend in the water column, although shallow-water

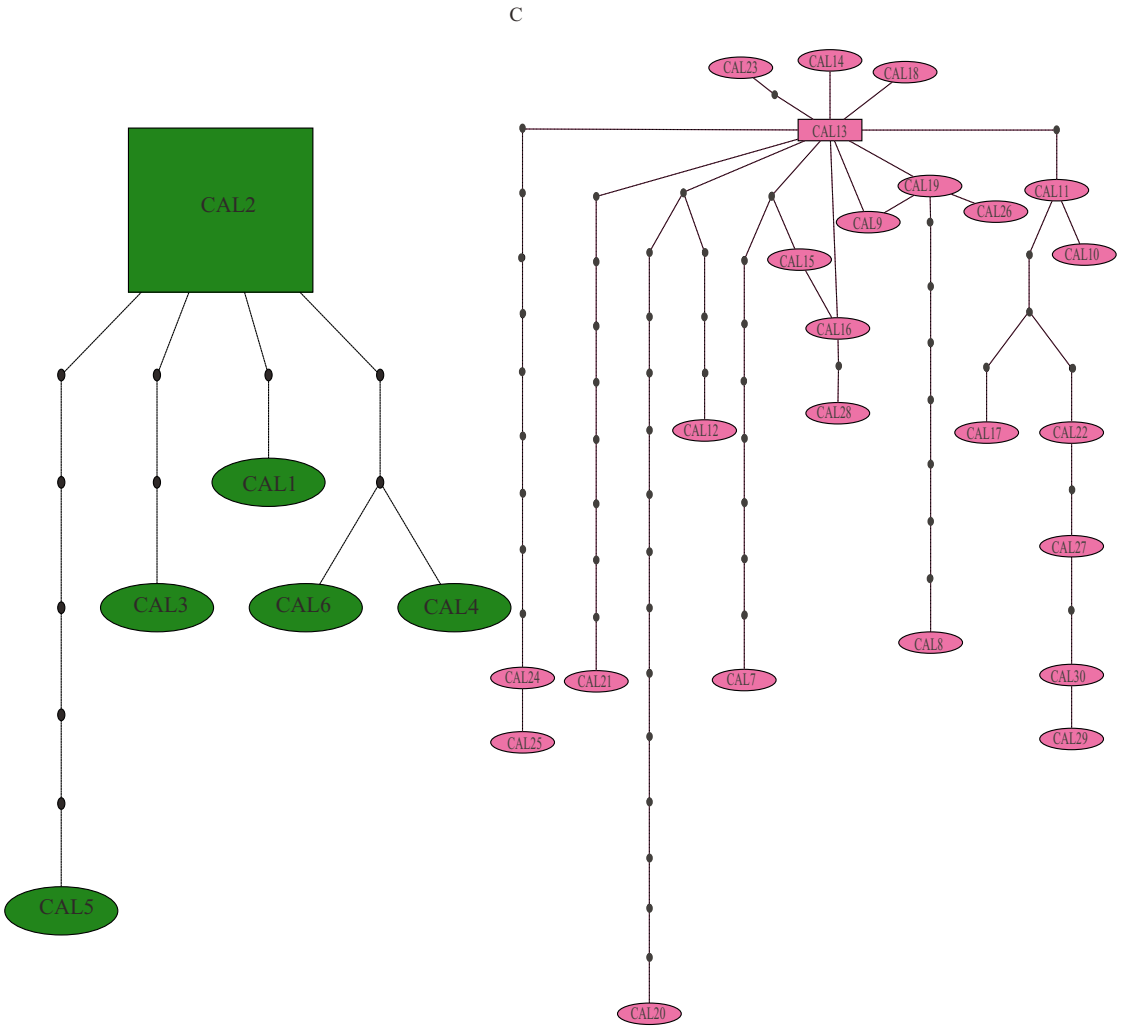

Figure 5. *Continued*

species tend to have relatively short pelagic phases (Zardus & Morse, 1998; Zardus, 2002).

Divergence along environmental gradients

The small scale over which divergence emerges, and the lack of obvious oceanographic or topographic features that could impede gene flow, suggests that selection may play an important role. The genetic break occurs at bathyal depths where the slope is relatively steep and environmental gradients are strong. A number of biotic and abiotic environmental conditions change across these depths, including temperature, pressure, oxygen, nutrient flux, sediment characteristics, calcite solubility, environmental heterogeneity, predation, species diversity, and trophic complexity (reviewed in Gage & Tyler, 1991). Strong environmental gradients can lead to population differentiation and speciation, even in the face of considerable gene flow (Irwin, 2012). Indeed, a growing body of evidence suggests that ecological forces may be much more important than previously thought in limiting gene flow and promoting diversification (reviewed in Nosil, 2012; Koutroumpa *et al*., 2013), especially in marine environments where allopatric constraints on gene flow appear to be limited (Bowen *et al*., 2013). Ecologically driven speciation could occur in the deep sea if adaption to local selective pressures along the depth gradient limits larval exchange among depth regimes as a result of immigrant inviability (*sensu* Nosil, Vines & Funk, 2005). Strong evidence for such a process exists in shallow-water corals (Prada & Hellberg, 2013) and may be even more likely in the deep sea where few other mechanisms are likely to impede gene flow on such small scales.

Several obvious phylogeographic and macroecological patterns are consistent with the notion that speciation in the deep sea is often driven by ecological changes along bathymetric gradients. Population differentiation is much greater for populations separated

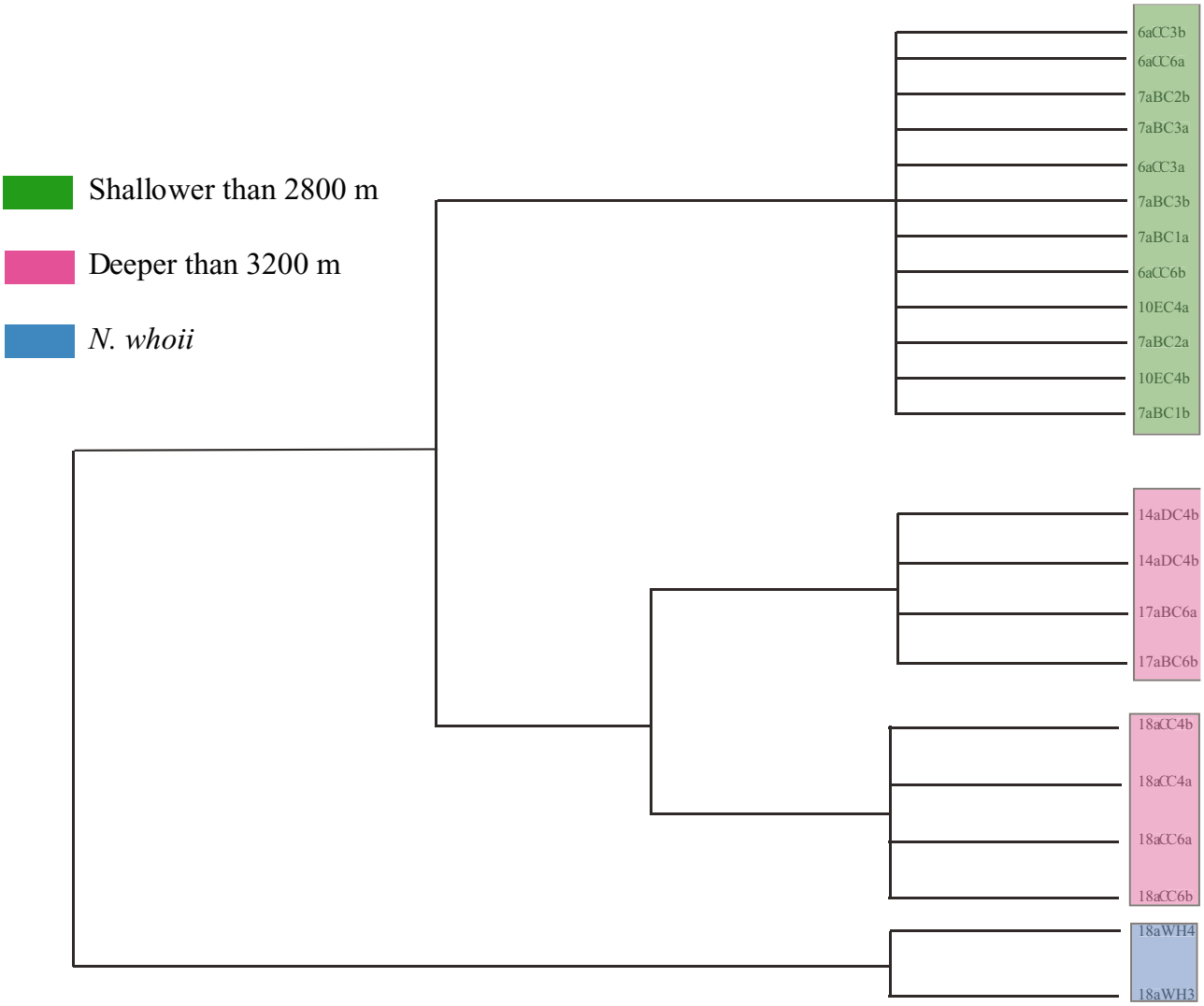

Figure 6. Phylogeny from heuristic search. Phylogeny reported from the heuristic search tool in Brownie. Putative species are represented as polytomies.

vertically (with depth) than for those separated horizontally (distance along isobaths) (Bucklin, Wilson & Smith, 1987; France & Kocher, 1996; Zardus *et al*., 2006; Raupach *et al*., 2007; Etter *et al*., 2011; Miller *et al*., 2011). For example, protobranch bivalves separated by 3 km in depth were considerably more divergent genetically than were those separated by over 10 000 km at the same depth (Zardus *et al*., 2006; Etter *et al*., 2011). The depth-related divergence is often sufficiently large to suggest the presence of cryptic species (France & Kocher, 1996; Chase *et al*., 1998; Etter *et al*., 1999; Held & Wägele, 2005; Reveillaud *et al*., 2010; Baird *et al*., 2011; Schüller, 2011). Further along the divergence spectrum, congeners and sibling species are often separated bathymetrically (e.g. Allen & Sanders, 1996a; Clague *et al*., 2011; White, Fotherby & Hoelzel, 2011; Castelin *et al*., 2012; Laakmann, Auel & Kochzius, 2012; Moura *et al*., 2012; Quattrini *et al*., 2013) and depth is the most frequently cited factor separating sibling species (Knowlton, 1993). Because species formation is a very dynamic process that occurs across a variety of timescales, we should expect a range of divergence levels reflective of various stages of speciation. The fact that these stages are commonly found along bathymetric gradients suggests depth, and the environmental gradients that attend changes in depth probably play a fundamental role in the diversification of the deep-water fauna.

Divergence and palaeo-oceanography

It is possible that the DWBC was much stronger in the past and disrupted gene flow between depth

regimes long enough for divergence to occur. Molecular clock estimates of the observed genetic divergence in COI between shallow and deep clades suggest that gene flow has been absent for more than 15 Myr (based on a COI clock of arcid bivalves from Marko, 2002), during which the DWBC varied considerably in intensity (Boyle & Keigwin, 1982; Keigwin & Pickart, 1999). If divergence in the past was sufficient to prevent recruitment of larvae from contrasting depth regimes, then even though the contemporary flows of the DWBC allow larval exchange between depths, gene flow would be precluded as a result of migrant inviability. Interestingly, another protobranch (*Nucula atacellana*) exhibits a strong genetic break among populations from different depths in the same general vicinity (Chase *et al*., 1998; Zardus *et al*., 2006), but multilocus estimates of divergence suggest a much more recent split (1 Mya, Jennings *et al*., 2013). As global climate shifted historically and thermohaline circulation waxed and waned, the DWBC may have periodically disrupted gene flow among populations at different depths, fostering repeated rounds of species formation. If true, we should expect other taxa with distributions that span the DWBC to exhibit diversification at similar times. In addition, if the waxing and waning of the DWBC is acting essentially as a speciation pump by repeatedly disrupting gene flow, it might also help to explain the well-known peak in diversity at bathyal depths in the western North Atlantic (Rex, 1981; Etter & Grassle, 1992).

Historical allopatry

Another possible explanation for the phylogeographic patterns is that the two lineages of *N. salicensis* diverged in allopatry and are coming back into proximity within the western North Atlantic. Although we cannot rule out divergence elsewhere within the Atlantic, there are few obvious mechanisms that would impede gene flow, and emerging phylogeographic patterns from a wide variety of taxa suggest that geographic divergence is much less likely than bathymetric divergence (France & Kocher, 1996; Chase *et al*., 1998; Etter *et al*., 1999; Reveillaud *et al*., 2010; Baird *et al*., 2011; Schüller, 2011).

CONCLUSION

Cryptic species appear to be much more prevalent along bathymetric gradients in the deep sea, which is consistent with the notion that environmental gradients that attend changes in depth play a key role in the diversification of the largely endemic deep-water fauna. The presence of cryptic species leads to underestimates of diversity and overestimates of geographic distributions, and can confound inferences about the ecological forces that regulate the structure and function of these communities. Understanding the frequency, geography, and taxonomic propensity of cryptic species will be essential to develop more effective strategies to manage deep-water ecosystems and mitigate the effects of increasing anthropogenic stresses.

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