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Multiple origins and incursions of the Atlantic barnacle *Chthamalus proteus* in the Pacific

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Abstract

***Chthamalus proteus*, a barnacle native to the Caribbean and western Atlantic, was introduced to the Pacific within the last few decades. Using direct sequencing of mitochondrial DNA (COI), we characterized genetic variation in native and introduced populations and searched for genetic matches between regions to determine if there were multiple geographical sources and introduction points for this barnacle. In the native range, we found great genetic differences among populations (max. F_{ST} = 0.613) encompassing four lineages: one endemic to Panama, one endemic to Brazil, and two occurring Caribbean-wide. All four lineages were represented in the Pacific, but not equally; the Brazilian lineage was most prevalent and the Panamanian least common. Twenty-one individuals spread among nearly every island from where the barnacle is known in the Pacific, exactly matched six haplotypes scattered among Curaçao, the Netherlands Antilles; St John, US Virgin Islands; Puerto Rico; and Brazil, confirming a multigeographical origin for the Pacific populations. Significant genetic differences were also found in introduced populations from the Hawaiian Islands (F_{CT} = 0.043, P < 0.001), indicating introduction events have occurred at more than one locality. However, the sequence, timing and number of arrival events remains unknown. Possible reasons for limited transport of this barnacle through the Panama Canal are discussed. This and a preponderance of Brazilian-type individuals in the Pacific suggest an unexpected route of entry from around Cape Horn, South America. Unification in the Pacific of historically divergent lineages of this barnacle raises the possibility for selection of 'hybrids' with novel ecological adaptations in its new environment.**

Keywords: ballast water, Caribbean, Hawaii, hull-fouling, introduced species, population genetic structure

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Introduction

Species boundaries in the sea can be complex due to cryptic isolation among populations (Palumbi 1994) and the prevalence of sibling species (Knowlton 1993); however, they are made even more so by the long-distance transport of alien species by ships. Maritime shipping has been responsible for transporting attached foulers on the hulls of vessels for at least the past several centuries and hauling larvae and other invaders in ballast water for the last 100 years or more (Carlton 1985, 1987; Williams *et al*. 1988; Wonham *et al*. 2000). These introductions have increased in recent decades as transit times have diminished and ballast-water volumes have risen (Carlton & Geller 1993). In the busiest harbours of the world, we are living with legacies of historical invasions (Coles *et al*. 1999) even as we are establishing new ones. The need to not only distinguish alien from native biota but also to discriminate among aliens themselves is becoming increasingly necessary.

Barnacles are quintessential hull-fouling organisms and several species have been introduced to new regions in the last century on the bottoms of ships (Crisp & Chipperfield 1948; Sandison 1950; Bishop & Crisp 1951). Larval barnacles have also been found live in ballast water transported over great distances and could be responsible for establishing some introduced populations (Carlton 1985). Several alien barnacles are known in the Pacific (Matsui *et al*. 1964; Newman 1986), the most recently introduced being the Atlantic barnacle, *Chthamalus proteus*, first documented from Hawaii in the mid-1990s (Hoover 1998; Southward

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et al. 1998). Native to the Caribbean, Gulf of Mexico, and western Atlantic (Dando & Southward 1980), it could have arrived as adults or larvae and has been observed on the hulls of interisland barges within Hawaii (Godwin 2003).

Chthamalus proteus had not been documented from the Pacific in barnacle surveys taken prior to the mid-1900s (Pilsbry 1927; Hiro 1939; Henry 1942; Edmondson 1946; Gordon 1970) nor was it found in a comprehensive survey of intertidal barnacles on the Hawaiian island of Oahu in 1973 (Matsuda 1973). However, by the time notice was taken of its arrival in 1995 populations were well established in harbours on Oahu, Maui, and Kauai (Southward *et al*. 1998). At that time it was also found in the northwestern Hawaiian Islands at the lagoon of Midway Atoll and in the eastern Pacific from Apra Harbor, Guam, along with a chthamalid of uncertain identity from the island of Pohnpei in Micronesia (Southward *et al*. 1998). Since that time, *C. proteus* has also been found in the South Pacific from the French Polynesian islands of Moorea and Mangareva along with a chthamalid of uncertain identity from the Micronesian island of Yap (A. Southward & G. Pauley, personal communication). Subsequent surveys in Hawaii have extended its range to other of the main Hawaiian Islands where it predominantly occurs in harbours and sheltered bays (J. Zardus, unpublished).

The genus *Chthamalus* comprises approximately 20 species that are difficult to separate morphologically, requiring genetic determinations in some cases (Dando *et al*. 1979; Hedgecock 1979; Dando 1987; Wares 2001; Southward & Newman 2003). In fact, *C. proteus* was not recognized as a species until it was distinguished from *Chthamalus fragilis* by enzyme electrophoresis (Dando & Southward 1980). Difficulty in discriminating one *Chthamalus* species from another raises the possibility that others have invaded the Pacific without our knowledge or that native *Chthamalus* species exist that have not been identified. Therefore, it is important that species boundaries for this barnacle in the Pacific be characterized genetically alongside genera-wide comparisons before they are obscured by additional introductions.

Molecular methods are in some cases the only approach to reconstructing the history of invasions (Geller 1996). Genetic analysis can help determine the origins of introduced species, reckon the timing or frequency of their arrival, and identify cryptic invaders (Ó Foighil *et al*. 1998; Geller 1999; Castilla *et al*. 2002; Holland *et al*. 2004). Accumulating data are revealing that introduced populations typically lack genetic structure but often retain much of the genetic variation from their source populations (Flowerdew 1984; Woodruff *et al*. 1986; Hebert *et al*. 1989; Boileau & Hebert 1993; Boom *et al*. 1994; Duda 1994; Geller 1996; Planes & Le Caillon 1998; Holland 2001). High genetic variation suggests successful invaders arrive in large numbers, arrive at high frequencies, or arrive from multiple points of origin. Invaders may also increase in genetic diversity if populations formerly isolated in the native range are reunited (Holland 2000). For these reasons, genetic characterizations of a species in both its native and alien range provide valuable insight on an introduction.

We sampled specimens of *C. proteus* from all known localities throughout the Pacific and from a number of areas scattered across its native range. Sequencing a portion of mitochondrial DNA (mtDNA), our objectives were to determine if this barnacle (i) arrived at multiple points in its introduced range and (ii) originated from multiple points in its native range. Both hypotheses were strongly supported on the evidence of population structure in the introduced range and genetic matches from nearly every native area sampled. Although our sampling in the native range was limited to relatively small numbers of specimens from widely separated geographical regions we discovered very great genetic structure among populations, sorting into geographical lineages that were also represented in the Pacific.

Materials and methods

Sample collection and sequence generation

Barnacles were collected from the 32 sites listed in Table 1. Sampling included all islands in the Pacific known for *Chthamalus proteus* or a chthamalid of uncertain identity (Fig. 1) and six localities across its native range in the Caribbean and western Atlantic (Fig. 2). Within Hawaii, samples were drawn from approximately 50% of all localities known for *C. proteus* in distributional surveys (Zardus, unpublished data) and represented all islands where this barnacle occurs. Samples were placed in 70–100% ethanol immediately upon collection. DNA was extracted from approximately 10 individuals per locality using a DNeasy Tissue Kit (QIAGEN). Under a stereomicroscope, muscle tissues were removed from large individuals for DNA extraction whereas the entire body was used for small individuals. Shell parts and any remaining tissues from each individual were archived in ethanol.

A ~650-bp fragment of the cytochrome *c* oxidase subunit I gene (COI) was amplified from the samples by the polymerase chain reaction (PCR) using 1–2 μL (~ 20–50 ng) of template DNA. In addition, each 25-μL reaction consisted of 2.5 μL 10× PCR buffer (QIAGEN), 1.0 μL $MgCl_2$ (25 mM), 0.5 μL dNTP's (10 mM), 0.2 μL *Taq* polymerase (5 U/μL), 1.0 μL each of the oligonucleotide primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3; 10 pM/μL; Folmer *et al*. 1994), and sterile-filtered H_2O to volume. PCR thermal profiles began with preliminary denaturing at 96 °C for 3 min followed by 35–50 cycles of the following steps: denaturing at 95 °C for 1 min, annealing at 40 °C for 1 min, and extension at 72 °C for 1.5 min. A final extension was performed at 72 °C for 5 min. Negative and positive controls

Table 1 List of sampling regions, island sites, place names, port-class codes, collection years, number of COI sequences obtained per site (*n*), and GenBank Accession nos for genetic samples of the barnacle *Chthamalus proteus* or congeners. Port class codes designate principal maritime traffic for Hawaiian sites only: M, military vessels; C, commercial vessels travelling internationally and to mainland USA; N, neighbour-island vessels travelling within Hawaii

Oceanic province and island group	Island and site no.	Place name	Port class	Year	*n*	Accession nos
Pacific:						
Hawaiian Islands	Midway Atoll	Midway Lagoon	M	2002	10	AY822764–822773
	Kauai 1	Niumalu Harbor, Nāwiliwili Bay	C	2002	12	AY822774–822785
	Kauai 2	Port Allen, Hanapēpē Bay	C	2002	10	AY822786–822795
	Oahu 1	Kahana Bay	N	2003	10	AY822796–822805
	Oahu 2	Pōhākea Point, Kāne'ohe Bay	N	2003	11	AY822806–822816
	Oahu 3	Kuli'ou'ou, Hawaii Kai	N	2003	10	AY822817–822826
	Oahu 4	Ke'ehi Lagoon	N	2001	10	AY822827–822836
	Oahu 5	Rainbow Bay Marina, Pearl Harbor	M	2003	11	AY822837–822847
	Oahu 6	Ford Island, Pearl Harbor	M	2001	10	AY822848–822857
	Oahu 7	Barbers Point Harbor	C	2003	11	AY822858–822868
	Molokai 1	Honouli Wai Bay	N	2002	10	AY822869–822878
	Molokai 2	Pūko'o	N	2002	10	AY822879–822888
	Molokai 3	Kaunakakai Harbor	N	2002	11	AY822889–822899
	Maui	Kahului Harbor	C	2003	10	AY822900–822909
	Hawaii 1	Waiākea Peninsula, Hilo Bay	C	2001	11	AY822910–822920
	Hawaii 2	Keaukaha Beach, Puhi Bay	N	2001	8	AY822921–822928
	Hawaii 3	Puakō Bay	N	2003	10	AY822929–822938
	Hawaii 4	Kawaihae Harbor	C	2003	11	AY822939–822949
Mariana Islands	Guam	Apra Harbor		1997	5	AY822950–822954
Caroline Islands	Pohnpei	Langer Island		2003	3	AY823026–823028*
	Yap	Tomil Harbor, Colonia		2003	3	AY823029–823031*
Society Islands	Moorea	Paopao, Cook's Bay		2001/04	0/10	AY822955–822964
Gambier Islands	Mangareva	Rikitea		1997	0	
Caribbean						
C. America	Panama 1	Galeta		2001	10	AY822965–822974
	Panama 2	Portobello		2001	4	AY822975–822978
	Panama 3	Colon		2002	5	AY822979–822983
Lesser Antilles	Curaçao 1	St Joris Bay		2000	3	AY822984–822986
	Curaçao 2	Spanish Water		2000	10	AY822987–822996
	Puerto Rico	Magueyes Island		2004	1	AY822997
Greater Antilles	St. John, USVI	Brewers Bay, Range Cay		2002	7	AY822998–823004
W. Atlantic						
S. America	Brazil 1	Caravelas, Bahía State		2003	3	AY823005–823007
	Brazil 2	Ubatuba, São Paulo State		2003	10	AY823008–823017

*Species identification uncertain.

were included with each batch of reactions. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN) and sent to Macrogen, Inc. for direct sequencing on an Applied Biosystems 3730xl capillary sequencer.

Sequencing was performed in one direction with primer LCO1490 for all samples and in the reverse with primer HCO2198 for approximately 50 samples with ambiguous base reads. Sequences were edited and aligned using SEQUENCHER 2.4 (Gene Codes) and trimmed to a common length. A sequence of *C. proteus* from GenBank was included as a reference (Table 2). To test for cryptic invasions or unidentified species, outgroup sequences from 15 other taxa in the genus *Chthamalus* were obtained either from GenBank or sequenced from donated specimens. They included all species known from the Caribbean as well as others from the Pacific and elsewhere (Table 2).

Phylogenetic analysis

Phylogenetic relationships were estimated using neighbour-joining methodologies as implemented in PAUP* 4.0b10 (Swofford 2000). Initially, the native-range samples were analysed apart from the Pacific samples to characterize baseline patterns of phylogeny; a combined analysis followed. The identical outgroup taxa were used with each analysis.

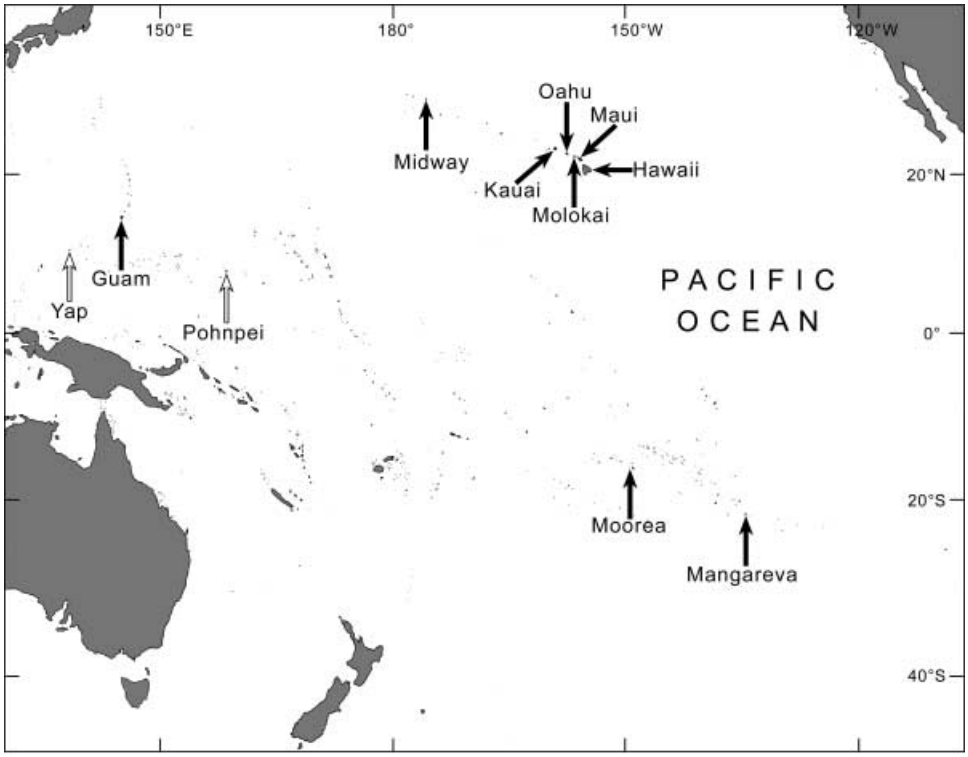

Fig. 1 Localities in the Pacific where the introduced barnacle *Chthamalus proteus* (solid arrows) or a chthamalid of uncertain identity (open arrows) has been reported.

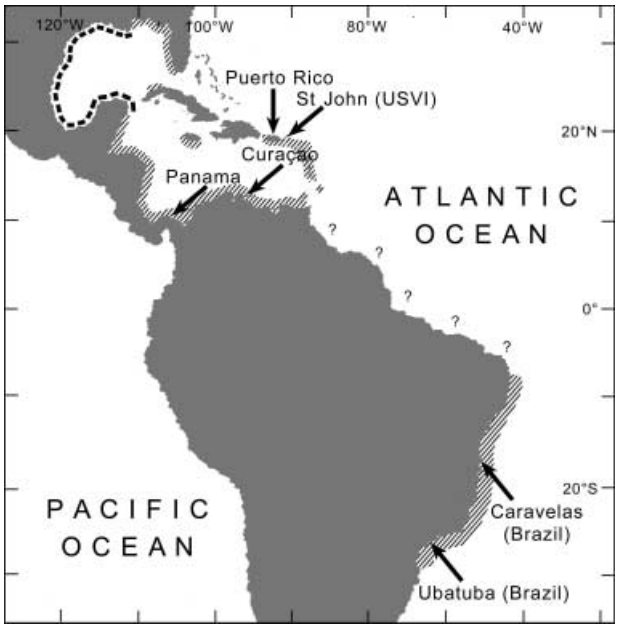

Fig. 2 Distribution of the barnacle *Chthamalus proteus* in its native range. Hatched areas along coastlines indicate occurrence of the barnacle in sheltered habitat, broken lines indicate areas of presumed occurrence, question marks indicate areas where occurrence of the barnacle is undetermined (after Dando & Southward 1980). Labelled arrows designate localities from which samples were obtained for genetic analysis in the present study.

Appropriate models of DNA substitution were selected by hierarchical likelihood-ratio tests performed by MODELTEST 3.5 (Posada & Crandall 1998). A temporal versions model (TVM) was selected for the native-range data with inclusion of the proportion of invariable sites (I) and gamma shape parameter (Γ) of rate heterogeneity across variable sites. The substitution rate matrix = 0.2, 11.9, 0.6, 0.1, 11.9, 1.0, I = 0.6043, and Γ = 0.9914. The same class of model was selected for the combined data (substitution rate matrix = 0.6, 11.7, 0.7, 0.5, 11.7, 1.0; I = 0.5729; and Γ = 0.9304). Bootstrap consensus values were obtained from 1000 pseudoreplicates. Clade structure and the distribution of geographical localities within them were compared between analyses from the two data sets.

Genetic variants or haplotypes of *C. proteus* were extracted using COLLAPSE 1.2 (©David Posada 1998–2004) and exact matches between native range and Pacific samples were enumerated. A maximum-parsimony network among haplotypes of the native range was constructed with TCS 1.13 (Clement *et al*. 2000). Lineages identified in the network were related to clustering patterns obtained from the phylogenetic analysis.

Analysis of population structure

Samples from the native range were grouped into four geographical regions for analysis of population structure:

Table 2 List of taxa, their provenance, collection locality, publication source, and GenBank Accession nos for members of the genus *Chthamalus* and other barnacle genera used as outgroups or reference sequences in phylogenetic analyses

Taxon	Region	Locality	Source	Accession nos
C. angustitergum	Curaçao	Santa Cruz	Wares 2001	AF234799
C. anisopoma	Mexico (Pacific)	Puerto Peñasco, Sonora State	Wares 2001	AF234816
C. bisinuatus	Brazil	Rio Grande do Sul, Tramandai State	present study	AY823018
C. challengeri	Japan	Komincuto, Kamogawoa Prefecture	present study	AY823019
C. 'cortezianus'	Mexico (Pacific)	Bahía Mazatlán, Sinaloa State	Wares 2001	AF234812
C. dalli	USA (Pacific)	San Francisco Bay, California	Wares 2001	AF239800
C. fragilis	USA (Atlantic)	Key Largo, Florida	Wares 2001	AF234813
C. malayensis	Hong Kong	Little Palm Beach, Clear Water Bay	present study	AY823020
C. malayensis	Malaysia	Mursing, East Peninsular Malaysia	present study	AY823021
C. 'mexicanus'	Mexico (Pacific)	Bahía de Tenacatita, Jalisco State	Wares 2001	AF234804
C. montagui	England	Plymouth Sound	present study	AY823022
C. neglectus	Hong Kong	Little Palm Beach, Clear Water Bay	present study	AY823023
C. proteus	Panama (Caribbean)	Portobello	Wares 2001	AF234806
C. panamensis	Panama (Caribbean)	Punta Culebra	Wares 2001	AF234802
C. stellatus	England	Plymouth Sound	present study	AY823024
unknown chthamalid	Hong Kong	HK University Sci. & Tech., Clear Water Bay	present study	AY823025

Panama, Curaçao, Puerto Rico/St John, and Brazil. Genetic differentiation among them was compared by pairwise F_{ST} measures and tested by analysis of molecular variance (AMOVA) using ARLEQUIN 2.001 (Schneider *et al*. 2000).

In the Pacific, population structure was analysed statistically using sequence data from only those islands with multiple localities for *C. proteus* (Kauai, Oahu, Molokai and Hawaii). AMOVA tests were used to compare genetic differentiation among localities grouped according to (i) island and (ii) type of boat traffic. The former tested the hypothesis that genetic patterns varied among islands, indicative of multiple points of introduction. The latter addressed the hypothesis that distributions within Hawaii varied according to local vs. beyond-island boat traffic. Localities associated with interisland vessels might be more similar than localities associated with vessels travelling ocean-wide. For the boat-traffic hypothesis, each locality was assigned to one of three travel classes according to its' predominant vessel type: commercial vessels travelling internationally or to the mainland, military vessels, and among-island personal craft (Table 1).

Results

Sequence characteristics

DNA was extracted, amplified, and sequenced from a total of 260 individuals from the Pacific, Caribbean, and Atlantic (Table 1). Sequence data could not be obtained for Mangareva Island, French Polynesia, because samples either did not amplify in PCR or were found by BLAST searches to be contaminated with DNA from a rissooid gastropod. Following editing and alignment, sequences were trimmed to a length of 558 bp. After removal of non-*Chthamalus proteus* sequences (described below) and the addition of one GenBank sequence, 255 sequences remained for analysis. Overall base frequencies in the data set were 23.37% A, 16.95% C, 18.95% G, and 40.73% T; and the ratio of transitions to transversions was 4.2353. A total of 152 polymorphic sites were found — 13.2%, 3.3%, and 83.6% at the first, second and third codon positions, respectively. There were a total of 102 parsimony-informative sites and 10 amino acid differences were found among nine individuals.

Among 54 sequences obtained from the native range (including the GenBank sequence) were 49 haplotypes (90.7% unique haplotypes). Among 201 Pacific samples were 162 haplotypes (80.6%) and 21 individuals exactly matching six native haplotypes. These six haplotypes were from all sampling regions in the native range except Panama (Table 3), the region with the highest sampling effort ($n = 20$). Haplotype no. 5 occurred at both sites in Brazil and was the dominant matching haplotype in the Pacific. Matching individuals in the Pacific were distributed on every island except Guam, where sampling effort in the Pacific was lowest ($n = 5$). Thus, haplotype diversity could have been under-represented at this site. This could have been true for the islands of Midway, Moorea, and Maui too, all of which had relatively small sample sizes ($n = 10$); nevertheless, exact matches were found at each.

Phylogenetic comparisons

Significant genetic structure was revealed in the native range by phylogenetic analysis. Four major clades were resolved with moderate support (Fig. 3). Bootstrap support

Table 3 Frequency and distribution of specimens of the barnacle *Chthamalus proteus* from localities of introduction in the Pacific exactly matching six haplotypes (*h1–h6*) from localities in the native range (see Table 1 for site number designations)

	Haplotypes and native localities					
Pacific localities	*h1* Curaçao 2	*h2* Curaçao 2 and St John	*h3* St John	*h4* Puerto Rico	*h5* Brazil 1 and 2	*h6* Brazil 2
Midway		1			1	
Kauai 1		1				
Kauai 2			1			
Oahu 1					1	1
Oahu 2					1	
Oahu 4					2	
Oahu 5				1		
Oahu 6					1	
Oahu 7	1				2	
Molokai 1			1		1	
Molokai 3						
Maui					1	
Hawaii 1					1	
Hawaii 2				1		
Hawaii 3				1		
Moorea 1					1	

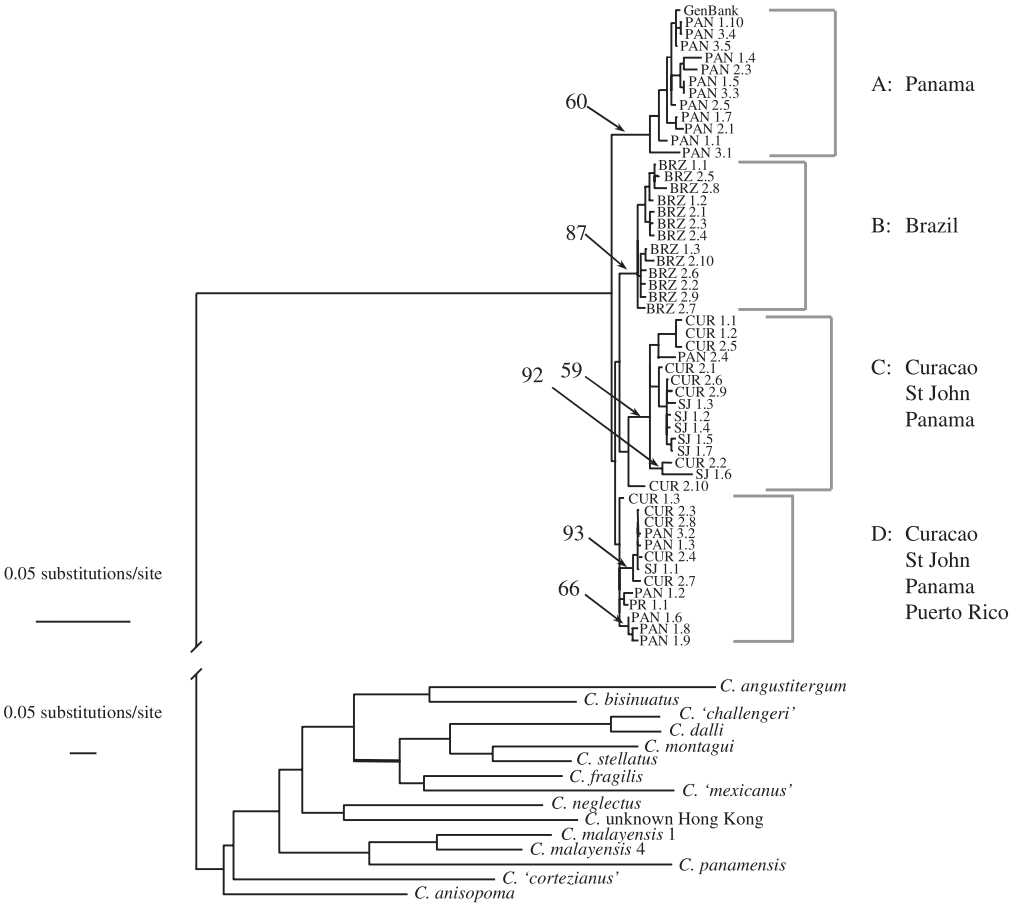

Fig. 3 Phylogenetic tree of the COI gene for 54 individuals of the barnacle *Chthamalus proteus* from localities in its native range. Clustering is by genetic distance using a neighbour-joining algorithm and the 15 outgroup sequences are the *Chthamalus* taxa listed in Table 2. Arrows indicate nodes with bootstrap support above 50%. Four major clades (A–D) are listed along with the geographical localities where they occur. Note the scale for branch length varies between ingroup and outgroup taxa.

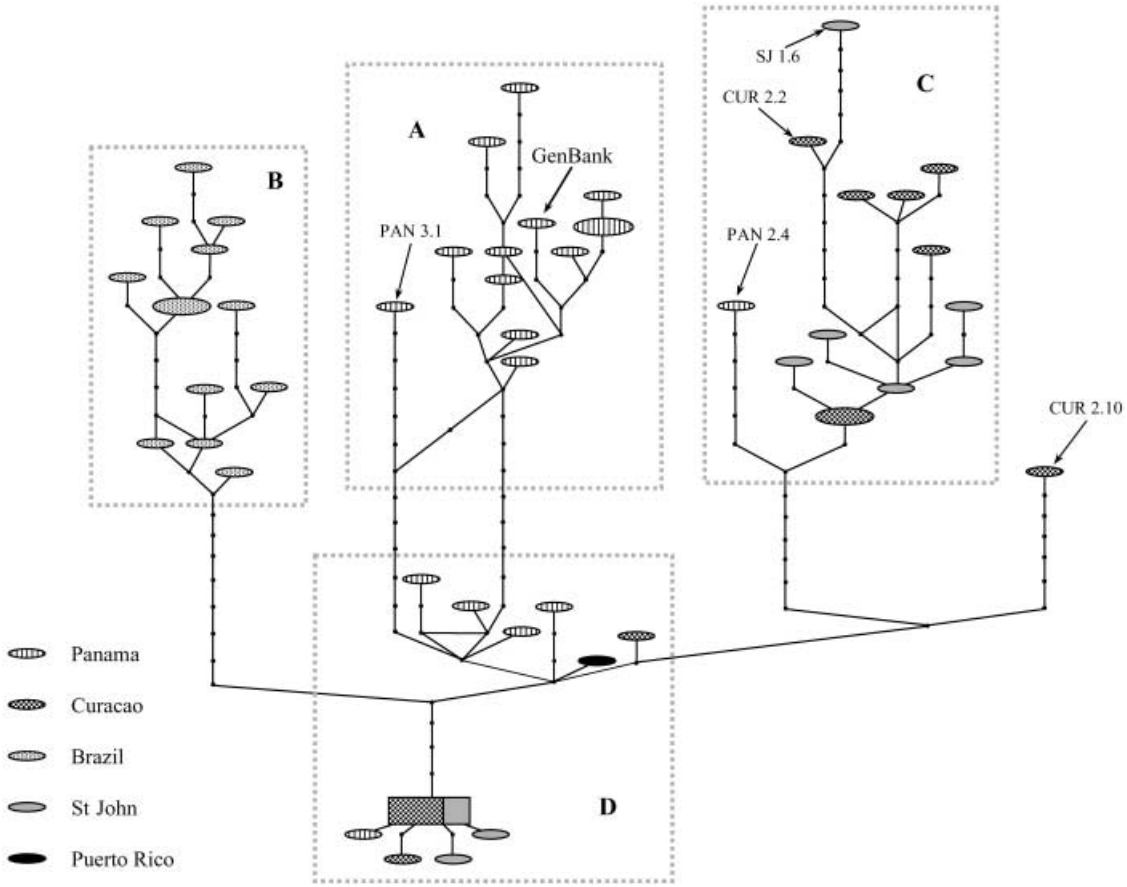

Fig. 4 Haplotype parsimony network for 49 COI haplotypes of the barnacle *Chthamalus proteus* from localities in its native range. Lines of most-parsimonious relationship connect ovals representing individual haplotypes. Nodes along each branch designate the number of base-pair differences between haplotypes. Fill patterns code for geographical locality and the size of the ovals corresponds to the number of individuals found matching that haplotype (range one to two). The rectangle corresponds to the ancestral haplotype, represented by three individuals from two localities. Dashed-line boxes with letter designations identify the four lineages defined by phylogenetic analysis from Fig. 3. Arrows and sequence labels indicate a reference GenBank sequence and five other individuals discussed in the text.

values of less than 50% were obtained for two clades (C and D) when an intermediate specimen (CUR 2.10) was included with either. These four clades, hereafter referred to as lineages A–D, corresponded with geography to varying degrees. Lineages A and B were endemic to Panama and Brazil, respectively. Panama also hosted lineages C and D, the only lineages that were found Caribbean-wide. Despite the sympatric distribution of these two clades they were resolved separately by the analysis; albeit with lower bootstrap support than lineages A and B. Notably, lineage B which is the most geographically isolated, was determined not to be a separate species based on similarity in genetic distance between it and other lineages and a lack of reciprocal monophyly.

A haplotype parsimony network corroborated the lineage designations identified by phylogenetic analysis and placed the ancestral haplotype within D, the most geographically widespread lineage (Fig. 4). The network topology consisted of haplotype lineages separated by many mutations, consistent with a scenario of early divergence and restricted gene flow among the lineages, reinforcing their evolutionary uniqueness. This also held true for the sympatric lineages C and D whose most similar haplotypes were only one base pair nearer to each other than to endemic lineages A and B.

Phylogenetic analysis of the combined Pacific and Atlantic data resulted in four clades matching almost precisely those from the native data set alone (Fig. 5). Bootstrap values measured above 50% for lineages A and B but fell below this value for clades C and D. The only topology changes included a reversal in position of clades C and D and sample CUR 2.10, originally peripheral to lineage C, clustered with lineage D. Importantly, no additional clades were resolved. Six samples from the two Micronesian localities of Yap and Pohnpei clustered with the outgroup taxa, matching most closely with the recently described species *Chthamalus neglectus* (Yan & Chan 2004). However, their level of divergence with *C. neglectus* makes it unclear whether these samples represent populations of this species or not.

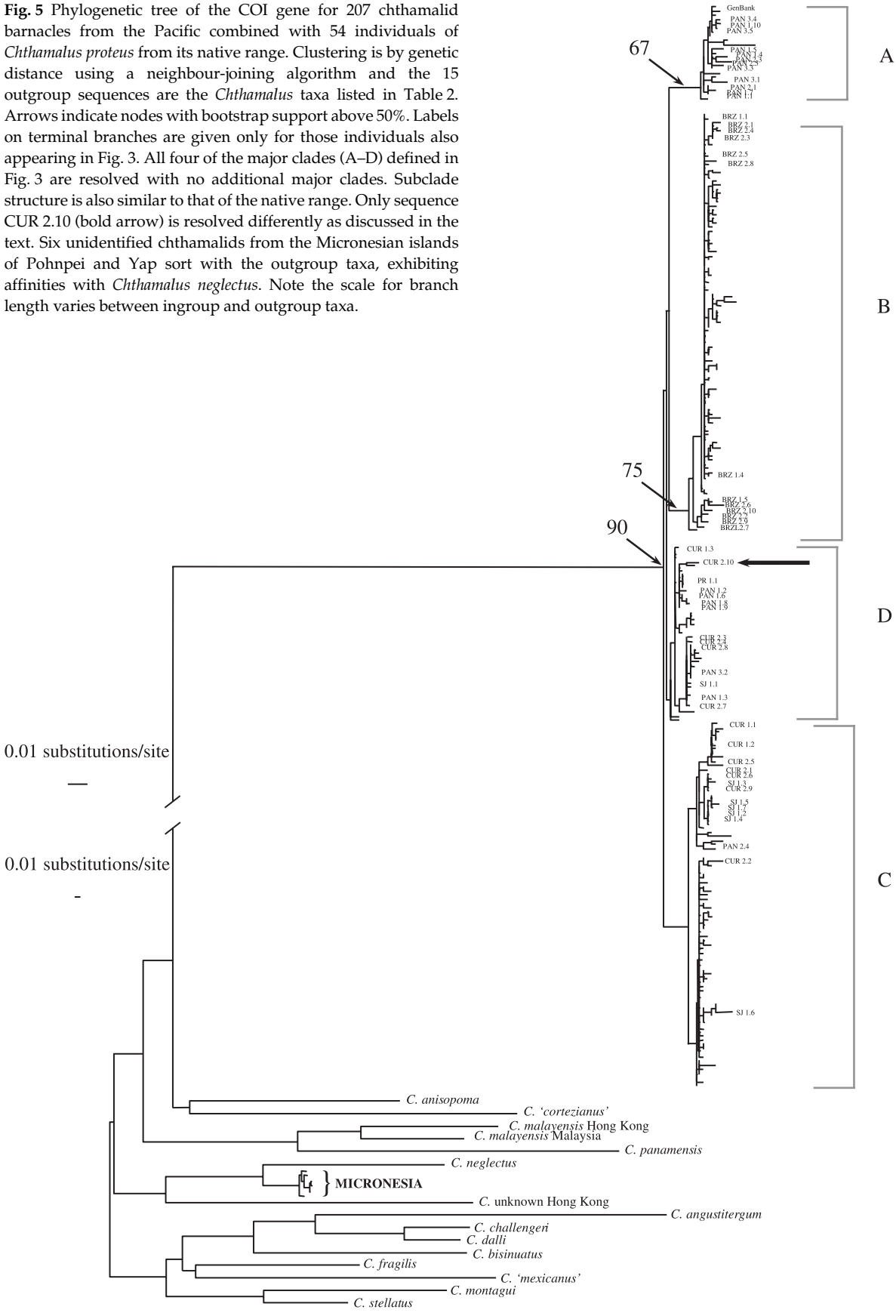

Fig. 5 Phylogenetic tree of the COI gene for 207 chthamalid barnacles from the Pacific combined with 54 individuals of *Chthamalus proteus* from its native range. Clustering is by genetic distance using a neighbour-joining algorithm and the 15 outgroup sequences are the *Chthamalus* taxa listed in Table 2. Arrows indicate nodes with bootstrap support above 50%. Labels on terminal branches are given only for those individuals also appearing in Fig. 3. All four of the major clades (A–D) defined in Fig. 3 are resolved with no additional major clades. Subclade structure is also similar to that of the native range. Only sequence CUR 2.10 (bold arrow) is resolved differently as discussed in the text. Six unidentified chthamalids from the Micronesian islands of Pohnpei and Yap sort with the outgroup taxa, exhibiting affinities with *Chthamalus neglectus*. Note the scale for branch length varies between ingroup and outgroup taxa.

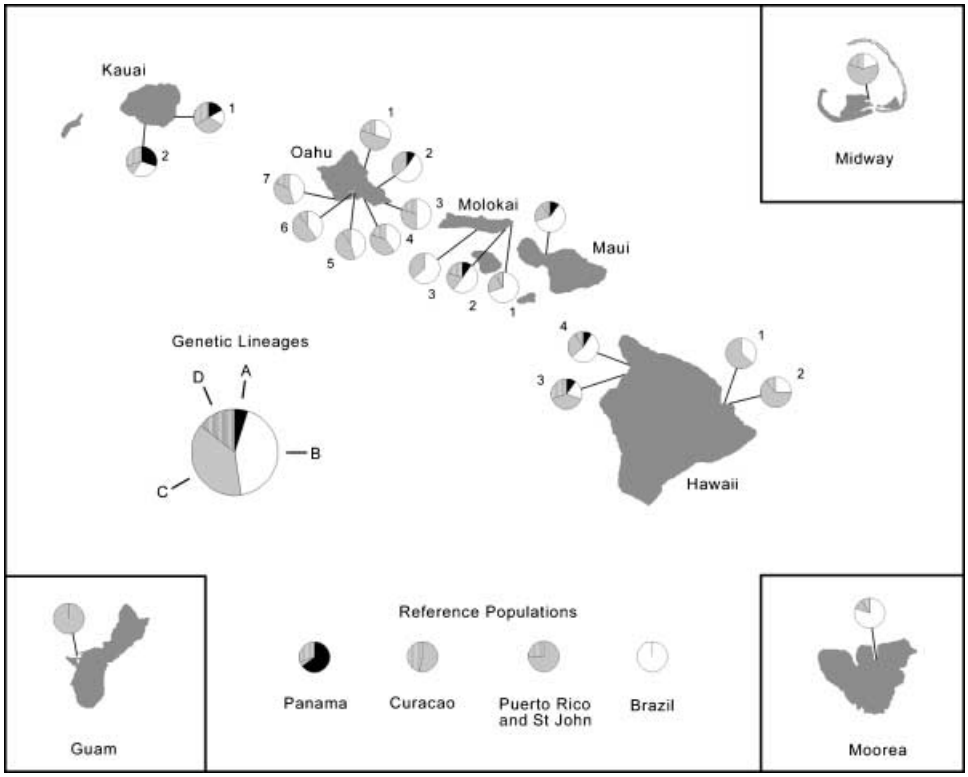

Fig. 6 Distribution of four genetic lineages defined by phylogenetic analysis for the barnacle *Chthamalus proteus* among 20 localities in its introduced range in the Pacific Ocean. The proportional occurrences of the lineages are represented for each locality. Localities are listed by number for each island and correspond to place names in Table 1. Proportions shown in the key reflect relative representation of the genetic lineages among all Pacific samples. Reference populations show the proportional occurrence of each lineage in the four regions sampled in the native range.

Distribution and occurrence of the four genetic lineages among the Pacific localities are shown in Fig. 6. Lineage B was marginally dominant (42.8%) and was represented at every locality except Guam. Lineage C was nearly as prevalent (37.8%) and was represented at all localities. Interestingly, a small subclade of lineage C, comprised of two peripheral individuals in the native data set, CUR 2.2 and SJ 1.6 (Fig. 4), and well supported by a bootstrap value of 92%, was also resolved in the combined analysis and accounted for 71.1% of lineage C in the Pacific (Fig. 5). Lineage D was only modestly represented in the Pacific (14.4%) but occurred at 80% of the localities. Lineage A was least represented among the samples (5.0%), occurring at 35% of the localities. Representation of the lineages suggests a gradient in the introduction of *C. proteus* to the Pacific, from the greatest contributing populations in the easternmost part of its range (Brazil) to the least contributing populations in the western Caribbean (Panama). Each of the main Hawaiian Islands hosted all of the lineages but not at all localities. Remarkably, the south shore of Oahu, the most intensively sampled area ($n = 52$) and the hub of shipping for Hawaii with the three most active harbours of Honolulu Harbor, Pearl Harbor and Barber's Point Harbor (Godwin 2003), lacked lineage A. As mentioned above, distributions for Guam, Midway, and Moorea should be viewed with caution due to small sample sizes. The single Maui location too represents a small sample size, yet all four lineages were found thereat.

Native range population structure

Samples from the native range when pooled into four regions (Panama, Curaçao, Puerto Rico/St John, and Brazil) were genetically diverse. Haplotye diversity was of similar magnitude among each of the four regions and nucleotide diversity was fairly constant with the exception of Brazil, which was approximately half that of the other regions (Table 4). Pairwise genetic distances ranged from 2.2% to 3.8%. Pairwise F_{ST} values were also 'very great' (Hartl & Clark 1997) and statistically significant except between the pair Curaçao vs. Puerto Rico/St John (Table 5). Parameters were highest for those pairwise comparisons involving Brazil. Whether the geographical distribution of *C. proteus* is continuous or disjunct along the northeastern portion of South America remains unknown (Fig. 2) but others have suggested biochemical differences may exist

Table 4 Sample number (n), haplotype diversity (h), and nucleotide diversity (π) for COI sequences of *Chthamalus proteus* from four regions in the native range

Region	n	h (± SD)	π (± SD)
Panama	20	0.995 (0.018)	0.022 (0.012)
Curaçao	13	0.974 (0.039)	0.023 (0.013)
Puerto Rico and St John	8	1.000 (0.063)	0.020 (0.012)
Brazil	13	0.987 (0.035)	0.011 (0.006)
Overall	54	0.996 (0.005)	0.030 (0.015)

Table 5 Pairwise comparisons of average percent divergence (below diagonal) and F_{ST} values (above diagonal) for COI sequences of the barnacle *Chthamalus proteus* among four regions in the native range

Region	Panama	Curacao	Puerto Rico and St John	Brazil
Panama	—	0.298*	0.391*	0.534*
Curacao	0.032	—	0.023	0.517*
Puerto Rico and St John	0.035	0.022	—	0.613*
Brazil	0.037	0.035	0.038	—

*Significant at $P < 0.0001$.

between populations from Brazil and the Caribbean (Dando & Southward 1980). That Brazil samples are genetically distinct is clear, but as mentioned above, they do not represent a separate species as measured by this gene. This conclusion is also supported by pairwise genetic distances with COI between *C. proteus* and eight other taxa in the genus, including its putative geminate partner *Chthamalus 'mexicanus'*, ranging from 14.7% to 24.4% (Wares 2001), approximately an order of magnitude greater than differences in the present study.

AMOVA comparison of the four native regions resulted in highly significant differences among regions and within sampling localities, but within each region localities did not significantly differ from one another (Table 6). Differences across all localities and among regions explained nearly all of the observed variation, indicating strong regional structure in the samples, echoing findings of the phylogenetic analysis.

Pacific population structure

In Hawaii, genetic differences among islands were significant, although they accounted for only a small amount of the total genetic variation observed, the great majority being explained by variability across all localities (Table 6). Within each island, localities did not significantly differ from one another.

Partitioning localities in the Hawaiian Islands into three ship traffic classes explained only a tiny fraction of the total variation and the effect was not significant (Table 6). The interaction effect of localities within ship class was also small and not statistically significant. Nearly all of the variation was explained by differences among localities although it too was not statistically significant (Table 6).

Discussion

Tracing origins of the Atlantic barnacle, *Chthamalus proteus*, in the Pacific by genetic methods, we found compelling evidence that it has arrived multiple times in the Pacific from several areas in its native range. Genetic architecture of its populations in the native range was surprisingly robust, imparting a strong geographical signal to Pacific invaders. Not only did we find individuals in the Pacific

Table 6 Results of AMOVA tests comparing variation in COI sequences of *Chthamalus proteus* grouped according to: (A) four regions in the native range (B) four Hawaiian islands in the introduced range, and (C) three classes of harbours within the Hawaiian Islands

Test	Source of variation	d.f.	SS	Variance components	% of variation	F_{ST}	F_{SC}	F_{CT}
A	Among regions	3	176.029	4.01910	42.4			0.424**
Native	Among sites w/in regions	5	29.338	0.11521	1.2		0.021	
Regions	Within sites	45	240.318	5.34039	56.4	0.436***		
	Total	53	445.685	9.47471				
B	Among islands	3	54.701	0.32879	4.3			0.043***
Hawaiian	Among sites w/in islands	12	67.299	–0.18958	–2.5		–0.026	
Islands	Within sites	150	1134.970	7.56646	98.2	0.018		
	Total	165	1256.970	7.70568				
C	Among classes	2	19.089	0.02696	0.4			0.004
Harbour	Among sites w/in classes	15	119.918	0.04168	0.5		0.006	
classes	Within sites	168	1270.870	7.56470	99.1	0.009		
	Total	185	1409.876	7.63333				

*$P < 0.05$, **$P < 0.005$; ***$P \ll 0.001$; statistical probabilities derived from 1023 permutations.

exactly matching haplotypes throughout the native range but also representatives of all major native lineages. We begin with an overview of phylogeographic patterns of this barnacle in its native range. Following this, we discuss genetic patterns in the Pacific and conclude with ruminations on the genus *Chthamalus* in the Pacific.

Native population characteristics

Significant genetic structure in populations of fish and invertebrates with pelagic dispersal has generally been found lacking in the Caribbean and adjacent regions (Mitton *et al*. 1989; Lacson 1992; Duffy 1993; Silberman *et al*. 1994; Shulman & Bermingham 1995; but see Taylor & Hellberg 2003). With samples of *C. proteus* collected from Panama to Brazil we uncovered remarkably high genetic differentiation and found four genetically distinct lineages. The geographical distribution of these lineages suggests a natural division of the range into eastern, western, and central sections. Lineages endemic to Brazil and Panama occur to the east and west, respectively, while two sympatric pan-Caribbean lineages dominate the central Caribbean. Although genetic subdivision of populations was not expected for this species in the Caribbean and western Atlantic, similar subdivisions have been found in two other *Chthamalus* species in the eastern Atlantic and Mediterranean (Pannacciulli *et al*. 1997) and may also be a feature of populations in this genus and others from the eastern Pacific (van Syoc 1994; Wares 2001; Sotka *et al*. 2004).

With additional sampling further structure may yet be detected for *C. proteus* in its native range. For instance, we lacked samples from the Gulf of Mexico where this barnacle occurs less abundantly (Dando & Southward 1980) and further divisions within the pan-Caribbean lineages C and D may also be found. Nevertheless, at least four distinct lineages exist and it must be emphasized that these same lineages were also resolved phylogenetically in the Pacific with no additions or significant rearrangements.

The apparent geographical discontinuity in *C. proteus* populations between Brazil and the Caribbean may be due to the interjection of freshwater plumes from the Amazon and Orinoco rivers. This outflow and its attendant effects on benthic habitats and larval dispersal is an isolating barrier for some marine fishes (Muss *et al*. 2001; Rocha *et al*. 2002; Carlin *et al*. 2003). At these localities lowered salinities may inhibit colonization of the substratum by adult barnacles and physical flow may transport larvae seaward. Despite its probable influence on the distribution of *C. proteus*, this outflow is not an absolute barrier to its dispersal but does appear to limit gene flow. Our results show that although genetically distinct, the Brazilian populations are not a separate species.

Pacific population characteristics

The occurrence of the four lineages in the Pacific and the presence of individuals matching haplotypes in Brazil and the Caribbean validate our assertion that introductions have occurred from throughout the native range. Panama, the geographically closest region and the most probable route of entry into the Pacific, appears to have contributed least to the introduction. Less than 7% of specimens in our study clustered with an endemic Panamanian lineage and no Pacific samples exactly matched any haplotype from Panama. Representatives of lineages C and D could have arrived from Panama where these lineages comprise a minor element; however, given that individuals were found exactly matching haplotypes from everywhere in the Caribbean except Panama argues otherwise. Also, most of lineage C in the Pacific matched a Curaçao/St John subclade. With the introduction occurring only decades ago the high number of unique haplotypes in the Pacific reflects our limited sampling depth in the Atlantic and Caribbean rather than recent evolution. Comprehensive sampling in the native range would undoubtedly account for most of the Pacific haplotypes. Given an average è value of 10.7 from our four native population groups, hundreds of haplotypes are likely to exist and using Ewens' sampling theorem for alleles (Ewens 1972; Hedrick 2005), scores of haplotypes are estimated to be found with even modest sample sizes. The model, as implemented in the program DNASP version 4.10.3 (Rozas *et al*. 2003), returns an estimate of 25 haplotypes in a sample of 100 individuals or 42 haplotypes in 500 individuals.

Perhaps most surprising of all is that the most common lineage in the Pacific has its origins in Brazil. The explanation for this is not immediately apparent but may have to do with the fact that *C. proteus* does not tolerate water of low salinity well (Dando & Southward 1980) and may not survive passage through the Panama Canal on boat hulls. Furthermore, we have found that *C. proteus* larvae reared in the laboratory are highly sensitive to culture conditions and food, failing to thrive on a wide-spectrum algal diet (Fread, unpublished). Thus, *C. proteus* larvae may also not survive long periods in ballast water. However, in Hawaii at least, *C. proteus* does have the advantage of reproducing year-round (Zardus, unpublished) suggesting that introductions may not be tied to a specific season. Life history data coupled with genetic data, suggest that invaders may have come on the hulls of Atlantic or Caribbean ships arriving in the Pacific from around Cape Horn, South America. If true, perhaps not Hawaii (Southward *et al*. 1998) but localities farther south in French Polynesia could have been invaded first and perhaps much earlier. For these reasons further investigation into shipping routes, temperature tolerance of adults, effects of temperature on reproduction, and this barnacle's distribution in the South Pacific is merited.

If not an initial entry point for this invasion in the Pacific, Hawaii is at least a major stepping stone for its spread. A much earlier arrival elsewhere in the Pacific would present the possibility of detecting genetic divergence between Atlantic and Pacific populations but, would probably require using markers that evolve much more quickly than COI and would necessitate greater sampling of the native range. Assigning dispersal polarities and arrival points can also be particularly difficult if cryptic stepping-stones are involved. Harbours that offer unsuitable habitat for adults may still be able to mediate ship-to-ship translocation of larvae as witnessed for the blue mussel in Pearl Harbor (Apte *et al*. 2000). However, this may not be an important consideration for *C. proteus* if its larvae do not survive ballast-water transport.

That we found significant population subdivision among the Hawaiian Islands indicates introductions have occurred at more than one point. Although differences among islands explain only a small portion of the overall variation, the palette of lineages varies at some localities. Differences are best seen with lineage A. It was found scattered throughout the main Hawaiian Islands but was best represented at the two large harbours on Kauai. However, it was absent all along the south shore of the nearest neighbouring island of Oahu, which encompasses the three most active harbours in the Islands. Differences were also found on the island of Hawaii, where lineage A was not found in the vicinity of Hilo Harbor on the east side but was present on the west side in the vicinity of the large harbour of Kawaihae, suggesting separate introductions for each side of the island.

Unique haplotypes dominated samples from the Pacific (81%) and were not useful in identifying linkages among localities. High haplotype diversity indicates that high numbers of invaders established the introduction, either arriving in large quantities simultaneously or in multiple events over time.

Not all islands in Hawaii are frequented by international or mainland vessels, thus the spread of *C. proteus* within the archipelago has to have been effected either by local boat traffic or larval dispersal in the plankton. We found no homogenization of lineages among localities as might be expected with island-wide dispersal in the plankton. Short-scale dispersal via the plankton is, however, clearly operating along protected shores and within bays on some islands. If planktonic dispersal is restricted, a 'gene shadow' representing the suite of lineages introduced at a particular site might then characterize a particular area. If dispersal is via small boats, then a pattern of linkage among distant harbours might be apparent. Elements of both short distance dispersal in the plankton and dispersal by boat can be interpreted from our data, but not unambiguously. Similarity in lineages along the south shore of Oahu could be due to either dispersal mechanism while similarities between Kauai and the west side of Hawaii may represent linkage by boat traffic.

An alternative, but less likely, explanation for genetic structure in the introduced Pacific populations is that selection is acting differentially on the various *C. proteus* lineages. Typical of most chthamalids, *C. proteus* resides high on the shoreline, occupying substratum exposed during low tides upward into the splash zone (Southward & Newman 1977), a habitat with great variation in environmental stress over short distances. *Chthamalus proteus* in Hawaii is primarily distributed in areas with protection from waves yet with exposure to high solar radiation. Across its native range from Brazil to the Caribbean it is expected to experience an even greater range in environmental conditions that may have resulted in different genetic lineages being regionally adapted. Adaptive divergence at the molecular level has been demonstrated in the barnacle *Semibalanus balanoides* under varying environmental conditions over small geographical scales (Bertness & Gaines 1993; Schmidt *et al*. 2000; Schmidt & Rand 2001). Selection affecting the frequency of mitochondrial lineages has also been demonstrated in a copepod exposed to toxicants (Schizas *et al*. 2001). It is possible that in *C. proteus,* lineage B dominates in the Pacific not because it has a higher rate of introduction but because it is better suited to the environment. Ecological studies are underway measuring the general fitness of *C. proteus* in Hawaii and testing its interactions with native species (Zabin & Hadfield 2002). Studies explicitly testing fitness among the various lineages of *C. proteus* are perhaps also warranted.

Chthamalus *in the Pacific*

Confluence in the Pacific of lineages that have long been separated in the native range raises the possibility for emergence of new variants differing in adaptive fitness from their progenitors. Mitochondrial DNA does not generally provide evidence of recombination among lineages as it is typically uniparentally inherited. However, nuclear markers might detect favourable 'hybrids' better suited to Pacific conditions. Multiple chthamalid species co-occur in other regions throughout the world and niche specialization among them appears to hinge on small differences in temperature, salinity, turbidity, and desiccation tolerance (Southward 1975; Dando & Southward 1980). The biogeography of the genus *Chthamalus* in general is remarkable in that multiple species overlap in distribution in many regions of the world (Southward 1975; Dando & Southward 1980; Poltarukha 2000; Wares 2001).

In the Caribbean, *Chthamalus fragilis*, *C. angustitergum*, and *C. bisinuatus* all overlap *C. proteus* in parts of its native range (Dando & Southward 1980; Poltarukha 2000). The question arises as to why *C. proteus* and not other

Caribbean chthamalids has become established in the Pacific; indeed, why haven't other Pacific *Chthamalus* species colonized Hawaii and other remote islands, either naturally or by anthropogenic transport? In the Pacific *Nesochthamalus intertextus* and *Euraphia hembeli* are the only chthamaloids that naturally range from oceanic islands in the south to Hawaii (Newman 1986). Introductions to Hawaii tend to originate from the western rather than from the eastern Pacific (Carlton 1987), and in the western Pacific there are five or six chthamalids from tropical latitudes (Poltarukha 2000) that could seemingly have been spread by ships. The barnacle in this study from Yap and Pohnpei Micronesia, bearing genetic similarity to *Chthamalus neglectus*, is perhaps the only chthamalid native to remote Pacific islands unless it too represents another anthropogenically mediated introduction. If the unexpectedly high structure of native *C. proteus* populations in the Caribbean is an indicator of dispersal ability of *Chthamalus* species generally, then the answer to some of these biogeographical riddles may lie in varying abilities of the larvae of different species to colonize ship hulls, survive in ballast water or disperse in the plankton.

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Michael Hadfield's research focuses on larval settlement and recruitment of benthic marine invertebrates, especially those inhabiting coral reefs and the tropical biofouling community. He also studies the demography of endemic Hawaiian tree snails, and the impacts of invasive species upon them.
