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PHYLOGEOGRAPHIC ANALYSIS OF THE BROODING BRITTLE STAR *AMPHIPHOLIS SQUAMATA* (ECHINODERMATA) ALONG THE COAST OF NEW ZEALAND REVEALS HIGH CRYPTIC GENETIC VARIATION AND CRYPTIC DISPERSAL POTENTIAL

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Abstract.—Direct development in benthic marine invertebrates is usually associated with narrow geographical range, low rates of colonization, and low levels of gene flow. Paradoxically, the small brittle star *Amphipholis squamata* broods its larvae to a crawl-away juvenile stage, yet has a cosmopolitan distribution. Using sequence and restriction-fragment-length-polymorphisms (RFLP) analyses of nuclear and mitochondrial DNA from 16 coastal populations throughout New Zealand, we tested whether the species is indeed a poor disperser, as may be expected from its brooding habit. We predicted that local and regional populations would be genetically structured according to isolation by distance. We also suspected that this ubiquitous ''species'' is composed of a variety of cryptic taxa in different geographic areas, as has been discovered in an increasing number of marine invertebrates. We found evidence of four genetically divergent and reproductively isolated lineages that can exist in syntopy. Lineages vary in abundance, haplotype diversity, and geographic distribution. The partitioning of genetic variation within the most common lineage, as well as the geographic distribution of the four lineages, suggest a north/south split. This pattern is consistent with known New Zealand marine biogeographic zones and appears to be linked to the regime of oceanic circulation, which is characterized by subtropical, southward-moving water masses in the north, and sub-Antarctic, northward-moving water in the south. We conclude that the dispersal ability of *A. squamata* is regionally restricted but with sporadic long-distance dispersal, which serves to increase local genetic variation. Our results support the idea that dispersal occurs through passive transport by drifting or rafting on macroalgae, which *A. squamata* commonly inhabits, and emphasize that poor dispersal ability is not necessarily a corollary of direct development.

Key words.—Biogeography, cryptic species, marine, rafting, 16S mtDNA.

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Amphipholis squamata (Delle Chiaje, 1828) is a small hermaphroditic brittle star in the family Amphiuridae. *A. squamata* is probably the most widespread of all coastal ophiuroids (Hyman 1955) because it exists in all of the world's oceans, from the sub-Arctic to the sub-Antarctic. It occupies a variety of habitats (algal turf, bryozoan colonies, coral rubble, gravel, boulders, sand, and mud) from the intertidal zone to at least 1300 m depth (Mortensen 1927; Fell 1958; Gage et al. 1983). *A. squamata* is an internal brooder (Fell 1946) and hence does not have free-living larvae. The cosmopolitan distribution is surprising given that benthic marine invertebrates, such as brittle stars, typically rely on larvae for dispersal.

Dispersal capabilities among benthic marine species vary widely, ranging from meters to thousands of kilometers, depending on larval duration and mode of development (e.g., Olson 1985; Grosberg 1987; Hellberg 1996; a more appropriate ecological multifactor classification of marine invertebrate developmental patterns has recently been suggested by Poulin et al. [2001]). Consequently, differences in larval developmental patterns have been causally linked to differences in geographic range, rates of colonization, and levels of gene flow (Scheltema 1971, 1977; Shuto 1974; Valentine and Jablonski 1983; Hedgecock 1986; Knowlton and Jackson 1993).

Recently, several studies have compared the population genetic structure of marine invertebrate species that have different modes of development. These studies generally conclude that population subdivision is higher in species with direct development than in those with planktonic development (e.g., Ward and Beardmore 1977; Hellberg 1996; Arndt and Smith 1998; Ayre and Hughes 2000; Collin 2001). In addition, geographic distance has a greater isolating potential in species with restricted gene flow (Hellberg 1996; Collin 2001).

We used DNA sequence data from *A. squamata* populations around the coast of New Zealand to test whether this species is a poor disperser, as might be expected from its brooding habit, or a good disperser, as suggested by its cosmopolitan distribution. In addition, we attempted to assess the effects of the physical environment and biogeography on the distribution of genetic variation.

New Zealand lends itself well to such a study given its linear coastline, extensive latitudinal range (the main islands span approximately 13 degrees of latitude), and thermal variation (mean surface water temperature range of 7°C). Furthermore, through New Zealand's geographical position, its populations of *A. squamata* are isolated from potential external sources that could confound interpretation of population structure.

Because dispersal in *A. squamata* is thought to be achieved solely through the (very restricted) motility of adult individuals, we predicted that local and regional populations are highly genetically structured and that population differentiation follows a model of isolation by distance. In addition, we tested whether the partitioning of genetic variation in *A. squamata* throughout New Zealand reflects biogeographic divisions already proposed for other marine groups (Pawson

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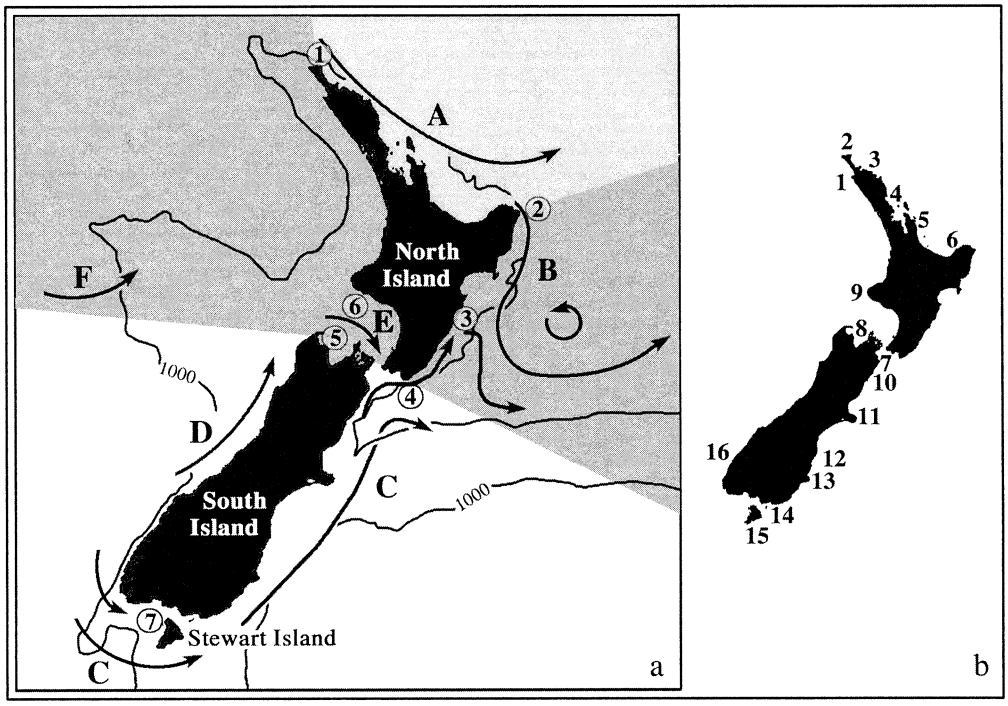

FIG. 1. Map of New Zealand. (a) Main surface ocean currents, sea floor contours of 1000 m (after Heath 1985; Parsons 1985), and marine biogeographic zones (after Pawson 1965; Francis 1996). Ocean currents and landmarks referred to in the text are: A, East Aukland Current; B, East Cape Current; C, Southland Current; D, Westland Current; E, D'Urville Current; F, East Australian Current; 1, Cape Maria Van Diemen; 2, East Cape; 3, Cape Turnagain; 4, Cape Palliser; 5, Cape Farewell; 6, Cook Straight; 7, Foveaux Straight. The light gray area represents biogeographic area I; dark gray, area II; and white, area III (after Francis 1996). (b) Sampling locations of 16 populations of *Amphipholis squamata* used in this study. The location names and sample sizes of populations are: 1, Ahipara (n = 12); 2, Cape Reinga (n = 14); 3, Cable Bay (n = 4); 4, Orewa (n = 14); 5, Hahei (n = 9); 6, Omaio (n = 11); 7, Wellington (n = 11); 8, Nelson (n = 4); 9, Opunake (n = 5); 10, Kaikoura (n = 12); 11, Banks Peninsula (n = 4); 12, off Otago Peninsula (45.51.139S / 170.50.82E), 70–100 m depth (n = 30); 13, Otago Harbour (n = 12); 14, Papatoetoe (n = 11); 15, Stewart Island (n = 4); 16, Fiordland (n = 17).

1965; Francis 1996) and is associated with environmental variables, such as temperature, depth, or currents. Previous population genetic studies of New Zealand marine species (based on allozyme data) have suggested that regional hydrography, the strong temperature gradient from the warm-temperate north to the cold-temperate south, as well as reproductive behavior and larval duration, affect population genetic structure (fish: Smith 1978; Smith et al. 1978; echinoderms: Mladenov et al. 1997; gastropods: Smith 1988; Smith et al. 1989; bivalves: Gardner et al. 1996; Apte and Gardner 2001; crustaceans: Smith et al. 1980; Stevens 1990, 1991; red algae: Intasuwan 1993).

Further, we predicted that *A. squamata* is composed of several cryptic taxa, as has been found in an increasing number of marine invertebrates (for a review see Knowlton et al. 1992; corals: Knowlton 1993; foraminifera: De Vargas et al. 1999; copepods: Lee 2000; Rocha-Olivares et al. 2001). Indeed, high genetic divergences were previously observed within a population of *A. squamata* from France (Sponer et al. 2001).

MATERIALS AND METHODS

Samples

A total of 173 individuals of *Amphipholis squamata* were collected at 16 locations around North and South Island, New Zealand (Fig. 1b), spanning a north-to-south distance (by sea) of approximately 1600 km. Collecting was done in intertidal and subtidal locations from algae, gravel, rocks, and kelp holdfasts. At each location, a surface area of approximately 10–100 m^2 was searched. One deep-water population was sampled by dredging at 70–100 m, off Otago Peninsula. Relatively few samples could be collected on the west coast of New Zealand, because this is generally a high-energy coast with little suitable habitat. In addition, *A. squamata* was often patchily distributed, whereby only a few specimens were found in some locations with seemingly suitable habitat (e.g., Opunake, Nelson, Stewart Island). All specimens were preserved in absolute ethanol. For use as outgroup comparisons, the congeneric species *Amphipholis gracillima* and the amphiurid *Amphiodia atra* were collected from South Carolina (USA).

DNA Extraction, Polymerase Chain Reaction, and Sequencing

A small piece of tissue (about 3mm of an arm) was added to 250 μl of 5% Chelex (Sigma St. Louis. MO) solution and heated at 65°C for 3 h, with the tissue constantly submerged in the Chelex beads (Walsh et al. 1991). Subsequently, the solution was vortexed, then boiled for 10 min and centrifuged at 13,500 g.

One to 5 μl of the supernatant of the cooled solution were used for each polymerase chain reaction (PCR) amplification. We generated sequence data from the mitochondrial DNA (mtDNA) gene 16S rRNA (16S). The universal primers LR-j-12887 (alias16Sbr) and LR-N-13398 (alias 16Sar; Simon et al. 1994) were used for PCR and sequencing of approximately 500 base pairs (bp) of the 16S gene. Amplification of double stranded product was achieved using the following conditions: initial denaturation for 2 min at 94°C, 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 50–53°C, extension for 1 min at 72°C, and final extension for 10 min at 72°C. Polymerase chain reaction products were cleaned for sequencing using silica-gel-based purification columns. Cycle sequencing was done with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Shelton, CT), using a temperature profile of initial denaturation of 2 min at 96°C, 25 cycles of denaturation for 15 sec at 96°C, annealing for 7 sec at 50°C and extension for 4 min at 60°C. Amplification products were run on an ABI 377 automated sequencer and verified in the program SeqEd (Applied Biosystems, Foster City, CA). Alignments were made using ClustalX (Thompson et al. 1997).

Restriction-Fragment-Length Polymorphism Analysis of the Internal Transcribed Spacer Region

In order to compare mitochondrial-and nuclear patterns, a subset of individuals ($n = 35$) was screened for restriction-fragment-length polymorphisms (RFLP) of the nuclear internal transcribed spacer (ITS) region. For amplification of ITS we used the primers 18d and 28u (Hillis and Dixon 1991), which bind in the 18S rRNA and 28S rRNA, respectively, and can be used to amplify approximately 2000 bp of DNA which include ITS-1, 5.8S rRNA, and ITS-2. PCR-amplification was carried out as for 16S (see above). Ten restriction enzymes were tested for their ability to digest the PCR-amplified fragment. The four-base cutter *Hae III* produced the highest number of easily scorable bands and was therefore chosen for all subsequent RFLP analyses. Digests were run on 8% non-denaturing poly-acrylamide gels and visualized with SYBR Green (Roche, Basel, Switzerland) staining.

Phylogenetic Analyses of 16S rDNA

Phylogenetic analysis of aligned sequences was carried out using PAUP* version 4.0b8 (Swofford 2000). For rooting of the tree we used the two amphiurid species *Amphipholis gracillima* and *Amphiodia atra*. Although the taxonomic status of the genus *Amphiodia* is uncertain, it is considered to be a close sister group to *Amphipholis* (Clark 1970).

To determine which model of DNA substitution best fitted our 16S sequence data we used the program Modeltest 3.0 (Posada and Crandall 1998), which uses hierarchical likelihood-ratio tests to calculate the goodness of fit of various general time reversible (GTR; Rodríguez et al. 1990) models of DNA evolution on the data. The selected model was TVM+G, a special case of GTR with the following substitution rates: A-C = 2.8517, A-G = 23.1061, A-T = 12.0486, C-G = 0.0001, C-T = 23.1061, G-T = 1.0000; and base frequencies: A = 0.2906, C = 0.2091, G = 0.1789, T = 0.3214. The proportion of invariable sites was found to be zero and the gamma distribution shape parameter was 0.1914. Using these parameters to estimate DNA distances, we constructed a neighbor-joining tree in PAUP*. Bootstrap confidence values were calculated in 1000 iterations.

We also explored relationships among a group of closely related 16S haplotypes (lineage A; see Results) by building an unrooted parsimony network using the computer program TCS (Clement et al. 2000). TCS calculates the most parsimonious network at the 95% confidence level.

Analysis of Molecular Variance (AMOVA)

We used a hierarchical approach (AMOVA; Excoffier et al. 1992) to investigate the distribution of molecular variance in New Zealand *A. squamata*. In this analysis a priori defined groups were evaluated for their contribution to the partitioning of the observed genetic variation. In order to increase resolution in a dataset containing many closely related haplotypes, we calculated genetic variation based on both genetic distance among haplotypes (Kimura 1980) and haplotype frequencies. The significance of fixation indices was tested by 10,000 permutations of groups, populations, and haplotypes. All calculations were carried out in the computer program ARLEQUIN version 2000 (Schneider et al. 2000).

The AMOVA was carried out on 123 samples from 16 populations throughout New Zealand, which were grouped according to known marine biogeographic areas. Two groups were tested: Group A reflects distribution patterns of reef fish (Francis 1996; Fig. 1a): (1) northeast North Island (populations 2–6); (2) northwest North Island, southeast North Island, and southwest North Island (populations 1, 7–9); and (3) the remainder of South Island and Stewart Island (populations 10–16). Group B was based on echinoderm distribution patterns (Pawson 1965): northern (populations 1–6), central (mixed, populations 7–10), and southern (populations 11–16) assemblages (Fig. 1b). Because the area from Cape Turnagain to Kaikoura is described as an area of mixing between North and South, we omitted the populations contained within this area (7–10) from the analysis and tested whether a north/south division exists among the remaining populations of *A. squamata*.

Multidimensional Scaling of Population Pairwise F_{ST} Values

AMOVA can only evaluate a priori determined groups and is therefore not suitable to resolve any unknown partitions in the data. In order to further explore the relationships among populations, especially those located in the proposed zone of mixing, we used a multidimensional scaling (MDS) approach. Population pairwise F_{ST} values were analyzed with MDS and plotted in two dimensions.

Isolation by Distance and Effects of the Physical Environment on Partitioning of Genetic Variation

We hypothesized that populations of *A. squamata* would be genetically structured according to isolation by distance. In addition, environmental factors such as temperature, ocean currents, and surrounding water depth may be important in shaping population structure. We used a multiple Mantel test

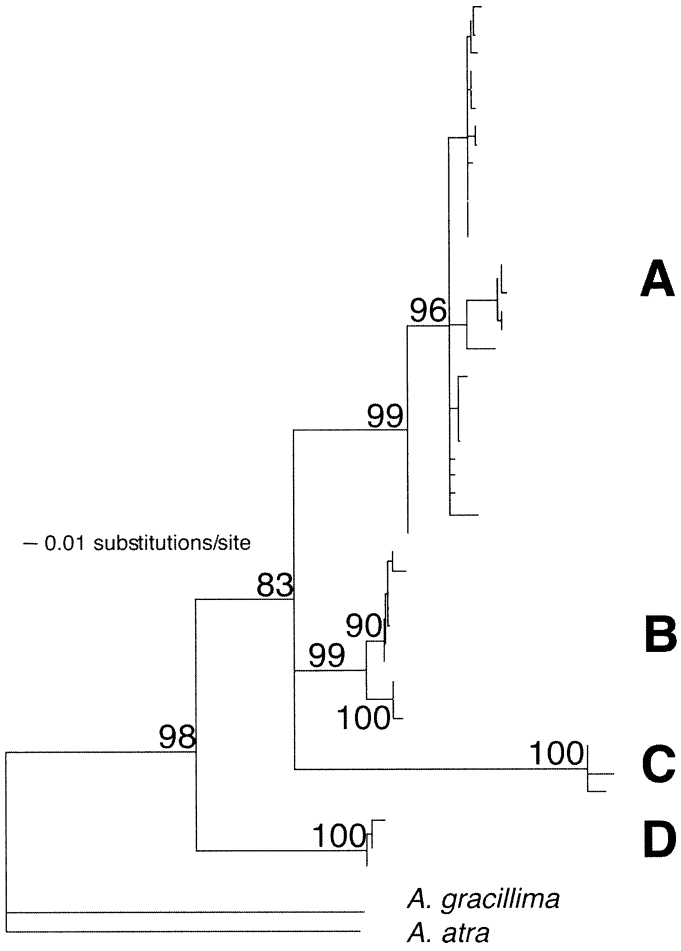

FIG. 2. Neighbor-joining bootstrapped consensus tree relating four major mtDNA lineages (A–D) of *Amphipholis squamata* in New Zealand. Numbers above branches indicate bootstrap confidence values, calculated from 1000 runs of random character resampling. Names of lineages are consistent with those used in Sponer et al. (2001).

(see Manly 1997a) performed with the program RT version 2.1 (Manly 1997b) to test for association between genetic distance among populations and geographic distance, temperature, depth, and surface ocean currents (data can be obtained from the author upon request). Analyses were carried out on two datasets: (1) among lineages (see Results) and (2) within a single lineage. In analysis 1, Euclidean distances were calculated from the frequencies of lineages in each population; for analysis 2, we calculated population pairwise genetic distances using Nei's D_A (Nei and Li 1979). Pairwise geographic distances were measured as direct distances by sea, mean annual surface temperatures were taken from Heath (1985), and shelf gradient (depth) reflected by distance to nearest shelf break were coded as (1) close, (2) medium, (3) far.

Surface ocean currents were coded from 1 to 0 to express the degree to which populations were located on the same water mass (1, on the same current; 0, on opposing currents; see Fig. 1a). North Island populations 1–6 lie on the East Auckland Current, which is fed with subtropical water from the East Australian Current and flows southeastward near the edge of the continental shelf. The South Island populations 11–16 are located on the Southland Current, which takes sub-Antarctic water along the east coast of the South Island and north as far as Cape Turnagain in the North Island. In the similarity matrix populations 1–6 were coded as 1 when compared to each other and 0 when compared to populations 11–16. Populations in the geographic center (7–10) are influenced by a combination of currents: (1) the southward-flowing East Cape Current (a branch of the East Auckland Current, most of which moves offshore in the vicinity of East Cape), (2) a branch of the northward-flowing Southland Current which meets the warmer water of the East Cape Current and (3) the warm D'Urville Current in the west. Populations 7–10 were coded as 1 when compared to each other, 0.75 when compared to the northern populations (1–6) and 0.25 when compared to the southern populations (11–16). Distance matrices for pairwise comparisons of temperature and surrounding depth were calculated as Euclidean distances. Significance levels were determined with 5000 randomizations. Populations with small sample sizes ($n < 5$) were combined with their nearest neighbor.

TABLE 1. Mean genetic distances within and between major lineages (A–D) in *Amphipholis squamata.* Distances are percent maximum-likelihood distances according to the model TVM+G and were calculated based on unique haplotypes only.

	A	B	C	D
A	*2.04*			
B	23.69	*2.78*		
C	34.33	31.08	*0.84*	
D	42.97	38.78	52.69	*0.11*

The multiple Mantel test is an adaptation of the classical Mantel test (Mantel 1967). It is based on the use of multiple regression methods with randomization of the order of the items in the dependent matrix. Oden and Sokal (1992), Manly (1997a), and Raufaste and Rousset (2001) noted that multiple (or partial) Mantel tests can be problematic with geographic data. Specifically, the effects of spatial autocorrelation may not be completely accounted for by including geographical distance in the regression equation. In these cases a randomization test may not be valid. To remedy this problem, Manly (1997a) suggested the use of restricted randomizations. To detect possible spatial autocorrelation, the regression residuals from the original analysis (regression of genetic on geographic and environmental distances) are regressed against geographic distance. Autocorrelation is indicated by a regression coefficient significantly different from zero. In such a case the randomization test is best restricted to local groups (Manly 1997a).

RESULTS

Descriptive Statistics and Phylogenetic Analysis

Diversity within *A. squamata* was extremely high and indels existed throughout the 16S region. Ingroup haplotypes comprised four well-supported and highly divergent lineages, hereafter referred to as lineages A–D (Fig. 2). Genetic distances between lineages were extremely high, measuring up to 52.69% (Table 1). Of the 173 specimens, 123 (71.1%) fell

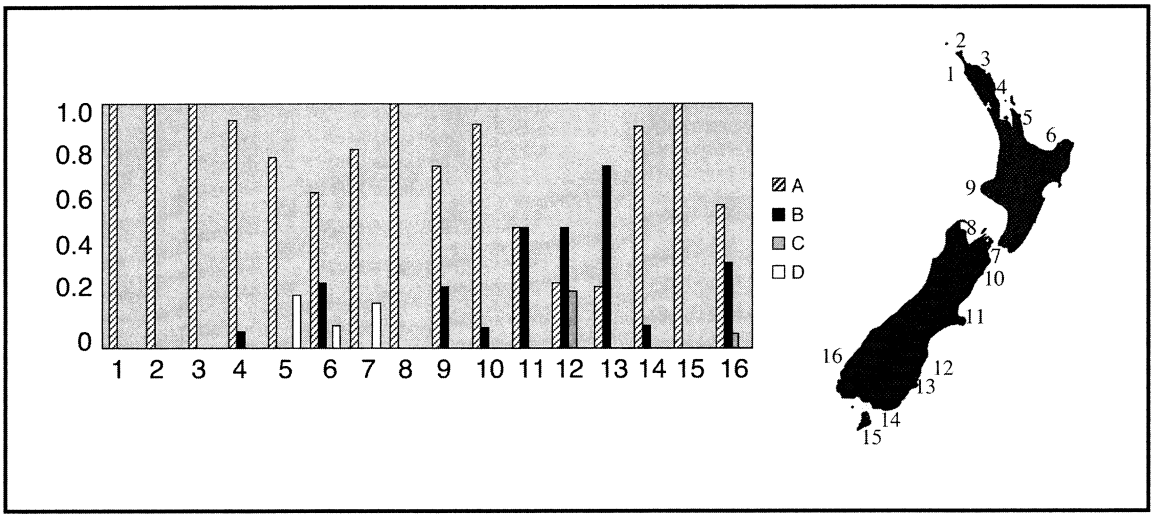

FIG. 3. Distribution and relative abundance of lineages A–D among sampling locations in New Zealand.

into lineage A (25 haplotypes), 32 (18.5%) fell into lineage B (nine haplotypes), 10 (5.8%) fell into lineage C (four haplotypes) and eight (4.6%) fell into lineage D (two haplotypes). Mean genetic distances (ML) between outgroups and all ingroup taxa was 46.06% for *Amphiodia atra* and 49.83% for *Amphipholis gracillima* (Table 1). Haplotype sequences have been submitted to GenBank with accession numbers AF522464 to AF522503.

RFLP of the ITS Region

Thirty-five individuals from the two populations found to harbor the greatest 16S nucleotide diversity (see below), the deep site (12) and Fiordland (16) (where lineages A, B, and C were found in sympatry), were used for RFLP analysis of the nuclear ITS region. *Hae III* digestion of the 2000 bp fragments produced three distinct patterns, containing 7–12 bands between 80 and 340 bp length. The three distinct RFLP patterns were termed A', B', and C' since each comprised exactly the same individuals as the mtDNA lineages A–C (Appendix 2). Thus, the nuclear polymorphism entirely supported the existence of the three divergent lineages in populations 12 and 16. In addition, complete congruence of nuclear and mitochondrial data imply that breeding between lineages is absent (or very rare). The ITS data suggest that lineages A' and B' are most similar to each other, as they shared 50% of bands, while A' and C' shared only 5.9% and B' and C' shared 6.7%. A close relationship between lineages A and B is further indicated by a maximum-parsimony phylogeny of the 16S dataset that groups the two lineages as sister taxa (tree not shown).

Distribution of Lineages A–D around New Zealand

Lineages A–D were found in varying proportions across the 16 sampling locations around New Zealand (Fig. 3). Lineage A was the most widespread, found at all 16 sites, followed by lineage B, found at nine sites, and lineages C and D were only found at two and three sites, respectively. Lineage A dominated in the north, from Ahipara (1) to Kaikoura (10), and also in Southland (Papatoetoe; 14) and Stewart Island (15) in the south. Lineage B was most prominent in the south, at Banks Peninsula (11), the deep site (12), Otago Harbour (13), and Fiordland (16) and was present at low frequency in four locations from Kaikoura (10) to Orewa (4). Lineage C was rare and restricted to the deep site and Fiordland, and lineage D was found in central locations, at Hahei, Omaio, and Wellington. In 11 of the 16 populations (69%) two or three divergent lineages were found in syntopy (in the very same sampling location).

Effects of Environmental Variables on the Partitioning of Genetic Variation among Lineages

Because a test for spatial autocorrelation (see Materials and Methods) found no significant association between the residuals of the regression analysis and geographic distance ($P = 0.267$; Table 2), subsequent significance testing was done by unrestricted Monte Carlo randomizations. Multiple regression analysis of Euclidean distances calculated from the frequency of lineages in each population on pairwise geographic distance, temperature difference, difference in surrounding depth, and currents found a significant association which explained 20% of the variance in the dependent variable ($R^2 = 0.201$, $F = 7.24$, $P = 0.01$; Table 2A). The partial regression coefficients of geographic distance and currents were significantly different from zero, indicating that both parameters are related to the distribution of lineages. The coefficients of temperature and depth were not significant. Since temperature is closely linked to currents we repeated the analysis twice, once by omitting the data on temperature and once the data on currents. Both regression functions were significant, with significantly large coefficients for temperature and currents, respectively (Table 2 B, C), suggesting that genetic structure is linked to variation in both temperature and currents. The coefficient for geographic distance, however, was only significant when the effects of currents were controlled for. Combining small populations ($N < 5$) with their nearest neighbors did not change the overall outcome ($R^2 = 0.395$, $F = 13.52$, $P = 0.009$).

TABLE 2. Multiple regression analysis for all lineages. Regression of frequency of lineages in populations on geographic distance (km) and difference in temperature (°C), depth (distance to shelf break: 1, near; 2, medium; 3, far), and currents (1, located on the same current, 0, located on different currents). In order to test for the linked effects of temperature and currents, the analysis was carried out including temperature and currents (A), temperature only (B) and currents only (C). Test of spatial autocorrelation: the *F*-statistic *P*-value of the regression of residual differences on geographic distances was 0.267. *P*-levels were determined with 5000 randomizations. Variables associated with significant *t*-values are in bold.

Variable	Partial *r*	*t*	*P*
A. Temperature and currents			
Distance	$\mathbf{-0.2937 \times 10^{-3}}$	**−3.55**	**0.012**
Temperature	0.3539×10^{-1}	2.85	0.093
Depth	-0.3989×10^{-1}	−1.11	0.361
Current	**0.2965**	**2.97**	**0.016**
$R^2 = 0.20$; overall $F = 7.24$ ($P = 0.010$)			
B. Temperature only			
Distance	-0.1349×10^{-3}	−2.07	0.156
Temperature	$\mathbf{0.4881 \times 10^{-1}}$	**4.09**	**0.025**
Depth	-0.3388×10^{-1}	−0.91	0.444
$R^2 = 0.140$; overall $F = 6.29$ ($P = 0.037$)			
C. Currents only			
Distance	$\mathbf{-0.2459 \times 10^{-3}}$	**−2.95**	**0.026**
Depth	-0.5078×10^{-1}	−1.37	0.237
Current	**0.4007**	**4.18**	**0.042**
$R^2 = 0.140$; overall $F = 6.53$ ($P = 0.017$)			

TCS Analysis of Lineage A

The maximum number of differences for parsimonious connections in the unrooted network was nine steps. Two divergent haplotypes in clade A could not be included in the network because they exceeded this maximum. The network consisted of three main groups (Fig. 4): group I contained mostly northern populations and was separated from group II by six steps. Groups II and III appeared widespread with several shared haplotypes between the two islands. At a local scale, populations generally harbored many diverse haplotypes.

Analysis of Molecular Variance of Lineage A

Molecular variance in *A. squamata* was geographically congruent with the echinoderm grouping of Pawson (group B; 1965), which partitioned northern and southern populations (11% of total variance explained; Table 3). The majority of variation was found within populations (51%); variation among populations accounted for 38% of the total (Table 3). In contrast, we found no evidence for a third, geographically central, phylogeographic region suggested from reef fish data (Francis 1996) or for mixing between northern and southern populations between Cape Turnagain and Kaikoura, as suggested from echinoderm data (Pawson 1965).

Multidimensional Scaling of Pairwise F_{ST} Values of Populations of Lineage A

Analysis of population pairwise F_{ST} values (see Appendix 1) by MDS illustrated the division between northern (1–6) and southern (12–16) populations, which make up discrete, nonoverlapping groups (Fig. 5). Geographically intermediate populations (7–10) clustered with northern populations, with the exception of a sample from the west coast of the North Island (9; Opunake), which was grouped with southern populations. The plot also suggests that a genetic break exists between the populations of Kaikoura (10) and Banks Peninsula (11), because these two populations were very distinct from each other and well embedded in their respective groups. Population 14 from Southland (Papatoetoe) was a clear outlier.

Isolation by Distance and the Effects of Environmental Variables on the Partitioning of Genetic Variation within Lineage A

Because a test for spatial autocorrelation (see Materials and Methods) found no significant association between the residuals of the regression analysis and geographic distance ($P = 0.542$; Table 4), subsequent significance testing was done by unrestricted Monte Carlo randomizations.

Multiple regression analysis of genetic distance on geographic distance and three parameters of the physical environment within lineage A revealed a significant association ($R^2 = 0.22$, $F = 8.16$, $P = 0.05$; Table 4A). The partial regression coefficients of genetic distance on geographic distance and currents were significantly different from zero, indicating that both of these parameters are related to the partitioning of genetic variation among populations. The coefficients for genetic distance on temperature and surrounding depth were not significantly high. Since temperature is closely linked to currents we repeated the analysis without temperature and currents, respectively. Again, currents were shown to have a significant effect, but temperature did not (Table 4 B,C). Combining small populations ($N < 5$) with their nearest neighbors again did not change the overall outcome ($R^2 = 0.262$, $F = 7.33$, $P = 0.018$).

DISCUSSION

Based on the brooding habit of *A. squamata* we set out to test whether this species is a poor disperser with high population genetic structure (possibly cryptic species) or, as its cosmopolitan distribution would suggest, a good disperser among distant geographic locations. Our data show that New Zealand harbors at least four highly divergent cryptic species of *A. squamata*, some of which are widely distributed throughout the area, lending some support to both of the competing hypotheses. We discuss the implications of our findings in detail below.

Extreme Genetic Diversity among Major Lineages

We identified four highly divergent lineages among *A. squamata* from New Zealand, separated by 12–53% sequence divergence, comparable to differences between ingroup and the morphologically highly differentiated outgroup taxa *Amphipholis gracillima* and *Amphiodia atra*. The distances among lineages A–D are extremely high compared to intraspecific mtDNA comparisons for other echinoderm taxa, which are typically below 3% (ophiuroids: Roy and Sponer 2002; sea urchins: Palumbi et al. 1997; Lessios et al. 1999;

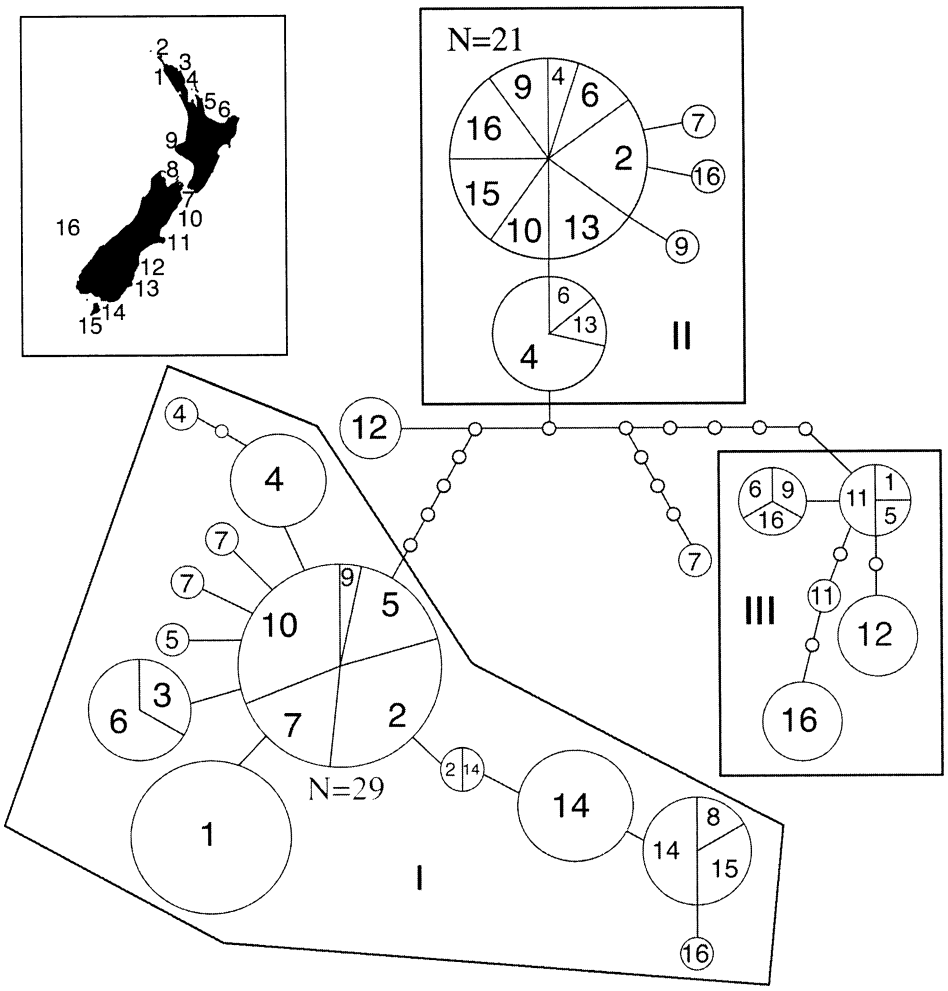

FIG. 4. Parsimony network of haplotypes from Clade A, constructed with the TCS computer program (Clement et al. 2000). Each big circle represents a unique haplotype and small empty circles indicate hypothetical, intermediate haplotypes not observed in the populations. Circle sizes and slices are drawn in proportion to relative frequencies of haplotypes and their occurrence in populations 1–16, respectively; numbering as in Fig. 1b.

McCartney et al. 2000; all these species have planktonic larvae).

There are three mutually nonexclusive explanations for the high levels of sequence divergence: (1) *A. squamata* may have an increased rate of mtDNA evolution compared to other taxa; (2) *A. squamata* may be an extremely old, single species; or (3) lineages A–D may represent distinct species. In order to investigate rate heterogeneity we compared 18S sequence data from a variety of taxa from all echinoderm classes (data taken from Littlewood et al. 1997) to *A. squamata* lineages

TABLE 3. Analysis of molecular variance of New Zealand *Amphipholis squamata* populations (lineage A). Groups were formed on the basis of biogeographic hypotheses from distribution patterns in reef fish (Francis 1996; group A) and echinoderms (Pawson 1965; group B; see Materials and Methods). Genetic distances between haplotypes were calculated according to the Kimura 2 parameter model. The significance of fixation indices was tested by 10,000 nonparametric permutations of groups, populations, and haplotypes.

Source of variation	df	Variance components	% variation	ϕ-statistic
Group A				
Among groups	2	−0.04364 Va	−1.35	−0.01353
Among populations	13	1.33797 Vb	41.48	0.40126***
Within populations	110	1.93136 Vc	59.87	0.40925***
Group B				
Among groups	1	0.39291 Va	11.25	0.11251***
Among populations	10	1.33123 Vb	38.12	0.42955***
Within populations	85	1.76793 Vc	50.63	0.49373***

*** $P < 0.0001$.

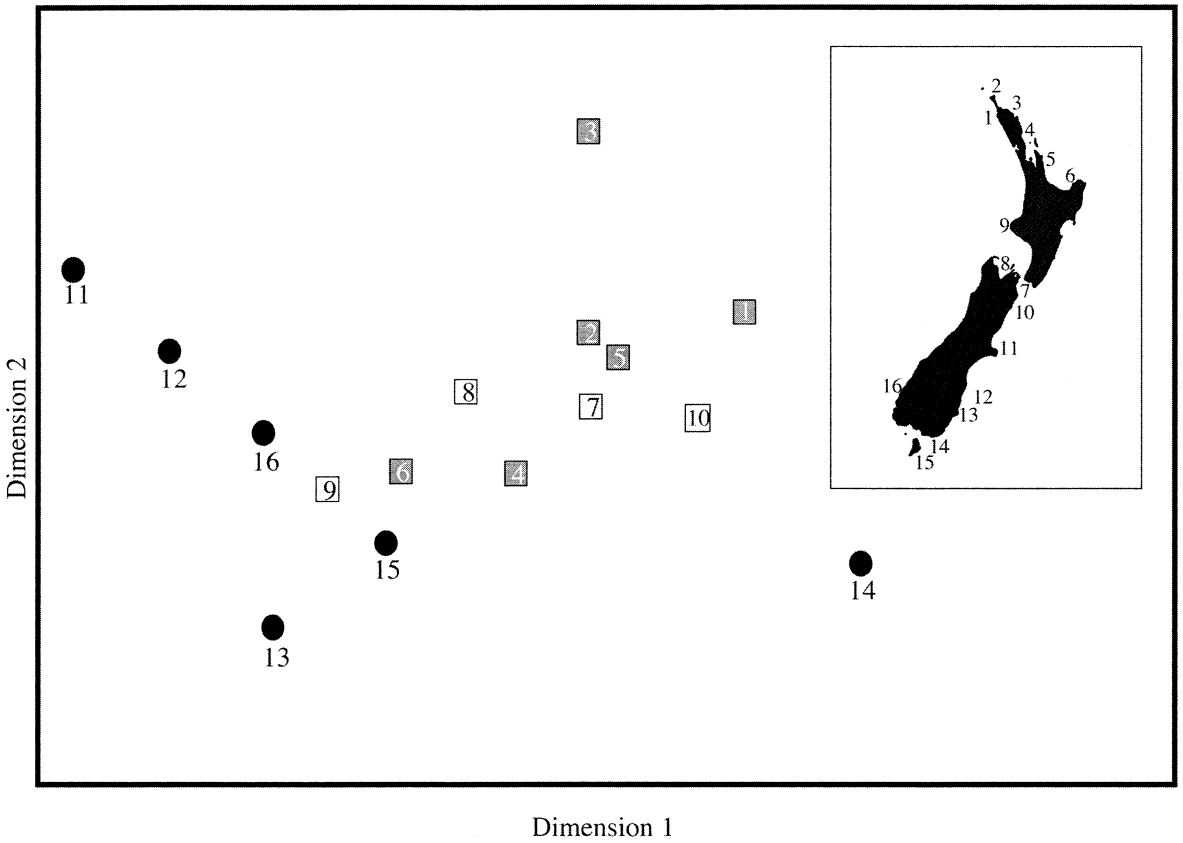

FIG. 5. Multidimensional scaling analysis of population pairwise F_{ST} values of lineage A (stress value = 0.12). Northern populations (1–6) are represented by gray squares, southern populations (11–16) by black circles, and intermediate populations (7–10) by clear squares.

TABLE 4. Multiple regression analysis for lineage A. Regression of genetic distance D_A (Nei and Li 1979) on geographic distance (km) and difference in temperature (°C), depth (distance to shelf break: 1, near; 2, medium; 3, far), and currents (1, located on the same current; 0, located on different currents). In order to test for the linked effects of temperature and currents, the analysis was carried out including temperature and currents (A), temperature only (B), and currents only (C). Test of spatial autocorrelation: the *F*-statistic *P*-value of the regression of residual differences on geographic distances was 0.542. *P*-levels were determined with 5000 randomizations. Variables associated with significant *t*-values are in bold.

Variable	Partial *r*	*t*	*P*
A. Temperature and currents			
Distance	$\mathbf{-0.3402 \times 10^{-2}}$	**−4.09**	**0.010**
Temperature	0.8381×10^{-1}	0.67	0.762
Depth	−0.5029	−1.39	0.288
Current	$\mathbf{0.4875 \times 10^{-1}}$	**4.85**	**0.002**
$R^2 = 0.22$; overall $F = 8.16$ ($P = 0.050$)			
B. Temperature only			
Distance	-0.3117×10^{-3}	−1.14	0.442
Temperature	0.3045	2.40	0.123
Depth	−0.4040	−1.02	0.432
Overall $F = 2.53$ ($P = 0.220$)			
C. Currents only			
Distance	$\mathbf{-0.3289 \times 10^{-2}}$	**−4.05**	**0.042**
Depth	−0.5287	−1.47	0.248
Current	$\mathbf{0.5122 \times 10^{-1}}$	**5.49**	**0.006**
$R^2 = 0.140$; overall $F = 10.78$ ($P = 0.003$)			

A–D (data not shown). These data corroborate the existence of the lineages recovered from 16S sequence. Furthermore, a relative-rates test of this combined dataset provided no evidence of an elevated evolutionary rate in *A. squamata*. These data strongly suggest that divergent lineages represent ancient evolutionary phenomena. In addition, it is unlikely that the lack of intermediate nodes in the *A. squamata* phylogeny is an artifact of insufficient sampling, since a comprehensive survey of populations worldwide has only revealed other highly divergent lineages (Sponer et al. 2001; Sponer 2002).

A growing number of widespread marine invertebrate taxa have been shown to consist of genetically divergent cryptic species existing in different parts of their overall geographic range (e.g., Knowlton et al. 1992; Knowlton 1993; De Vargas et al. 1999; Lee 2000; Colborn et al. 2001; Rocha-Olivares et al. 2001). A lack of morphological differentiation among sibling species may be common in the marine environment and *A. squamata* may represent another case of morphological stasis in the face of genetic divergence. In concordance with the phylogenetic species concept (Cracraft 1983), *A. squamata* lineages represent different species. Furthermore, nuclear ITS data provide compelling evidence for reproductive isolation of lineages in sympatry and therefore represent ''good species'' under the biological species concept (Mayr 1982). Together, these data provide compelling evidence for the existence of cryptic species within *A. squamata*.

Cryptic radiations have been characterized as ''nonadaptive'' or ''morphostatic,'' for their lack of anatomical and niche diversification. As a consequence, cryptic species should not be able to exist in sympatry, due to competition (Gittenberger 1991; Davis 1992). Indeed, in *A. squamata* morphological stasis persists despite the fact that divergent lineages (individually and together) exist in a wide range of habitats, climates, and depths. However, there are two aspects that make cryptic species in *A. squamata* particularly intriguing. First, lineages A–D in *A. squamata* are not allopatric. In fact divergent lineages commonly exist in syntopy (in the same aggregation) displaying no apparent microhabitat specialization and so presumably compete for resources. Although we have not specifically investigated the microspatial distribution of lineages in these aggregations, there is no indication of differential habitat use. It is conceivable that resources are plentiful and competition among *A. squamata* lineages is low, allowing them to use the same ecological niche. Alternatively, it remains possible that there are physiological, behavioral, or very small morphological differences that remain undetected (e.g., Deheyn and Jangoux 1999; but see Sponer et al. 2001). Second, in the great majority of other reported cases of sibling species, distinguishing morphological characters were found upon closer inspection (for a review, see Knowlton et al. 1992). In *A. squamata* this does not seem to be the case since a global morphometric study, using scanning electron microscopy, found no diagnostic differences among biogeographic groupings (Sponer 2002). Although the study did not include genetic data, it did include several specimens from New Zealand, now known to belong to lineages A, B, and C.

Explanations for morphological stasis have ranged from low mutation rate, to developmental constraints and long-term environmental stability (reviewed in Eldredge and Stanley 1984). We have shown that the mutation rate in *A. squamata* is comparable to that of other echinoderms. Given the wide ecological and geographic range of *A. squamata*, it is difficult to argue that stabilizing selection plays a role. Based on a case study from the fossil record, Lieberman and Dudgeon (1996) questioned the prominent role of stabilizing selection as a mechanism for stasis and predicted that if a species exists in several different environments it will be buffered against net change. It was further suggested that stabilizing selection may play an important role in maintaining stasis only if communities are at equilibrium or if the physical environment is stable over long periods of time. Neither of these requirements are likely to have been fulfilled throughout the (presumably) long evolutionary history of *A. squamata*. Sheldon (1996), on the other hand, has argued that morphological stasis should occur in unstable, changing environments, such as coastal habitats, while long-term stable habitats should promote gradualistic morphological evolution. His ''plus ça change-model'' predicts that initial major environmental perturbations (e.g., cycles of glaciation, with particularly high impact in coastal habitats) select for generalist phenotypes that are well adapted to subsequent environmental fluctuations. If true, this process could help explain how morphostasis could occur in a taxon as widespread as *A. squamata*, both in terms of geography and habitats.

Phylogeography and Population Genetics of A. squamata

In accordance with expected adult mobility, *A. squamata* was assumed to have low dispersal potential. We therefore posited that there would be significant local population genetic structure and isolation by distance. However, genetic structure was only observed at a regional level, between North and South Islands. It is intriguing that this division was observed in both the distribution of divergent lineages as well as the population genetic structure within lineage A. The congruence of phylogeographic patterns could be the result of a common vicariance process over several time periods, such as sea level changes during glacial and interglacial cycles. In fact the three distinct haplotype groups within lineage A (Fig. 4) appear to support a scenario of historic fragmentation and isolation. However, we believe that divergence of lineages in situ in New Zealand is unlikely since lineages A, B, and D have also been sampled in other regions of the world (Sponer 2002). Of these, lineage A is the most widespread, existing worldwide (Sponer 2002). Interestingly, sequence divergence (K2P) between haplotypes of lineage A from New Zealand and South Africa and between New Zealand and the North Atlantic was as low as 0% and 1.4%, respectively (Sponer 2002), suggesting unexpectedly high population connectivity over large distances. Indeed, within New Zealand we observed only weak local genetic structure and widespread existence of shared haplotypes, despite its assumed low dispersal potential. Surprisingly, currents were found to have a significant effect on the genetic structure of populations around New Zealand as highlighted by the location of the genetic break that demarcates North and South Island populations. The break, which is centered around Kaikoura and Banks Peninsula, coincides with the divergence of the Southland Current offshore towards the Chatham Islands (Fig. 1), which appears to represent a major marine barrier on the east coast of New Zealand, because a similar biogeographic pattern has also been found in other echinoderms, reef fish, and seaweeds (Pawson 1965; Parsons 1985; Nelson 1994; Francis 1996).

The distribution of lineages was also found to be correlated to temperature, suggesting that lineages may have different temperature optima (Table 2B). A strong correlation between the geographic distribution of *A. squamata* lineages and temperature patterns has also been found along the coast of South Africa (Sponer 2002), where temperature clines are steep, due to coastal upwelling. In comparison to South Africa, New Zealand experiences a narrower range of temperatures. Furthermore, the existence of divergent lineages in syntopy suggests that temperature plays a minor role in lineage distribution.

In conclusion, we believe that haplotypes of different lineages have sporadically colonized New Zealand by interoceanic dispersal (see section below). Their distribution has been subsequently shaped through isolation by distance, ocean currents, and temperature. The distribution pattern of haplotypes therefore depends, in part, on where the initial site of introduction occurred.

Cryptic Dispersal Mechanisms

There are a number of cryptic mechanisms that may allow species without free-living larvae to disperse. We believe that

the most plausible dispersal mechanism for *A. squamata* is by passive transport with drifting macroalgae or debris (epiplanktonic dispersal). There is a general lack of direct evidence that species can successfully disperse to new locations and establish new populations by this mechanism (Highsmith 1985; Jackson 1986). Epiplanktonic transport is nevertheless thought to play an important role in shaping the distribution of benthic marine organisms (e.g., Fell 1967; Miller 1968; Arnaud et al. 1976; Vermeij 1978), especially those that lack other dispersal methods (e.g., species with direct development or clonal reproduction). Algal epifauna can be transported by rafting whereby organisms cling to buoyant objects (driftwood, seaweeds, etc.) or by drifting inside benthic (unattached) algae on the sea floor (Holmquist 1994). Rafting appears to be restricted to cooler areas, where drift algae are abundant and float for long distances before deteriorating (Highsmith 1985; Johannesson 1988; Parker and Tunnicliffe 1994). Brittle stars may therefore be particularly prone to rafting and drifting as they commonly live within seaweeds (Hyman 1955). *A. squamata* has been observed in mats of floating algae (Washington, USA; Highsmith 1985) and in holdfasts of drifting *Macrocystis pyrifera* kelp (Tasmania; Edgar 1987). Both observations were made close to shore (maximum 1.2 km). In a field experiment it was also found that *A. squamata* can survive inside detached *Macrocystis pyrifera* kelp holdfasts for at least 191 days at sea (Edgar 1987). Although these observations do not provide direct evidence that *A. squamata* can effectively disperse by drifting or rafting, they demonstrate its potential to do so.

New Zealand has a rich seaweed flora that could serve to transport rafting brittle stars. We have collected *A. squamata* from many types of seaweed, including kelp holdfasts. If epiplanktonic transport is the predominant dispersal mechanism of *A. squamata* we might predict that population structure would reflect ocean current flows and geographic distance (since the likelihood of death and transport offshore increase with travel distance and time). The genetic structure we report is congruent with major current patterns in New Zealand and with isolation by distance. The genetic evidence of long-distance dispersal in *A. squamata* is consistent with occasional long-distance rafting events. We therefore believe epiplanktonic transport to be an important dispersal mechanism in *A. squamata* in New Zealand, which may also account for its worldwide distribution.

From the conclusion that rafting is an important mode of dispersal in *A. squamata*, we should expect that gene flow among shallow water populations is higher than between populations at depth. Unfortunately, we cannot test this prediction adequately in this study because only one deep-water population was sampled (12). Comparing this deep-water population to all shallow water populations yields inconclusive results: in lineages A and C, population 12 contains several unique and divergent haplotypes and does not have any haplotypes in common with other populations. However, in lineage B, population 12 shares the most common haplotype with a number of populations, throughout New Zealand. Future studies may test whether deep-water populations are indeed more genetically structured than their shallow water counterparts.

Alternative Scenarios

We cannot discount the possibility that *A. squamata* may be inadvertently transported through human activities, such as mariculture or possibly shipping (Roy and Sponer 2002). It is possible that individuals living in commercial mussel or oyster stands may be transported between sites, mimicking the effects of natural dispersal. There may be reason for suspicion in the case of shared haplotypes between the far south and the far north. Even if it is possible to raft the distance, half of the journey (in either direction) must be traveled against the major ocean currents. Interestingly, the genetic similarity of these southern populations (Stewart Island [15] and Papatoetoe [14]) with the north is mirrored in the distribution of lineages. Foveaux Strait (between Southland and Stewart Island) sustains an intensive greenshell mussel (*Perna canaliculus*) fishery that is known to introduce stock from elsewhere in New Zealand, including Northland (Hickman 1983). It is possible that these southern *A. squamata* populations have been affected by introductions of individuals from other (likely northern) areas.

Conclusions

Amphipholis squamata has been regarded to be a single cosmopolitan species. However, a host of recent studies suggest that the reportedly vast geographic distributions of many marine species are artifacts of poor taxonomy. Although these hypotheses remain to be tested for *A. squamata* worldwide, the present study confirms the existence of cryptic species, yet it appears that rafting, which has long been suggested to be a potent dispersal mechanism, can afford very large (possibly worldwide) distributions to some of those species.

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Appendix 1

Population pairwise F_{ST}-values for Clade A. Populations are numbered 1–16 as in Figure 1b. Significance levels associated with F_{ST}-values (from 10,000 permutations) are indicated by asterisks.

	1	2	3	4	5	6	7	8
1	0.0000							
2	0.2752***	0.0000						
3	0.4841**	0.3014*	0.0000					
4	0.3629***	0.0632	0.3712**	0.0000				
5	0.1765	0.0205	0.2349**	0.1534	0.0000			
6	0.4647***	0.1647	0.4220	0.0854	0.2451	0.0000		
7	0.2056***	−0.0357	0.2065	0.0958	−0.0576	0.2004*	0.0000	
8	0.3963**	0.2456*	0.2790*	0.2259*	0.1698*	0.1992*	0.1766*	0.0000
9	0.5497***	0.2496	0.5806*	0.1456	0.3258	−0.1618	0.2777	0.2116*
10	0.2633***	−0.0555	0.3431	0.1286	−0.0343	0.2782*	−0.0655	0.2670*
11	0.8222**	0.7359**	0.9389*	0.6589***	0.6963*	0.4834**	0.6974**	0.5043*
12	0.6675***	0.5691***	0.6799***	0.4946***	0.5168**	0.3087*	0.5330**	0.4138*
13	0.7766***	0.5124*	0.9680*	0.3567*	0.6467***	0.0228	0.5461**	0.3931*
14	0.5798***	0.4126***	0.8575**	0.4937***	0.4060***	0.6178***	0.3664***	0.3389*
15	0.5924***	0.2340	0.6649*	0.1358	0.3807**	−0.1208	0.2863*	0.1430
16	0.5602***	0.4185***	0.5451**	0.3406***	0.3962**	0.1036	0.4044***	0.3186**

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Appendix 2

Internal transcribed spacer restriction-fragment-length polymorphism scores of 35 *Amphipholis squamata* from Fiordland (population 17) and the deep site off Otago Peninsula (population 12).

Sample ID	mtDNA	ITS
883 FIORD	A	A′
901 DEEP	A	A′
967 DEEP	A	A′
968 DEEP	A	A′
969 DEEP	A	A′
970 DEEP	A	A′
976 DEEP	A	A′
982 DEEP	A	A′
1751 FIORD	A	A′
1752 FIORD	A	A′
1763 FIORD	A	A′
887 FIORD	B	B′
971 DEEP	B	B′
972 DEEP	B	B′
974 DEEP	B	B′
975 DEEP	B	B′
977 DEEP	B	B′
978 DEEP	B	B′
979 DEEP	B	B′
980 DEEP	B	B′
981 DEEP	B	B′
983 DEEP	B	B′
985 DEEP	B	B′
987 DEEP	B	B′
988 DEEP	B	B′
1759 FIORD	B	B′
1760 FIORD	B	B′
1762 FIORD	B	B′
898 DEEP	C	C′
899 DEEP	C	C′
900 DEEP	C	C′
904 DEEP	C	C′
905 DEEP	C	C′
906 DEEP	C	C′
995 DEEP	C	C′

APPENDIX 1. Extended.

	9	10	11	12	13	14	15	16
1								
2								
3								
4								
5								
6								
7								
8								
9	0.0000							
10	0.3857*	0.0000						
11	0.4779	0.8071**	0.0000					
12	0.2722*	0.6223***	0.1958	0.0000				
13	−0.0456	0.6817**	0.8747*	0.5207*	0.0000			
14	0.7227***	0.4639***	0.9473**	0.7759***	0.9444***	0.0000		
15	−0.1660	0.4035**	0.6649*	0.4298*	−0.0528	0.7567**	0.0000	
16	0.0292	0.4786***	0.1816	0.1195	0.2181	0.6553**	0.1580	0.0000