

## Two Genetically Distinct Populations of Bobtail Squid, *Euprymna scolopes*, Exist on the Island of O'ahu<sup>1</sup>

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**Abstract:** Population structure of the endemic Hawaiian bobtail squid, *Euprymna scolopes*, was examined using both morphological and genetic data. Although allozyme polymorphism was negligible, measurements of eggs, juveniles, and adults, as well as genetic data sequences of mitochondrial cytochrome oxidase I, demonstrated highly significant population structuring between two populations found on the northeastern and southern coasts of the island of O'ahu. These data suggest that extremely low levels of gene flow occur among these populations. Population subdivision of marine shallow-water invertebrates in Hawai'i is not expected based on earlier surveys, but may reflect a more general pattern for organisms, both marine and terrestrial, that exhibit limited dispersal. The subdivision also provides insight into the pathway through which coevolution between *E. scolopes* and its internal symbiont, *Vibrio fischeri*, may proceed.

THE HAWAIIAN ARCHIPELAGO presents unique opportunities for the study of evolutionary biology because of its relative isolation, high levels of endemism, and the explosive radiation of a number of taxa (Simon 1987, Wagner and Funk 1995). For example, the terrestrial invertebrate biota, which has been particularly well studied in genera such as *Drosophila* flies, *Tetragnatha* spiders, and Achatinellinae tree snails, typically exhibits extensive adaptive radiations comprising species endemic to the archipelago (Roderick and Gillespie 1998, Thacker and Hadfield 2000). These patterns are

thought to result from the extremely heterogeneous nature of the terrestrial island landscape, which presents an abundance of spatially limited, yet distinct, niches ripe for adaptive radiation (Gillespie et al. 2001).

Marine invertebrates, however, have much lower rates of endemism than their terrestrial counterparts, with endemism ranging from 2 to 20% for nearshore gastropods, brachyuran decapods, and certain polychaetes and echinoderms of the central Pacific (Scheltema 1986, Kay and Palumbi 1987). These benthic marine invertebrates appear instead to represent attenuated Indo-Pacific fauna, and the marine endemic species that do exist are usually distributed throughout all of the islands of the archipelago (Scheltema 1986). The wide geographic distributions of these marine invertebrate species indicate that fairly high gene flow must be occurring between populations within species (Palumbi 1992). Such gene flow is presumably facilitated by long planktonic larval phases, which ensure high dispersal. Yet, within such widespread species some population structure may nevertheless persist (Barber et al. 2000).

Although much is known about marine invertebrate species with extended larval life histories, less is understood about species that do not have broadly dispersing larval or juvenile stages. One such species is the bobtail squid, *Euprymna scolopes*, which is endemic to

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the Hawaiian Islands (Singley 1983). Female *E. scolopes* lay clutches of eggs on hard substrates in the shallow waters of the back reef. No true larval stages occur in this species (i.e., the juveniles hatch with a morphology and behavior very similar to those of the adult [Arnold et al. 1972]). Specifically, after a brief planktonic stage of hours to a few days, the juvenile squid take on the diel pattern of behavior characteristic of the species (i.e., they bury in the sand during the day and forage in the water column at night).

Previously, it was observed that *E. scolopes* is notable for its habitat loyalty, and it has been suggested that two reproductively isolated populations exist on northeastern and southern coasts of the island of O'ahu (Singley 1983). The *E. scolopes*-*Vibrio fischeri* association has become a model for animal-bacterial symbiosis and in particular the persistent colonization of animal epithelia by extracellular bacteria (McFall-Ngai and Ruby 1998). Recent studies of the squid-vibrio symbioses at the level of species have provided strong evidence for coevolution of the host and symbiont (Nishiguchi et al. 1998). Whether population subdivision of *E. scolopes* exists is critical to understanding the coevolution of this species with its bacteria partners—how isolated are populations of *E. scolopes* and at what scale would population-specific strains of *V. fischeri* be expected to evolve? In this study we tested the hypothesis of population subdivision of *E. scolopes* with both morphological and genetic data. Our results indicate that populations of *E. scolopes* on O'ahu are indeed distinct. Further, the results demonstrate that at least some species of Hawaiian marine invertebrates are more subdivided than previously realized, likely as a consequence of a life history lacking dispersive egg or larval stages.

#### MATERIALS AND METHODS

##### *Capture and Maintenance of Specimens*

Adult *Euprymna scolopes* were collected in 1997 and 1998 from two sites, the shallow sand flats off Niu Valley along leeward O'ahu and along the shores of Kane'ohē Bay on

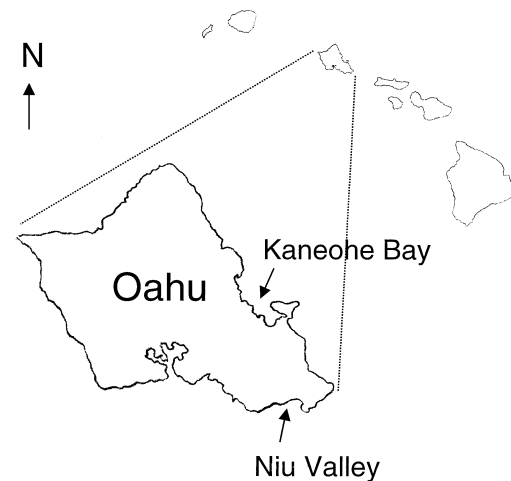


FIGURE 1. Collection sites on O'ahu, Hawaiian Archipelago, for specimens examined in this study.

windward O'ahu (Figure 1). Specimens were maintained in running seawater tables at 24°C at the Kewalo Marine Laboratory, University of Hawai'i.

##### *Morphometric Data*

Measurements of morphological characters were performed to determine whether significant phenotypic differences occur between Kane'ohē Bay and Niu Valley animals. Three characters were measured on specimens from each of these two sites: female mantle length, egg diameter, and hatchling mantle length. The latter two measurements were made using a dissection microscope (Wild M5) at 10× with an ocular micrometer. Eggs were measured on the day that they were laid. The hatchlings were first anesthetized in a 50:50 solution of 0.37 M MgCl<sub>2</sub>:seawater before the mantle-length measurements were taken. Approximately 75 specimens were examined for each character per locality.

##### *Allozymes: Cellulose Acetate Gel Electrophoresis*

Variation at allozyme loci was examined through cellulose acetate gel electrophoresis, a method that requires very little tissue (Richardson et al. 1986, Hebert and Beaton

1989). Squid tissue was frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until used in analyses. Mantle tissue, because it is of low yield, and light organs, because they contain symbionts, were removed before homogenization. Tissue samples (0.5 g) were placed on ice and homogenized in an equal volume of distilled water. The homogenates were centrifuged for 5 min at  $12,000\times g$  at  $4^{\circ}\text{C}$  to pellet tissue debris. Supernatant fluids were placed in microcentrifuge tubes on ice until use. Allozyme electrophoresis was run on cellulose acetate gels in electrophoresis chambers (Zip-zone, Helena Laboratories Inc., Beaumont, Texas). The histochemical staining recipes used were given in Hebert and Beaton (1989). Two buffer systems resulted in the best resolution: "tris-glycine"—0.4 M Tris with 2 M glycine, pH 8.5, with gel/buffer dilution 1:9; and "tris-maleate"—0.1 M tris-maleate, pH 7.8. Of the 18 presumed enzymes assayed 7 yielded acceptable enzyme activity and clear resolution: isocitrate dehydrogenase (IDH; E.C. 1.1.1.42), malate dehydrogenase (MDH; E.C. 1.1.1.37), malate dehydrogenase NADP<sup>+</sup> (ME; E.C. 1.1.1.40), glucose-6-phosphate isomerase (GPI; E.C. 5.3.1.9), mannose-6-phosphate isomerase (MPI; E.C. 5.3.1.8), phosphoglucosmutase (PGM; E.C. 5.4.2.2), and aspartate amino transferase (AAT; E.C. 2.6.1.1). The tris-glycine buffer was used for PGM and ME, and the remaining loci were run with tris-maleate. Horizontal gels were run at 180 V and 3 mA for 45 min at  $4^{\circ}\text{C}$ . Approximately 30 alleles were scored at each locus for 20 individuals from each locality.

#### *Mitochondrial DNA: Cytochrome Oxidase I*

Squid DNA was extracted from frozen testis tissue (25 mg) with a Qiagen QIAamp Tissue Kit (cat. no. 29304). The isolated genomic DNA was reprecipitated with 95% ethanol, washed with 70% ethanol, and resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). A polymerase chain reaction (PCR) was used to produce DNA templates for the sequencing of the mitochondrial cytochrome oxidase I (COI) gene. Each 50- $\mu\text{l}$  PCR reaction contained 4  $\mu\text{g}$  of template

DNA, 0.4  $\mu\text{M}$  of each primer, 5  $\mu\text{l}$  of 8 mM dNTPs, and 0.12  $\mu\text{l}$  of Taq polymerase (Perkin Elmer, Norwalk, California). Amplifications were performed using a thermocycler (GeneAmp PCR 9600). The PCR conditions were as follows: 35 cycles of  $94^{\circ}\text{C}$  for 30 sec,  $47^{\circ}\text{C}$  for 30 sec, and  $72^{\circ}\text{C}$  for 60 sec. The primers used to amplify COI were C1-J-1718 (26 mer, 5'-GGAGGATTTGGAAATTGATTAGTTCC-3') and C1-N-2191 (alias "Nancy" 26 mer, 5'-CCCGGTAAAAT-TAAAATATAAACTTC-3') (primer designations given in Simon *et al.* [1994]). The resulting PCR products were purified with a GeneClean Kit (Bio 101 Inc., La Jolla, California). Seven microliters of the resultant DNA and 4  $\mu\text{l}$  of 1  $\mu\text{M}$  C1-J-1718 primer were used for cycle sequencing using the PRISM Ready Reaction Terminator Cycle Sequencing Kit with an ABI Model No. 377 automated sequencer (PE Applied Biosystems, Foster City, California). A total of 18 COI sequences was obtained from 10 individuals from Kāne'ōhe Bay and 8 individuals from Niu Valley. All DNA sequences were examined and aligned using Sequencher 3.1 (GeneCodes, Ann Arbor, Michigan).

#### *Phylogeographic Analysis*

The evolutionary history of the alleles at a locus can be represented in a phylogenetic tree, which can then be interpreted in light of the geographical distribution of those alleles; this approach is termed phylogeography (Avice 2000). The interpretation of such an analysis can be complicated for nuclear loci because of recombination (Begun and Aquadro 1992, Hudson 1994) and for both nuclear and mitochondrial loci because of the persistence of ancestral alleles (Templeton and Sing 1993). There is no evidence that mitochondrial sequences in this species recombine, yet persistence of ancestral alleles is an issue. Templeton *et al.* (1992) and Templeton and Sing (1993) provided a method for examining the recent history of alleles at a locus. The method focuses phylogenetic reconstruction on the most recent substitutions before attempting to resolve deeper nodes. Assuming that more recent changes are less likely to in-

clude homoplasy or recombination, sequences separated by one substitution are linked first, the resulting groups form the basis for linking alleles separated by two substitutions, and so on. Details of the method are provided by Templeton and Sing (1993), Templeton (1998), Davies et al. (1999), and Posada and Crandall (2001).

#### *Population Analysis*

Population structure was determined through the analysis of molecular variation (AMOVA) method of Excoffier et al. (1992) as implemented by the computer program Arlequin 2.0 (Schneider et al. 2000). Indices of population subdivision analogous to standard  $F$  statistics (Wright 1951, 1965) were calculated with significance assessed using permutation procedures that randomly redistribute individuals among populations. This general approach was used to estimate  $F$  statistics based upon (1) allele frequencies only, and (2) the genetic distance among alleles as well as their frequencies. The  $F$  statistics used were (1)  $\theta$  (Weir and Cockerham 1984, see Excoffier et al. 1992), and (2)  $\Phi$ , calculated using the average Kimura 2-parameter distance observed between alleles. Exact tests were performed based on the distribution among populations of (1) alleles and (2) phylogenetically derived allele groups (TFPGA, Miller 1998). In the latter case, alleles in the same one-step clade of the Templeton networks were pooled.

#### RESULTS

Significant differences were found among populations for both morphological and genetic characters. Niu Valley females were highly significantly larger and produced highly significantly larger eggs and hatchlings than Kāne'ohe Bay females (Figure 2), supporting Singley's (1983) earlier observations. Although phenotypic differences between the two populations do not provide direct evidence of genetic, or inherited, differences between the two populations, such data are certainly consistent with this hypothesis of genetic differentiation. The life history im-

plications of differing egg and female body sizes among these populations have not yet been examined.

The seven enzyme stains resolved nine enzyme-coding loci (IDH and AAT each stained for two loci). All loci examined were monomorphic. Though surprising, this result is not unexpected for squid—other intra/ interspecific studies of squid population structure have yielded negligible allozyme polymorphism and in some cases monomorphism (Brierley et al. 1993, 1995, Izuka et al. 1996). However, other allozyme studies have distinguished among closely related squid species (Izuka et al. 1994) and seasonal cohorts (Kang et al. 1996). Although allozyme data collected here contribute no information concerning the hypothesis of distinct populations, they do raise a question for future work: to what extent does selection contribute to the lack of variation in squid electrophoretic proteins?

Partial COI sequences of approximately 430 base pairs from both Niu Valley and Kāne'ohe Bay animals were obtained. Ten sequences from Kāne'ohe Bay and eight from Niu Valley were resolved. Measures of gene diversity, mean number of pairwise differences, and base composition were very similar in the two populations (Table 1). Our results yielded six distinct haplotypes distinguished by seven polymorphic sites, with only one haplotype being shared among the two populations (Figure 3).

Statistical analysis revealed significant population structure between the populations. Both frequency- and distance-based  $F$  statistics were highly significant, with the distance-based estimates,  $\Phi$  (0.60), being considerably larger than the frequency-based  $\theta$  (0.49) (Table 2), illustrating that the sequence data do provide additional information with regard to population structuring in this species.

The values of both  $F$  statistics are extremely high, especially considering the small distance between populations. These data, coupled with the observation of only one shared haplotype between the populations (frequency of approximately 10% in each population), suggest that gene flow is minimal between these

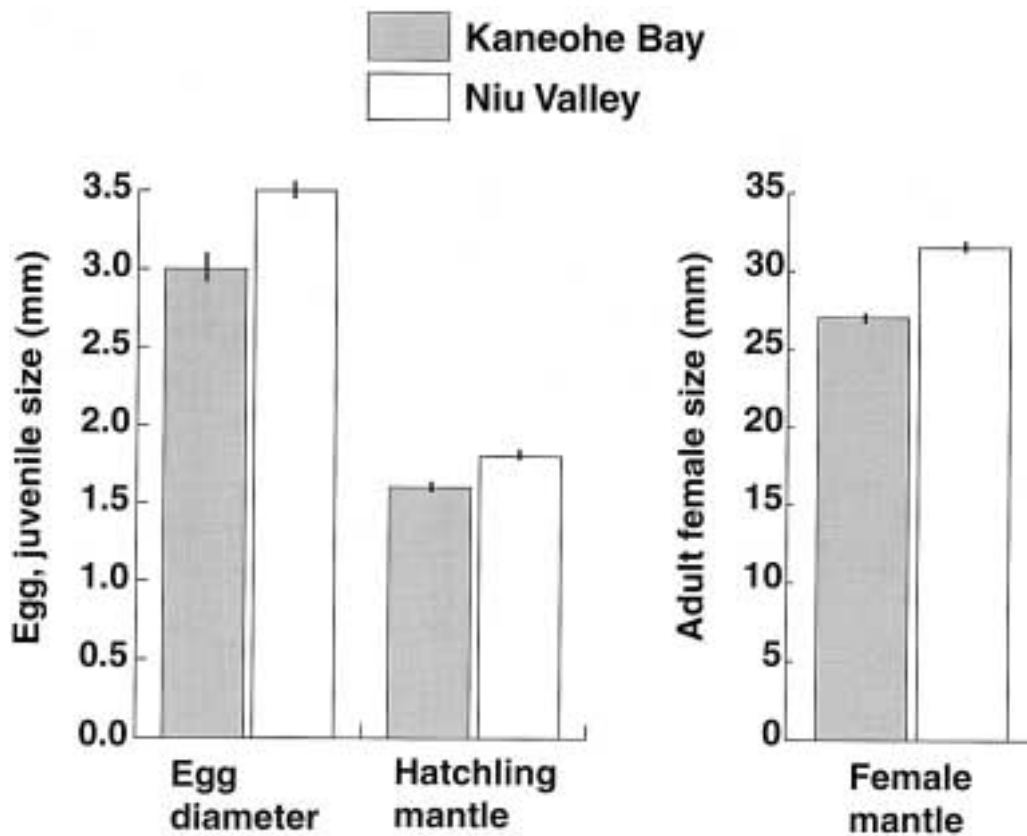


FIGURE 2. Morphological differences among individuals collected in Kāne'ohe Bay and Niu Valley locations. Sample sizes were for eggs, hatchlings, and females (78, 62, and 30, respectively) for each population. All comparisons were highly statistically significant ( $t$ -tests,  $P < 0.001$ ). Female age was uncertain; females were measured the day after dying in the laboratory.

two populations. Using the standard migration estimator for mitochondrial data (Slatkin 1994), migration between these two populations is calculated as approximately one migrant exchanged between the populations only every two to three generations, with a generation time of 3 to 4 months. This effective rate of migration is very low, yet it is still sufficient to prevent complete divergence between the populations by genetic drift alone. It should be noted that this estimate of gene flow should be considered an upper estimate—the two populations may also show some genetic similarity, if they share a recent history (see Bohonak 1999, Bohonak and Roderick 2001).

#### DISCUSSION

The analysis of both morphological and genetic data indicates that populations of *E. scolopes* on O'ahu are extremely subdivided, likely exchanging very few, if any, migrants, each generation. The extent to which other populations of *E. scolopes* are so subdivided in Hawai'i is clearly important and well worth investigating. Kāne'ohe Bay is surrounded by the only true barrier reef in the Hawaiian Islands (Kay and Palumbi 1987), and it may be that the well-studied Kāne'ohe Bay population is an anomaly. Yet, water does flow actively in and out of the bay.

Subdivision of the *E. scolopes* populations

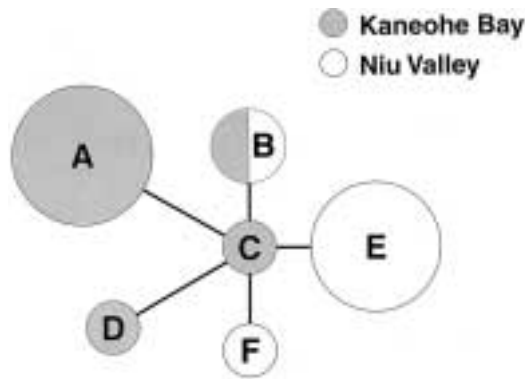


FIGURE 3. Mitochondrial haplotype network and localities as estimated by the method described by Templeton (1998). Haplotype identities are noted within circles with a letter, and distances between haplotypes are proportional to the number of base pair differences (one or two steps) between haplotypes. The size of the circles is proportional to the number of individuals ( $n = 18$ ) sharing a particular haplotype.

has implications beyond the species. If the level of subdivision observed here is found elsewhere, one can also ask, at what level of divergence does one see a concomitant change in the symbiont *Vibrio fischeri*? At the mechanistic level, under these very refined con-

ditions, one may be able to pinpoint those traits (likely to be cell surface determinants on the host or symbiont) that change as the partners coevolve and become, as a unit, genetically distinct from their congeneric squid-bacteria counterparts. For example, symbionts of Japanese squid *E. morsei* infect Hawaiian *E. scolopes* easily by themselves, but quickly lose in competition with the native Hawaiian strain (Nishiguchi et al. 1998). This competitive difference was easily determined because *E. morsei* strains of *V. fischeri* are brightly luminous in culture, whereas *E. scolopes* strains are dim in culture. Thus, when symbiotic organs of such competition experiments are plated on agar, the ratio of the two strains is easily determined. Unfortunately, all isolated strains of *V. fischeri* from Hawai'i are dim in culture and, therefore, to compete the symbionts of Hawaiian populations of *E. scolopes* would require genetic engineering of *V. fischeri* strains to introduce distinguishing genetic markers.

The extent of population structure is also important in determining whether host isolation occurs before symbiont isolation or vice versa, or whether host and symbiont track each other closely. The numbers of *V. fischeri*

TABLE 1  
Within- and Between-Population Comparisons of Mitochondrial COI DNA Sequences

Haplotype Data	Locality		Base Position <sup>a</sup>						
	Kāne'ohe Bay ( $n = 10$ )	Niu Valley ( $n = 8$ )	101	158	263	296	377	389	392
	Frequency	Frequency							
A	0.700	0.000	G	G	G	A	T	T	A
B	0.100	0.125	.	.	A	.	C	.	.
C	0.100	0.000	.	.	.	.	.	C	.
D	0.100	0.000	.	.	.	G	.	.	.
E	0.000	0.750	A	.	.	.	.	.	G
F	0.000	0.125	.	A	.	.	.	.	.
Gene diversity	$0.53 \pm 0.18$	$0.46 \pm 0.20$							
Mean no. pairwise differences	$1.32 \pm 0.89$	$0.93 \pm 0.71$							
Nucleotide composition (%)									
C	20.7	20.4							
T	35.8	36.0							
A	27.4	27.3							
G	16.1	16.2							

<sup>a</sup> Differences in base positions are shown relative to a reference haplotype (A), with identities shown by “.”.

TABLE 2  
F Statistics and Exact Tests for Population  
Differentiation

Method	F	P
Frequency ( $\theta$ )	.492	<0.00001
Distance ( $\Phi$ )	.604	<0.00001

Note: Statistics are presented that are based solely on the frequency of alleles ( $\theta$ ) and that also consider the genetic distance among alleles ( $\Phi$ ).

in the water are strongly dependent on the density of host squid in the environment (Lee and Ruby 1994). Thus, at the mouth of Kāne'ōhe Bay, where there are few hosts, there are very few *V. fischeri*. There may also be little genetic exchange between symbiont populations.

In sum, populations of *E. scolopes* on two sides of O'ahu are more distinct than previously realized, suggesting that gene flow may be substantially limited in some marine species in Hawai'i. This result likely reflects the lack of long-lived pelagic larvae. Further, the Hawai'i populations of *E. scolopes* and their symbionts offer some interesting opportunities to gain insight into the coevolution of bacteria and their animal hosts. Evidence that the host populations can be extremely subdivided is an intriguing start.

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