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Global population genetic structure of the starlet anemone *Nematostella vectensis*: multiple introductions and implications for conservation policy

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Abstract Distinguishing natural versus anthropogenic dispersal of organisms is essential for determining the native range of a species and implementing an effective conservation strategy. For cryptogenic species with limited historical records, molecular data can help to identify introductions. *Nematostella vectensis* is a small, burrowing estuarine sea anemone found in tidally restricted salt marsh pools. This species' current distribution extends over three coast lines: (i) the Atlantic coast of North America from Nova Scotia to Georgia, (ii) the Pacific coast of North America from Washington to central California, and (iii) the southeast coast of England. The 1996 IUCN Red List designates *N. vectensis* as "vulnerable" in England. Amplified fragment length polymorphism (AFLP) fingerprinting of 516 individuals from 24 *N. vectensis* populations throughout its range and mtDNA sequencing of a subsample of these individuals strongly suggest that anthropogenic dispersal has played a significant role in its current distribution. Certain western Atlantic populations of *N. vectensis* exhibit greater genetic similarity to Pacific populations or English populations than to other western Atlantic populations. At the same time, *F*-statistics showing high degrees of genetic differentiation between geographically proximate populations support a low likelihood for natural dispersal between salt marshes. Furthermore, the western Atlantic harbors greater genetic diversity than either England or the eastern Pacific. Collectively, these data clearly imply that *N. vectensis* is native to the Atlantic coast of North America and that populations along the Pacific coast and in England are cases of successful introduction.

Keywords AFLP · Anthropogenic dispersal · Asexual · Conservation · Introduction · *Nematostella vectensis*

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Introduction

Species introductions in coastal habitats have increased exponentially over the past century. In North America alone, 298 species of introduced marine organisms have established throughout coastal environments (Ruiz et al. 2000). Patterns of introduction are diverse. The west coast of North

America has experienced multiple introductions from the Indo-Pacific, and the east coast of North American has experienced multiple introductions from Europe. In addition, there is evidence of numerous intra-continental introductions among the Pacific, Atlantic, and Gulf coasts of North America (e.g. Wasson et al. 2001). The historical literature implicates intercontinental shipping as a principal explanation for the intensification of marine introductions in recent decades (Ruiz et al. 2000). Species that successfully establish in novel geographic regions can have dramatic, often detrimental effects on local ecosystems, both by decreasing organismal diversity and by reshaping habitats (e.g. Ruiz et al. 1999; but see Gurevitch and Padilla 2004). Although a number of studies have identified introduced marine species, few have characterized the avenue of introduction (Puth and Post 2005) or the pattern of establishment once a species has arrived (Sakai et al. 2001). Analyzing species introductions is critical to understanding how anthropogenic activities reshape natural ecosystems. In addition, the introductions themselves represent "natural" experiments that may reveal the dynamic processes involved in colonization events (Lee 2002).

Our understanding of coastal introductions is deficient in three important areas. First, the evidence required to reconstruct a species' native range is typically not available because the invasion pre-dates extensive habitat surveys or because the species is cryptic (Ruiz et al. 2000). Molecular data can compensate for the absence of a historical record, but relatively few molecular studies have addressed coastal introductions (e.g. Geller 1996; Bachelet et al. 2004; Roman and Palumbi 2004; Dawson et al. 2005; Ben-Shlomo et al. 2006). Second, disproportionate attention has been paid to animals with exclusively sexual reproduction. Many coastal invertebrates can reproduce asexually, which may significantly influence the pattern of colonization and the resulting impact on resident fauna and flora (Vrijenhoek 1998; Ting and Geller 2000; Brown and Eckert 2005). Indeed, in a variety of marine invertebrates, asexual reproduction has been shown to be a significant factor influencing the genetic structure of natural populations (Hoffmann 1986; McFadden 1997; Uthicke and Conand 2005; Zilberberg et al. 2006). Furthermore, numerous studies in terrestrial and aquatic systems have revealed that asexual reproduction can play an important role in the introduction and subsequent invasion of plant species (e.g. Amsellem et al. 2000; Eckert et al. 2003; Bossdorf et al. 2005; Lui et al. 2005; Li et al. 2006). Third, the importance of scale has been largely neglected; broad patterns of invasion have received greater emphasis than local dynamics of colonizing populations (Wasson et al. 2001; Roman and Palumbi 2004). Because local population dynamics and organismal dispersal ability have dramatic impacts on the geographic spread of a species (Palumbi 2004), a thorough understanding of local population structure is required to understand the forces that impact a species' regional or global distribution, particularly for introduced species (Ben-Shlomo et al. 2006; Pauchard and Shea 2006; Theoharides and Dukes 2007). A better understanding of gene flow and reproductive strategies at finer spatial scales can help inform hypotheses about likely factors influencing connectivity over broader geographic scales.

The starlet sea anemone, *Nematostella vectensis* Stephenson 1935, is an informative species for investigating the effects of natural and anthropogenic dispersal on the distribution of an estuarine organism at multiple levels of spatio-temporal resolution. *N. vectensis* is a small (typically <1 cm), infaunal organism inhabiting salt marshes, saline lagoons, and other sheltered estuarine environments. *N. vectensis* undergoes both sexual and asexual reproduction (Hand and Uhlinger 1992, 1994; Reitzel et al. 2007). Laboratory populations will readily undergo the entire ontogenetic repertoire, so ontogenetic factors that may influence population demographics and dispersal are amenable to experiment (Hand and Uhlinger 1992, 1994; Darling et al. 2005). Completely or largely clonal populations exist throughout the range of *N. vectensis* (Pearson et al. 2002; Darling et al. 2004). Reproductive plasticity is also likely to influence local population dynamics. Studies of reproductively plastic species offer some of the best opportunities to examine the adaptive significance of sexual versus asexual reproduction, particularly in the process of colonization (Vrijenhoek 1998).

Sexual reproduction and larval development have been described in some detail (Hand and Uhlinger 1992, 1994; Reitzel et al. 2007). The sexes are separate, and fertilization is external. Females package eggs into negatively buoyant, gelatinous egg

masses that are expelled through the oral opening. Several hours after fertilization, the ciliated planula larva emerges from the egg mass. The planula rapidly undergoes development into a juvenile polyp, which settles in soft substrate approximately 1-week after fertilization. In long-standing laboratory cultures (single-sex and mixed-sex), no evidence has been found for environmental sex determination, sex reversal in adults, or apomictic reproduction. Therefore, we assume that the production of larvae requires union of egg and sperm from males and females with genetic sex determination.

Asexual reproduction occurs through two distinct forms of transverse fission (Hand and Uhlinger 1992; Darling et al. 2005; Reitzel et al. 2007). In physal pinching, a small fragment of the aboral portion of the body column is cleaved, and it then regenerates missing oral structures over a period of several days. In polarity reversal, the aboral portion of the adult forms an oral crown, leading to the production of an individual with a mouth and tentacles at both ends of the primary body axis. Over a period of weeks, the animal grows in length until transverse fission midway between the two oral crowns separates two fully formed adult polyps (see Reitzel et al. 2007 for details). In both instances, the asexual propagules display very limited mobility, comparable to that of the adult polyp.

Nematostella vectensis's current global distribution is strongly suggestive of recent anthropogenic dispersal; despite apparent limitations on its natural dispersal ability (Darling et al. 2004), the species' range currently includes geographically isolated regions in the eastern Pacific, western Atlantic, northern English Channel, and western North Sea (Hand and Uhlinger 1994). Previous research suggests that North America may be the origin of English *N. vectensis* populations (Sheader et al. 1997; Pearson et al. 2002). The geographic isolation of *N. vectensis* in southern England and the prevalence of single-sex populations are consistent with recent introduction followed by asexual reproduction (Sheader et al. 1997). North American shellfish imports have been suggested as a probable vector (Sheader et al. 1997).

Previous molecular studies have raised the possibility that the English populations may have originated from recent anthropogenic introductions. Pearson et al. (2002) used RAPD markers to characterize the population genetic structure of *N. vectensis* populations in five estuaries in the south of England. Darling et al. (2004) used AFLP markers to characterize the population genetic structure of *N. vectensis* populations in nine New England estuaries. English populations were found to exhibit low levels of genetic diversity with no significant genetic structure between populations. A single genotype accounted for 61% of individuals sampled. By contrast, New England populations were found to exhibit greater genetic diversity with highly significant genetic structure between populations (as indicated by two estimates of F_{ST}). Unfortunately, different molecular markers were used in these two studies, precluding a direct comparison. *N. vectensis* populations on the Pacific coast of North America have not yet been studied using molecular markers. However, despite occupying a wide distribution from Washington to central California, many of the Pacific coast populations have been reported to harbor only females, suggesting that these populations are the result of introduction followed by extensive clonal reproduction, mirroring the situation found in England (Hand and Uhlinger 1994).

Information on global population structure may be required to formulate effective conservation policy for species with broad or cosmopolitan distributions (Paz et al. 2003; Nobrega et al. 2004; Block et al. 2005). Despite the existing evidence that it may have been introduced from North America, *N. vectensis* remains a matter of conservation concern in Britain. This species is protected under the Wildlife and Countryside Act and UK Biodiversity Action Plan (Anon 1995), and it maintains its designation as a vulnerable species in the IUCN Red Data Book (last assessed in 1996). To confirm the introduced status of *N. vectensis* in England and to identify possible source populations, additional genetic data are required from throughout the rest of the species current range including the mid-Atlantic and southeastern United States, regions that are known to have supplied shellfish to English consumers in the late 19th century (*Crassostrea gigas*, Yonge 1960). In this respect, *N. vectensis* may be emblematic of a broader pattern. The trans-Atlantic shellfish trade has been implicated in the introduction of several North American species to England (e.g., *Urosalpinx cinerea*, *Crepidula*

fornicata and *Asterias forbesi*, Yonge 1960; *Mercenaria mercenaria*, Mitchell 1974).

This study used molecular markers (AFLPs and *mt*DNA sequences) to characterize the population structure of *N. vectensis* at local and global scales in order to understand the relationship among far-flung populations in England, the western Atlantic, and the eastern Pacific. Natural dispersal between these coastlines appears unlikely given no evidence for circumarctic populations. Molecular data comparing populations between these two regions as well as from England can directly address which geographic areas comprise the native range of *N. vectensis*. If the animal's limited natural dispersal ability is responsible for the establishment of these far-flung populations, then they must represent ancient divergences, and therefore large genetic distances should separate English, western Atlantic, and eastern Pacific populations. Alternatively, if populations are the result of recent anthropogenic dispersal, we would expect introduced populations to be more closely related to geographically distant populations and that the greatest genetic distance would separate populations within the one coastline that encompasses the native range. Similarly, populations in introduced locales should show limited diversity due both to extreme founder events and the ability of *N. vectensis* to establish stable populations through clonal expansion by asexual reproduction.

The combination of AFLPs and *mt*DNA sequences has been successfully employed for studying genetic structure of both native and introduced populations (e.g. Timmermans et al. 2005; Grapputo et al. 2005). The abundant polymorphic markers provided by AFLP analysis are useful for estimating genetic diversity, for identifying population structure, and for inferring interpopulation relationships (Vos et al. 1995; Bensch and Åkesson 2005). The *mt*DNA sequences provide an independent genetic marker, and because the homology of nucleotide polymorphisms can be determined with greater confidence than the homology of restriction fragments, the *mt*DNA sequences are considered more reliable for the construction of rooted phylogenetic trees that may be used to pinpoint introductions and their sources (Bensch and Åkesson 2005).

Materials and methods

Sample collection

Nematostella vectensis was collected from estuarine pools and tidal creeks by sifting top sediments over nylon screens ($\sim$1 mm mesh size) from 2003 to 2004. Where possible, animals were collected from multiple pools at each site and from multiple points within each pool in order to minimize bias from spatially restricted clones. Animals were transported to the lab in marsh water and then transferred to fresh, low salinity artificial seawater ($\sim$12 ppt Instant Ocean, Aquarium Systems). Cultured anemones were maintained as described by Hand and Uhlinger (1992).

The Rhode River (MD) population used in this study was collected approximately 15 years ago. This particular population, which has been used for all molecular and developmental work previously reported for *N. vectensis* (reviewed in Darling et al. 2005), was initiated from dozens of founders and has been maintained at population sizes ranging from hundreds to thousands of individuals. The primary mode of reproduction for this population during the course of laboratory culture has been sexual. Given the large founding population and consistently high population size, it is unlikely that this population has experienced significant bottlenecks affecting neutral genetic diversity. Despite several attempts, we were unable to collect *N. vectensis* from the Rhode River site in 2003 or 2004 due to an apparent local extinction, thus we were unable to compare genetic diversity of the laboratory population with the source population originally collected 15 years ago.

In this study, we report on 24 populations, including nine New England populations sampled previously (Darling et al. 2004) and 15 additional populations representing portions of *N. vectensis*' current reported range that we had not previously sampled (e.g., England, Nova Scotia, the mid-Atlantic and southeastern United States, and the Pacific coast of the United States; Fig. 1). Altogether, these 24 populations account for nearly the entire reported range of the species (Hand and Uhlinger 1994) and include one previously unreported location (Halifax, NS). Our sampling does not include any populations previously identified along the Gulf Coast of the United States. We attempted to collect *N. vectensis*

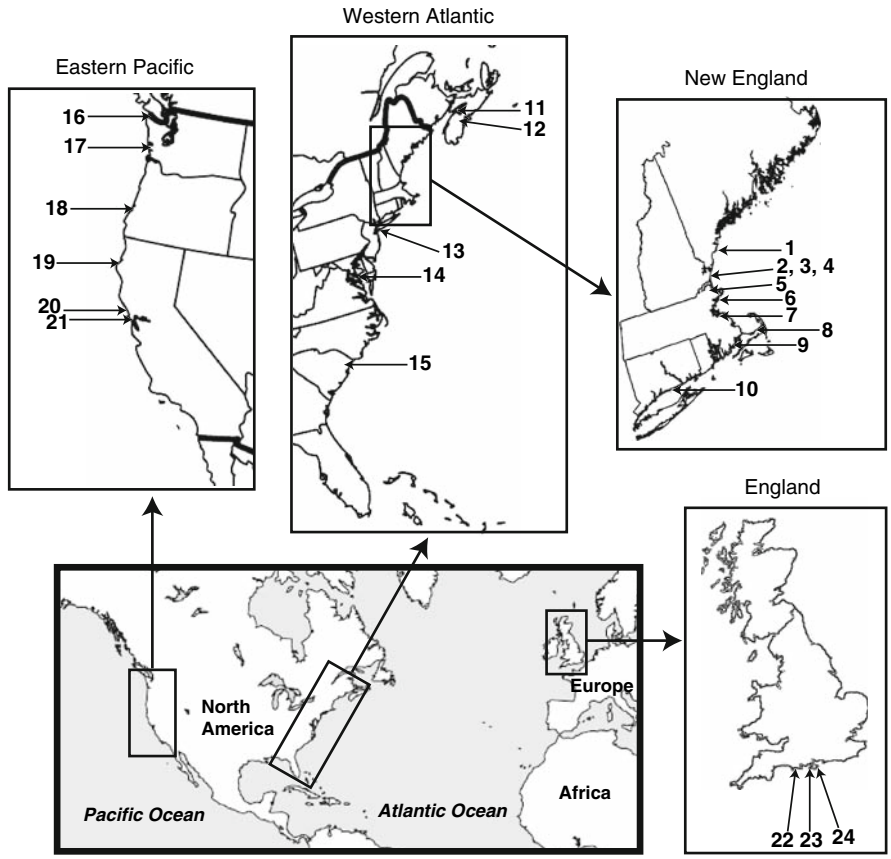

Fig. 1 Geographic distribution of *Nematostella vectensis* populations collected in this study. Populations were collected from three distinct regions, Eastern Pacific coast of the United States, Western Atlantic coast of the United States and Canada, and the southern coast of England. Site numbers correspond to populations in Table 1

from many estuaries in this region in 2005, some previously identified as containing *N. vectensis* (St. Marks, FL, Ocean Springs, MS, and Golden Meadow, LA), but were unsuccessful. For some sites composed of extensive salt marsh habitat, we sampled multiple subpopulations in order to obtain a more complete representation of the local genetic diversity (Table 1). Sample sizes varied according to population density, site accessibility, and the number of individuals provided by collaborators (Table 1).

AFLP fingerprinting

Nematostella vectensis DNA was extracted using the DNeasy kit (Qiagen) from small tissue fragments removed from the pedal end of each individual. Individuals used for DNA extraction were starved for a minimum of 5 days prior to extraction minimizing the risk of spurious amplification of contaminating DNA from undigested prey items. AFLP fingerprints were generated utilizing commercially available restriction site adaptors and fluorescently labeled AFLP primers (PE Applied Biosystems). Protocols for producing AFLP fingerprints, including restriction-ligation and PCR reactions, were as described in Darling et al. (2004).

Fingerprints were visualized on 5% Long Ranger XL polyacrylamide gels (BioWhittaker Molecular Applications), 36 cm well-to-read distance, using an ABI-377 automated sequencer with GENESCAN analysis software. Bands were sized using internal GENESCAN-500 ROX size standards, and scored semi-automatically using GENOTYPER 2.5 software. Within GENOTYPER, significant electropherogram peaks (>500 intensity units) in the range of 100–500 bp were used to define categories (all peaks within ±0.5 bp) for potential AFLP markers. In some cases, neighboring categories showed irresolvable overlap, such that GENOTYPER was unable to consistently assign a peak to one of the two categories; these categories were eliminated from the final data analysis. Datasets were generated by labeling all peaks recognized by GENOTYPER, and then using

Table 1 Summary of *Nematostella vectensis* sampled populations (see Fig. 1 for geographic locations) and AFLP results

Name of site		N	N_S	N_G	F_G	P	H_E
1. Spurwink River	ME	31	1	31	–	45.1	0.18266
2. Odiorne Point	NH	8	1	8	–	20.3	0.11802
3. Wallis Sands	NH	24	1	24	–	24.2	0.10575
4. Rye Harbor	NH	9	1	9	–	21.6	0.12244
5. Old Town Hill	MA	6	1	2	83	3.3	0.02359
6. Crane	MA	24	1	11	64	7.8	0.02779
7. Neponset River	MA	36	1	12	69	26.1	0.05291
8. Pocassett	MA	20	1	20	–	29.4	0.12886
9. Sippewissett	MA	36	4	36	–	58.2	0.17780
10. Clinton	CT	24	2	24	–	33.3	0.09560
11. Halifax	N.S.	17	1	17	–	24.2	0.11981
12. Kingsport	N.S.	34	3	34	–	34	0.09140
13. Meadowlands	NJ	18	1	18	–	26.1	0.13622
14. Rhode River	MD	16	1	16	–	68.6	0.18034
15. Baruch	SC	36	5	36	–	49	0.29120
16. San Juan Island	WA	33	4	24	18[a]	23.5	0.04580
17. Willapa Bay	WA	5	1	4	40[a]	11.1	0.08226
18. Coos Bay	OR	36	1	36	–	43.1	0.17956
19. Humboldt Bay	CA	35	4	32	6	24.8	0.05080
20. Bodega Bay	CA	14	1	14	–	19	0.12005
21. Tomales Bay	CA	18	2	18	–	25.5	0.10750
22. Gilkicker	Eng	12	1	5	67[b]	3.9	0.02000
23. Near Salterns	Eng	14	1	5	61[b]	1.3	0.00915
24. Salterns	Eng	10	1	5	67[b]	2.6	0.00832
Total		516	41	440	2.9	98	0.10870

A total of 153 AFLP markers were used for data analysis of 516 individuals

N, Number of individuals; N_S, number of sub-populations; N_G, number of distinct genotypes; F_G, frequency of the most common genotype (% of total); P, percent polymorphic loci; H_E, average expected heterozygosity

[a] Most common genotype shared between San Juan Islands and Willapa Bay

[b] Most common genotype shared between all three English populations

the table functions of GENOTYPER to record any of these peaks that fell within the defined categories. All data were checked by visual inspection of electropherograms to correct peak calls missed by GENOTYPER. GENOTYPER data tables were exported to Microsoft Excel and converted to binary form (presence/absence) for subsequent analysis. For this study, 516 individuals were genotyped using two different primer pairs. These two primer sets have been shown to generate highly robust and reproducible AFLP genotypes for *N. vectensis* (Darling et al. 2004).

The widespread occurrence of clonal reproduction provided a natural test of the repeatability of the AFLP methods used in this study (Darling et al. 2004). For example, the 28 individuals collected from Crane Reservation, MA, were found to exhibit identical AFLP fingerprints—clearly the result of clonal reproduction. One-hundred percent of the electropherogram peaks obtained in these 28 AFLP fingerprints were repeatable across all reactions using both primer sets. In addition to fingerprint consistency between individuals from clonal populations, we also generated replicate AFLPs, beginning from DNA, for ten randomly chosen individuals. In this case, the two replicated electropherograms were also identical for the peaks used in our data set, again supporting repeatability of the AFLP reactions for this species.

Data analysis

Allele frequencies at all loci were estimated from dominant AFLP marker data using the software AFLP-SURV 1.0 and applying a Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky 1999). Genetic diversity within populations was assessed by estimating unbiased expected heterozygosities (Nei 1972) and percent polymorphic loci. It is important to note that estimates of heterozygosity based on dominant markers can be subject to substantial biases if the population deviates markedly from Hardy–Weinberg equilibrium (Zhivotovsky 1999). Genetic diversity was compared between the three geographic regions representing *N. vectensis*'s range (i.e. Pacific coast, Atlantic coast and England) with Mann–Whitney *U*-tests. Significance of regional differences in genetic diversity was determined based on two measures of genetic diversity (expected heterozygosity and percent polymorphic loci). The potential impact of genetically distinctive populations on regional population structure was examined by removing these populations from the analysis (e.g. Baruch removed from Atlantic and Coos Bay from Pacific).

Estimates of Wright's F_{ST} were obtained using AFLP-SURV 1.0 to determine the degree of genetic

differentiation between populations at various spatial scales. For global and regional F_{ST}, each collection site was considered a separate population; for local F_{ST}, each individual pool was considered a subpopulation within the collection site. To determine the significance of F_{ST} values, observed values were compared to a null distribution of values for 1,000 pseudo-random permutations of individuals. Differentiation between regional and local populations was also assessed by conducting analysis of molecular variance (AMOVA) using ARLEQUIN version 2.0 (Schneider et al. 2000). As clonal reproduction by distinctive genotypes could unduly influence analyses of population structure, we replicated the analyses for AMOVA and F_{ST} after excluding duplicate clonal genotypes.

Unbiased genetic distances (Nei 1978) were calculated between pairs of individuals and between pairs of populations using AFLP-SURV 1.0. Neighbor-joining trees were generated from these distance matrices using the NEIGHBOR program of PHYLIP (version 3.6; Felsenstein 2004). Trees were drawn and edited using TREEVIEW (Page 1996). Support for particular nodes on the population-level neighbor-joining tree was determined using 1,000 replicates of the bootstrap. Bootstrap proportions $\geq$0.50 are indicated at the respective nodes on the tree.

We tested for isolation by distance within each coastline (Pacific, western Atlantic, England) and for combinations of western Atlantic plus England and western Atlantic plus Pacific using IBD v1.52 (Bohonak 2002) on distances obtained from the AFLP data. Geographic distance was calculated by estimating coastal distance between sites along the same coastline. For comparisons between coastlines, we determined the shortest straight-line distance. These analyses were repeated after removing duplicate, clonally related genotypes from the dataset.

*mt*DNA sequence

For a subsample of anemones from each genetically distinct population (54 individuals from 23 populations), a 487-bp fragment of cytochrome oxidase I mtDNA was amplified using the primers LCO_1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO_2198 (5′-TAAACTTCAGGGTGACCAAAAA TCA-3′, Folmer et al. 1994). The amplifications were carried out in a total volume of 20 µl, with 1× PCR buffer (New England Biolabs), 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 2 pmol each primer, and 1 U Taq polymerase (New England Biolabs). PCR amplifications were performed in a PTC-200 thermocycler (MJ Research) with the following temperature cycling profile: 2 min denaturation at 94°C followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 60 s at 47°C, and extension for 30 s at 72°C. Gel-purified products were sequenced directly with BigDye Terminator (Applied Biosystems). DNA sequences were aligned using Clustal W (Thompson et al. 1994).

Results

Nuclear variability

The AFLP analyses identified 156 distinct bands of which 153 (98%) were polymorphic. Based on these 153 polymorphic markers, the 516 individuals assayed exhibited 440 unique genotypes. Genetic diversity within sites was measured as both percent polymorphic loci (P) and average expected heterozygosity (H_E). Both of these measures varied extensively between locations (Table 1). The three English populations and two of the Massachusetts populations (Old Town Hill and Crane) exhibited the lowest values for P and H_E. Each of these populations was dominated by a single genotype. Populations along the Atlantic Coast of North America (e.g. Rhode River, MD, Sippewissett, MA, and Baruch, SC) exhibited the highest values for P and H_E.

The Atlantic coast of North America exhibited significantly higher genetic diversity than England or the eastern Pacific (Table 2). Removal of geographic or genetic outliers within regions does not affect conclusions about regional genetic diversity. Even after excluding Baruch, SC, the southernmost Atlantic population, the Atlantic region still exhibited higher genetic diversity than the Pacific or England. Likewise, after excluding Coos Bay from the Pacific coast data set, the Pacific region still exhibited significantly lower genetic diversity than the Atlantic region. Based on a neighbor-joining analysis (see below, Fig. 2), Coos Bay does not appear to be closely related to other Pacific coast populations.

Table 2 Mann–Whitney *U*-tests for significance of difference in genetic diversity for *Nematostella vectensis* populations based on percent polymorphic loci (P) and average expected heterozygosity (H_E) from AFLPs among three geographic regions: North America Atlantic coast (NA Atlantic), United States Pacific coast (US Pacific), and England

	Average value for region
NA Atlantic versus US Pacific	
P:	(39.484 vs. 12.715)***
H_E:	(0.139 vs. 0.0728)**
NA Atlantic versus England	
P:	(39.484 vs. 2.600)***
H_E:	(0.139 vs. 0.0125)***
NA Atlantic (no Baruch, SC) versus US Pacific	
P:	(33.145 vs. 12.715)***
H_E:	(0.119 vs. 0.0728)**
NA Atlantic (no Baruch, SC) versus US Pacific (no Coos Bay, OR)	
P:	(33.145 vs. 10.183)***
H_E:	(0.119 vs. 0.0639)**
NA Atlantic (no Baruch, SC) versus England	
P:	(33.145 vs. 2.6)**
H_E:	(0.110 vs. 0.0125)***

Subsets of the total populations were also analyzed to test for effects of outlying populations within a region (see Materials and methods for description). Table lists average values for measure of genetic diversity and significant differences (*P* values) for testing whether the more diverse region has significantly greater diversity than the less diverse region

** $P < 0.01$

*** $P < 0.001$

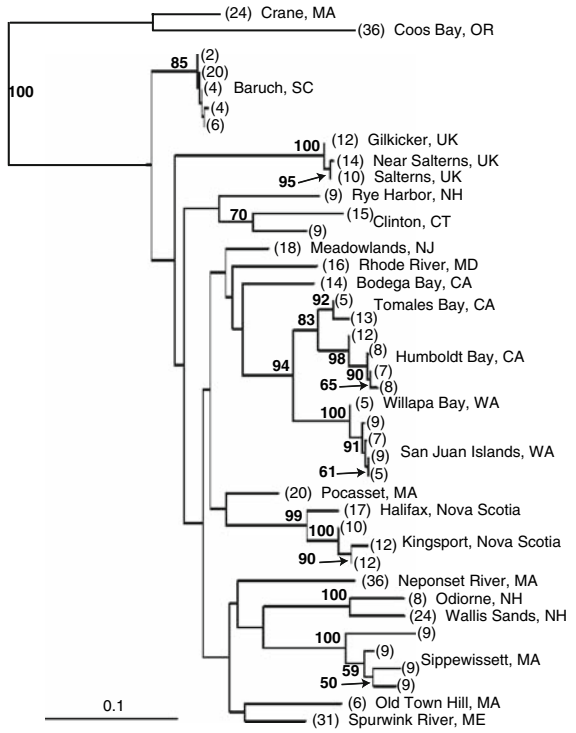

Fig. 2 Neighbor-joining tree for sampled *Nematostella vectensis* populations. All 516 individuals were included separately in the analysis, but each individual terminal branch corresponds to a sub-population or population. Geographic subpopulations were sampled at Baruch, Sippewissett, Kingsport, San Juan Island, Humboldt Bay, and Tomales Bay. The number of individuals represented by each terminal branch is shown in brackets. Bootstrap values over 50% are shown next to corresponding nodes. Scale bar at lower left is Nei's genetic distance. Tree was arbitrarily rooted with Crane and Coos Bay as they represent the most genetically distant populations

Hierarchical AMOVA indicated that a majority of the variation (52.66%) was attributable to differences between populations within regions (Table 3). Genetic variation within populations (25.17%) was also significant ($P < 0.0001$) as was variation among geographic regions (28.72%, $P < 0.0001$). The removal of duplicate identical AFLP profiles (presumably attributable to clonal reproduction) did not substantially alter the partitioning of genetic variation: similar proportions of the overall variation could still be attributed to differences among populations within regions (45.21%), differences within populations (29.84%), and differences among regions (24.96%).

A high degree of genetic divergence among populations and among subpopulations was also supported by F_{ST} values (Table 4). Across all populations under study, the F_{ST} value was extremely high ($F_{ST} = 0.6123$, $P < 0.0001$) indicating extensive genetic differentiation between populations. The removal of duplicate identical AFLP profiles from the dataset had little impact on the overall F_{ST} for all populations ($F_{ST} = 0.6055$, $P < 0.0001$). F_{ST} values also reveal a high degree of genetic divergence within particular populations (e.g. Nova Scotia: $F_{ST} = 0.2253$, $P < 0.0001$).

For six of seven sites where multiple subpopulations were sampled within a site, genetic differentiation was also high between individual subpopulations (Table 4). Baruch was the only population that did not display significant genetic divergence at the subpopulation level ($F_{ST} = \sim 0$, $P = 0.9813$). Consistent with the analysis from all

Table 3 Hierarchical AMOVA for *Nematostella vectensis* populations

Source of variation	Degrees of freedom	Sum of squares	Variance components	% Total	*P* value
Among regions	4	3073.01 (2696.72)	7.105 (5.965)	28.72 (24.96)	<0.0001 (<0.0001)
Among populations					
Within regions	19	4963.70 (3528.31)	12.772 (10.806)	52.66 (45.21)	<0.0001 (<0.0001)
Within populations	475	3003.61 (2973.97)	6.104 (7.132)	25.17 (29.84)	<0.0001 (<0.0001)
Total	515	11040.31	24.255		

AFLP variance was partitioned into three groups: variance among regions, variance among populations within regions, and variance among subpopulations within populations. Five regions were defined: England, North American Atlantic, U.S. Pacific, Baruch, and Coos Bay. Baruch and Coos Bay were treated separately due to their status as either geographic or genetic outliers. Values in parentheses were calculated after the removal of duplicate clonal genotypes

Table 4 Wright's F_{ST} calculated over all *Nematostella vectensis* populations, for subpopulations at seven sites, and for three broader geographic regions (Nova Scotia, England, and US Pacific)

Population	All individuals		Clones removed	
	F_{ST}	*P*	F_{ST}	*P*
All populations	0.6123	<0.0001	0.6055	<0.0001
Sippewissett	0.2046	<0.0001	–	–
Clinton	0.4919	<0.0001	–	–
Kingsport	0.0831	<0.0001	–	–
Nova Scotia	0.2253	<0.0001	–	–
Baruch	–	0.9813	–	–
San Juan Island	0.0934	0.0030	0.1075	0.0420
Tomales	0.0541	0.0180	–	–
Humboldt	0.1420	<0.0001	0.1388	<0.0001
England	–	0.3137	–	0.1734
US Pacific[a]	0.4641	<0.0001	0.4428	<0.0001

Analyses for only unique genotypes, when present, were repeated for all populations as well as any region or location where clonal genotypes were identified. Subpopulations represent those individuals collected from isolated pools within sites. For the number of subpopulations at each site see Table 1

[a] Not including Coos Bay

populations, excluding duplicate identical AFLP profiles from the analysis of genetic differentiation did not impact F_{ST} values for analysis of populations or regions (Table 4).

The neighbor-joining analysis does not resolve the populations from different regions into three distinct groups (Fig. 2). While the English populations are clustered together with high bootstrap support (BP = 100%), the tree cannot be rooted so that either the Pacific region or the western Atlantic region comprises a distinct subtree. The populations from California and Washington formed a cluster, with particularly high bootstrap support (BP = 94%) for a group uniting two California populations (Tomales Bay and Humboldt Bay) with two Washington populations (Willapa Bay and San Juan Islands). The population from Coos Bay, OR does not appear closely related to any other Pacific coast population in our data set. In 100% of bootstrap replicates, the highly divergent populations from Coos Bay and Crane, MA clustered together. The clustering of these two populations does not appear to be an artifact of long branch attraction as the genetic distance between Crane and Coos Bay was approximately half the distance between Crane or Coos Bay and the next closest population, Baruch, SC (pairwise distance: 0.021 vs. 0.035). In addition, a close inspection of the AFLP electropherograms revealed several genetic makers shared by Crane and Coos Bay that were not present in the other sampled populations (Fig. 3).

The English populations do not appear particularly closely related to any of the North American populations in the neighbor-joining tree. Pairwise comparison of genetic distances indicated that populations from England were most similar to the population from Baruch, SC, with the smallest distance between the English populations and a Baruch subpopulation (pairwise distance: 0.1494 for Gilkicker, 0.1534 for Salterns, 0.1522 for near Salterns). Populations from California and Washington grouped most closely to populations from the Mid-Atlantic coastline (Maryland, NJ).

At the level of individual anemones, the neighbor-joining analysis generally clustered individuals collected from the same population into discrete subtrees exclusive of individuals from other populations

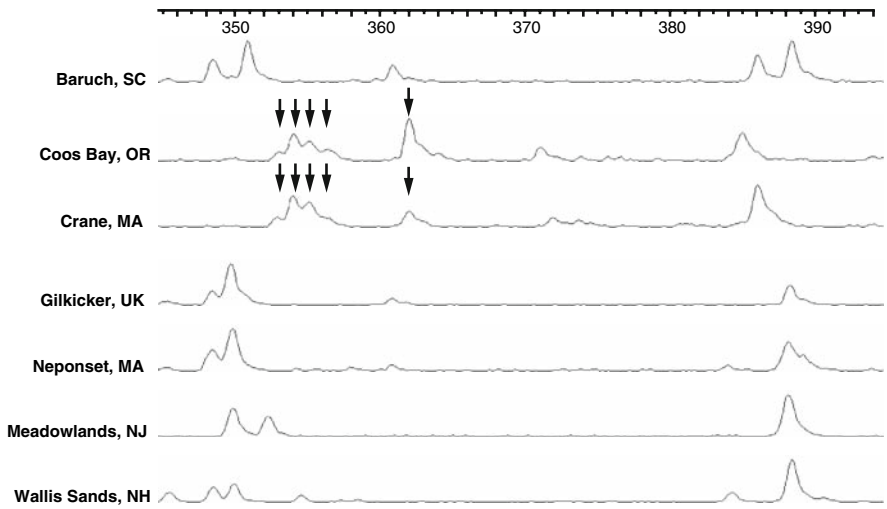

Fig. 3 Electropherograms showing some of the genetic markers that are unique to populations of *Nematostella vectensis* from Coos Bay, OR and Crane, MA. These markers (indicated by arrows), as well as pairwise genetic distance and the clustering of these two populations in Fig. 2, support the conclusion that these populations are more closely related to one another than to other populations despite their geographic distance. Overall, the results suggest that these populations have been anthropogenically dispersed, potentially from the same source location that was unsampled in our data set

(Fig. 4). Excluding those cases where a single genotype was shared among two or more populations (Washington and English populations), this general pattern was disrupted by only ten individuals (out of 516) that fail to group with other individuals collected at the same location. All ten of these individuals represent relatively long branches—not one of these individuals appears particularly closely related to individuals from either its own location or any other location.

At a finer scale, the neighbor-joining analysis revealed individuals from some subpopulations to group together to the exclusion of individuals from other subpopulations at the same site (Fig. 5). For example, at Sippewissett, MA, the eight individuals from one subpopulation (pool four) constitute a discrete subtree despite the proximity of individuals from neighboring pools only a few tens of meters distant. Similarly, at Clinton, CT, the nine individuals from pool one constitute a discrete subtree. The clustering of genotypes at this fine scale suggests that gene flow among subpopulation within these sites is relatively low. This is consistent with the high degree of within-site genetic differentiation indicated by AMOVA and F_{ST} (Tables 3 and 4, respectively).

In all cases, there was no support for significant isolation by distance. Mantel tests indicate no significant correlation between genetic and geographic distance across all populations ($r = 0.1387$, $P = 0.140$) or within each coastline (Pacific: $r = -0.1664$, $P = 0.700$; Atlantic: $r = 0.1338$, $P = 0.201$; England: $r = 0.8564$, $P = 0.341$). Exclusion of duplicate clonal genotypes did not impact the results of these comparisons—Mantel tests were still insignificant when all populations were compared ($r = 0.1273$, $P = 0.154$) or when each coastline was analyzed separately (Pacific: $r = -0.1702$, $P = 0.707$; Atlantic: $r = 0.1209$, $P = 0.627$; England: $r = 0.8126$, $P = 0.330$).

We observed evidence of clonal reproduction in 9 of the 24 sampled populations (Table 1). In six of these populations (three Massachusetts populations—Old Town Hill, Crane and Neponset—and the three English populations), a single genotype represented a majority of the sampled individuals from that location. The recovery of the same genotype at more than one geographically isolated population was observed only in Washington, where the same genotype was found at both San Juan Island and Willapa Bay, and England, where the same genotype was found at Gilkicker, Salterns, and near Salterns. We found no evidence of asexual reproduction—two or more individuals exhibiting the same genotype—in any of the other 15 locations sampled.

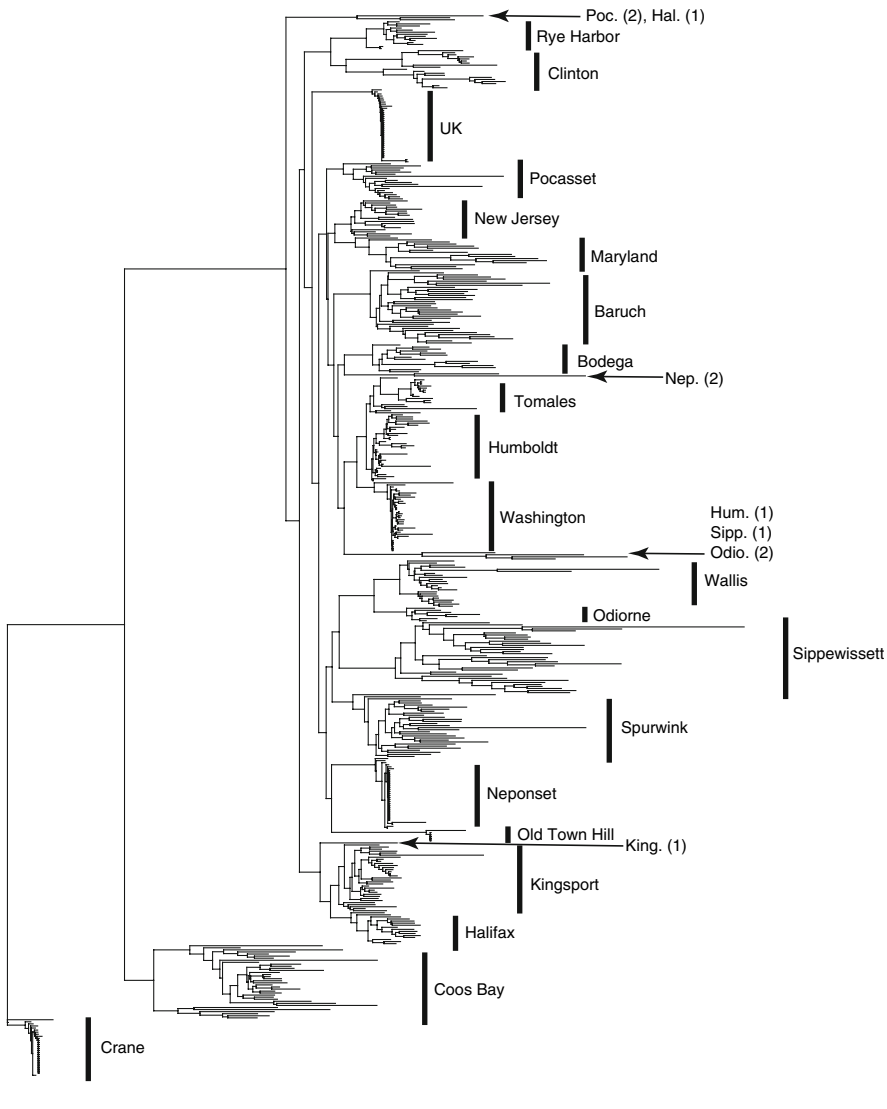

Fig. 4 Neighbor-joining tree representing the relationship of all *Nematostella vectensis* individuals. A majority of individuals (506 of 516) grouped with other individuals from the same location. In rare cases, some individuals did not cluster with either their respective population or any other population. Arrows indicate these individuals. Abbreviations for these individuals are Poc (Pocasset), Hal (Halifax), Nep (Neponset), Hum (Humboldt), Sipp (Sippewissett), Odio (Odiorne), and King (Kingsport)

mtDNA variability

Sequencing of a 487-nucleotide fragment of cytochrome oxidase I from 54 individuals from 23 populations identified 2 haplotypes (GenBank accession numbers DQ538492 to DQ538493). Fifty individuals shared the dominant haplotype. Four individuals in New England differed by a single point mutation at position 337. All individuals sequenced from the Pacific coast ($n = 16$) and England ($n = 7$) shared the dominant haplotype found in 27 of 31 individuals from the western Atlantic.

Discussion

Global population structure and instances of introduction

The data presented here rule out the existence of deep genetic divergences between populations from the western Atlantic, England, and the eastern Pacific. While no single line of evidence is decisive, in aggregate, the data strongly suggest that both the English and Pacific populations are the result of recent introductions from the western Atlantic (Table 5). The Pacific and English populations

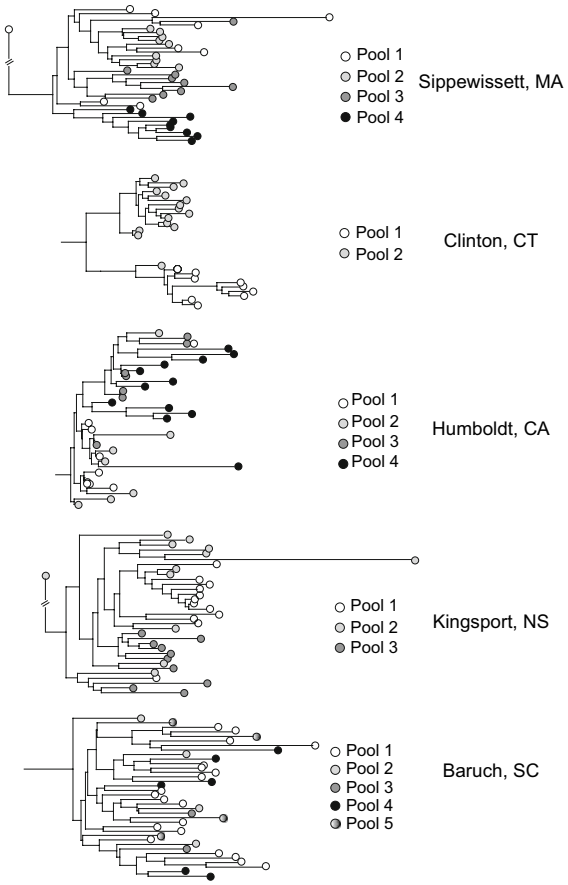

Fig. 5 Portions of a neighbor-joining tree for populations of *Nematostella vectensis* where two or more subpopulations were sampled. Terminal branches with circles at the ends represent individuals from pools within a location

exhibit significantly less genetic diversity than western Atlantic populations, and they appear more closely related to particular western Atlantic populations than are these western Atlantic populations to other western Atlantic populations. A similar pattern has been reported in introductions of other coastal species from Europe to North America (Berg et al. 2002; Roman 2006), although persistently reduced genetic diversity may not be as a common feature of introduced populations as previously assumed (Roman and Darling 2007). According to the AFLP data, the Atlantic coast of North America exhibits significantly higher genetic diversity than either the Pacific or English coasts (as assayed by either percent polymorphic loci or expected heterozygosity). Our findings provide the first comparative genetic data to corroborate previous suggestions that *N. vectensis* has western Atlantic origins (Sheader et al. 1997; Pearson et al. 2002).

Anemone populations in the Pacific and England share a single mitochondrial haplotype that is also found in populations along the Atlantic coast of North America. Despite the general utility of mitochondrial genes for population genetic analysis (Avise 2004), the overall lack of mtDNA variation in *N. vectensis* populations may not be surprising given evidence for slow mtDNA evolution in other anthozoans (Shearer et al. 2002). However, despite the limited utility of mtDNA sequence variation for resolving population structure in *N. vectensis*, the fact that all of the haplotype diversity we observed is expressed within a handful of western Atlantic populations is consistent with the AFLP analysis, supporting the conclusion that Pacific and English populations are the result of anthropogenic introduction. The alternative hypothesis, that Pacific and English populations are ancient populations native to their respective regions, is not supported. If these were native populations, one would expect unique polymorphisms contained in populations from these coastlines that would differentiate them from populations along the Atlantic coast of North America.

Excepting Coos Bay, OR, the neighbor-joining analysis of the AFLP genotypes supports a close relationship between Pacific coast and Mid-Atlantic populations (Rhode River, MD and Meadowlands, NJ), suggesting a potential source region for these Pacific populations. Four of the five Pacific coast populations clustered together with high bootstrap support, suggesting either a single introduction that has subsequently spread along the coast or repeated introductions from the same source population. A previous synopsis of *N. vectensis* sex ratios within Pacific populations suggested that all individuals collected from California and Washington estuaries were female (Hand and Uhlinger 1994), again supporting a singular introduction with lateral spread. However, a laboratory culture derived from Tomales Bay (CA) has produced embryos that became adults, indicating that males are present at this site. In addition, there is evidence that males may also reside at low densities in a population at Padilla Bay, WA (Harter 1997; Harter and Matthews 2005), a population not sampled for this study. Therefore, we think that it is likely that males are present in more of these Pacific coast populations, but they may be rare.

Table 5 Evidence favoring anthropogenic over natural dispersal as a cause for the current distribution of *Nematostella vectensis* in discontinuous coastal regions (western Atlantic, Pacific, and England)

	Potential causes of current distribution			
	Natural dispersal from unknown ancestral native range		Anthropogenic dispersal to England and Pacific from native range in western Atlantic	
Predicted consequences				
Age of divergence between populations on different coastlines	Millions of years	No	Hundreds of years	Yes
Genetic distances between different coastlines	Extremely high; much higher than genetic distances between populations within coastlines	No	Extremely low; on the same order as genetic distances between populations within coastlines	Yes
Coastline specific polymorphisms	Common	No	Rare or absent	Yes
Genetic diversity within coastlines	Comparable	No	Greater diversity within the western Atlantic	Yes
Isolation by distance	Present	No	Absent	Yes

The current study contradicts all the predictions of the natural dispersal hypothesis (no) and supports all the predictions of the anthropogenic dispersal hypothesis (yes)

Sexual reproduction would account for the observed genetic diversity and population structure of Pacific coast populations (as determined by F_{ST} values). However, the introduction of genetically diverse female founders from western Atlantic source populations or a relatively higher mutation rate in the Pacific populations cannot be ruled out as explanations for the observed genetic diversity.

Neither the genetic data presented here nor the collection data published elsewhere allow us to identify the original site(s) of introduction for *N. vectensis* on the Pacific coast. The first Pacific report of *N. vectensis* was from San Francisco Bay (Hand 1957), with subsequent reports in other Pacific estuaries in the 1980s and 1990s (Humboldt Bay in 1992, Barnhart et al. 1992; Tomales Bay in 1989, Hand and Uhlinger 1994; Washington state in 1980s, Kozloff 1983). The chronology of field collections cannot establish the chronology of the species' geographic spread because *N. vectensis* is a small, cryptic, burrowing animal that occupies little studied high marsh habitats. New populations of *N. vectensis* along the Pacific continue to be identified (Willapa Bay, Bodega Bay, this study; Columbia River, J. Chapman pers. comm.). Additional genetic data from *N. vectensis* and other introduced estuarine species may allow us to reconstruct estuarine invasion routes and to better understand the dynamics of estuarine introductions.

English populations appear most closely related to the population from Baruch, SC based on pairwise genetic distance. Our data and data from Pearson et al. (2002) indicate it is possible that a single individual was introduced to England, and its asexual progeny subsequently colonized a number of estuaries in south and southeast England. Alternatively, multiple genotypes may have been introduced with only a single genotype expanding due to competitive exclusion, frequent population bottlenecks, or a combination of both. All anemones collected from England have been reported as female (although males may be cryptic as in Pacific populations), and individuals from even the most distant sites in England share the same predominant genotype (Pearson et al. 2002). The type specimen originally described by Stephenson was collected at Bembridge, Isle of Wight by G. F. Selwood of Municipal College, Portsmouth "some time" prior to 1935 (Stephenson 1935). Therefore, we can assume that this species was reasonably abundant in southern England by the early 1930s. At present we cannot substantiate a likely source region for the *N. vectensis* introduction in England. As the distance analysis suggests, English populations may be most closely related to populations from the southeastern United States, but our sampling currently includes only a single population from this area. Further analysis of additional populations from the southeastern United States (North

and South Carolina and/or Georgia) could address this hypothesis.

Two of the most genetically distinctive populations (Coos Bay, OR and Crane, MA) grouped together on the neighbor-joining tree to the exclusion of all other populations. As mentioned previously, their apparent association cannot be ascribed to long-branch attraction because these two populations were roughly half as distant from each other as from the next nearest population, and they shared unique markers not found in any other population, i.e. these two populations group together based on their similar genotypes, not their substantial dissimilarity to other populations. While the anemones at these two locations are genetically most distant from other populations, they are undoubtedly members of the same species based on species-specific morphological traits (possession of nematosomes, Stephenson 1935) and interfertility with other *N. vectensis* populations (Coos Bay: Hand and Uhlinger 1994; Reitzel unpublished data; Crane: Reitzel unpublished data). Because the grouping of Coos Bay and Crane appears quite distant from other western Atlantic lineages on the neighbor-joining tree, and because the Crane population exhibits signs of having been introduced itself, it is premature to hypothesize a region of origin for the presumably introduced animals in Oregon. *N. vectensis* was first reported in Coos Bay in the early 1980s (Rudy and Rudy 1983). Our genetic evidence suggests that the population at Coos Bay resulted from a separate introduction event than populations along the rest of the Pacific coast. At Crane, the observed presence of only a single genotype over 2 years of collections is consistent with a recent introduction or colonization at this site that occurred after the marsh underwent tidal restoration in 1999 (Hutchins et al. 2001).

We observed two cases where an identical genotype was observed in more than one site. In the two Washington populations, the same genotype comprised 18% and 40% of genotyped individuals for San Juan Island and Willapa Bay, respectively, despite a separation of more than 250 km. For the English populations, an identical genotype was represented by more than 60% of sampled individuals in each of the three habitats, a result similar to that reported by Pearson et al. (2002). In no other circumstance, including samples from subpopulations taken from pools as little as 5 m apart within one marsh, did we observe the same genotype in distinct locations.

The occurrence of a single genotype in distant locations suggests two potential explanations: adult dispersal between adjacent marshes or anthropogenic introduction (asexual production of larvae has never been reported in *N. vectensis*). Two arguments favor an anthropogenic explanation. First, we observed the sharing of genotypes among distant estuaries only in regions where *N. vectensis* appears to have been introduced (in England and the Pacific). If adult dispersal were common, neighboring sites should harbor identical genotypes throughout the entire range. Second, the organism's natural dispersal ability appears quite limited. Fieldwork on colonization rates in salt marsh pools found that *N. vectensis* seldom if ever colonized pools despite being abundant in an adjacent mudflat (Stocks and Grassle 2001). This finding is corroborated by clustering of individuals from populations in neighbor-joining trees conducted for this study. In England, the conservation plan for *N. vectensis* has explicitly called for the (re)introduction of adult animals to higher quality estuarine habitats (Williams 1976)—adult *N. vectensis* may have been intentionally introduced into suitable and previously unoccupied estuarine habitats. By contrast, in Washington, it is more likely that the introduction of adults from one estuary to another was inadvertent, potentially as a result of marsh restoration efforts, shipping, or recreational boating.

Implications and considerations for conservation policy

Nematostella vectensis has been a species of conservation concern in England for almost three decades in response to declining populations observed in the 1970s and 1980s (Williams 1976, 1983, 1987). In the 1983 IUCN Invertebrate Red Book, *N. vectensis* was designated as "vulnerable" (facing a high risk of extinction in the wild), and it has retained this designation to the present. This species received additional protection in 1988 under the Wildlife and Countryside Act 1981 (Sheader et al. 1997) and in 1995 as part of the UK Biodiversity Action Plan (Anon 1995, 2002 action plan available at: http://www.ukbap.org.uk/UKPlans.aspx?ID=-471). The current genetic data, as well as other complementary data (dominance of females, majority of

individuals from geographically distant populations represented by one genotype), strongly suggest that the English populations are not native to this region, but were introduced from the western Atlantic. Given its apparent introduced status, efforts to conserve *N. vectensis* in England may need to be reconsidered.

The conservation of introduced *N. vectensis* populations in England appears to be motivated by its misidentification as a native species and to protect vulnerable coastal habitats. The recognition of introduced status eliminates the former motivation, but it does not undermine the latter. Introduced populations may receive protection for many reasons, e.g., if the species is threatened or extinct in its native range (see, for example, Donlan 2005) or mistakenly identified as native (cryptogenicity; e.g. Gouin et al. 2001). In deciding whether to promulgate, preserve, ignore or actively eliminate *N. vectensis*, it is important to consider past, present, and future impacts on native ecosystems. Scant existing evidence suggests that *N. vectensis* is a weakly competitive species due to both small size and limited prey capture (Frank and Bleakney 1978). Although reports of adult densities at certain times of year are high (2,500 m^{-2}, Sheader et al. 1997), our collections and previous *N. vectensis* surveys (e.g. Nixon and Oviatt 1973; Kneib 1988; Posey and Hines 1991; Stocks and Grassle 2001; D. Knott pers. comm.) suggest that typical field densities are considerably lower. Given that a large individual (body length, 1 cm) has a dry weight of approximately 0.5 mg (Sullivan, Reitzel, and Finnerty, unpublished data), even the highest reported density (2,500 m^{-2}) would represent only 1.25 g of biomass per square meter, suggesting that *N. vectensis*' impact on the estuary community is likely to be negligible (Parker et al. 1999). Given its likely modest impact on native ecosystems and its apparently limited ability to disperse between adjacent habitats, the conservation of naturalized English populations of *N. vectensis* is unlikely to have negative ecological consequences, and their conservation does serve to protect dwindling estuarine habitat (Bertness et al. 2002). We therefore suggest that the "vulnerable" status of *N. vectensis* in England be retained, and that populations continue to receive modest protection, with the understanding that such protection is serving the more general goal of protecting a threatened habitat. Efforts to establish new *N. vectensis* populations in England ought to be discontinued. Active conservation efforts in the species' native range should await more complete understanding of local population viability and geographic distribution of genetic diversity.

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