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RESEARCH ARTICLE

# Genetic evidence for the cryptic species pair, *Lottia digitalis* and *Lottia austrodigitalis* and microhabitat partitioning in sympatry

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**Abstract** It has been proposed that the common West Coast limpet, *Lottia digitalis*, is actually the northern counterpart of a cryptic species duo including, *Lottia austrodigitalis*. Allele frequency differences between southern and northern populations at two polymorphic enzyme loci provided the basis for this claim. Due to lack of further evidence, *L. austrodigitalis* is still largely unrecognized in the literature. Seven additional enzyme loci were examined from populations in proposed zones of allopatry and sympatry to determine the existence of *L. austrodigitalis* as a sibling species to *L. digitalis*. Significant allele frequency differences were found at five enzyme loci between populations in Laguna Beach, southern California, and Bodega Bay, northern California; strongly supporting the existence of separate species. Both species exhibit two microhabitat morphotypes, a gooseneck barnacle morph in the mid-intertidal zone and a rock morph in the high-intertidal zone. In sympatry, *L. austrodigitalis* was more abundant higher in the intertidal on rocks, whereas *L. digitalis* was more abundant lower in the intertidal on barnacles. This finding supports earlier claims of microhabitat partitioning in this sibling species pair. In addition to this finding, the transition zone between the species was found to have shifted substantially northward in only two decades, from Monterey Peninsula, CA to near Pigeon Point, CA, where *L. digitalis* previously dominated.

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## Introduction

Sibling species are defined as sister species that are impossible or extremely difficult to distinguish based on morphological characters alone (Mayr and Ashlock 1991). Marine sibling species are ubiquitous, found from the poles to the tropics, in most known habitats, and at depths ranging from intertidal to abyssal (Knowlton 1993). We will refer to species that are indistinguishable morphologically, whether or not they are sister species, as "cryptic species" and will restrict our use of "sibling species" to cryptic species for which there is phylogenetic evidence for sister species status. Cryptic species that are not sibling species could exhibit similar characteristics due to either convergence or the retention of more ancient plesiomorphic similarity. Although this definition of sibling species is somewhat more restrictive than "Modern Synthesis" vintage and more recent definitions (Mayr and Ashlock 1991; Knowlton 1993), we believe that it is helpful for distinguishing between alternative situations where there might or might not be phylogenetic estimates available. Cryptic species are often overlooked in the marine environment as a result of several factors: (1) the difficulty of discovering reproductive isolation when it occurs, (2) scarcity of well preserved soft tissues in museum collections, (3) the confounding factor of normal intraspecific geographic variation, and (4) the assumed potential for long-distance dispersal along coastlines being equated with expectations for broad geographic ranges (Knowlton 1993). Sibling species are common in various marine taxa such as poriferans (Sole-Cava and Thorpe 1986; Boury-Esnault et al. 1992), cnidarians

(McCommas and Lester 1980; Bucklin and Hedgecock 1982; Buss and Yund 1989), mollusks (Mastro et al. 1982; Moyse et al. 1982; Ward and Janson 1985; Chaney 1987), crustaceans (Mathews et al. 2002; Strasser 2003; Asakura and Watanabe 2005; MacPherson and Machordom 2005) and echinoderms (Manwell and Baker 1963; Lessios 1981; Nishida and Lucas 1988; Matsuoka and Hatanaka 1991).

Estimates of marine biodiversity are increasing as more sibling species are being discovered. For example, the copepod genus *Tisbe* has grown from a few to over 63 species (Marcotte 1984) as a result of discovering significant allozyme differences. Further, eight sibling species of polychaetes were identified within what was formerly a single species, *Capitella capitata*, based on differences in chromosome number (Grassle et al. 1987). Analysis of mitochondrial gene regions for limpets that at one time had all been referred to as *Lottia strigatella* (Carpenter 1864) confirmed the validity of the more northern *L. paradigitalis* (Fritchman 1960) and also revealed previously unrecognized species in the south (Simison and Lindberg 2003).

The scientific evidence for establishing a cryptic species complex can be somewhat controversial, like that of the limpet pair, *Lottia digitalis* (Rathke 1833) and *L. austrodigitalis* (Murphy 1978). *L. digitalis* is a common temperate northeastern Pacific limpet species that occupies a broad band within the rocky intertidal habitat, from the mid to high tide zone, and has conventionally been thought to exhibit a large geographic range spanning from the Aleutian Islands, Alaska to Baja California Sur, Mexico (McLean 1978; Morris et al. 1980; Lindberg 1981; Lindberg et al. 1987; Shanks 1998; Holyoak 1999). Limpets identified as *L. digitalis* from Santa Cruz, California, were shown to be morphologically plastic (Lindberg and Pearse 1990) exhibiting a lower-profile broad morphotype with mottled brown, black, green, or golden coloration when it inhabits vertical rock faces and a taller and narrower morphotype that is white with black stripes when it dwells on gooseneck barnacles (*Pollicipes polymerus*). These "rock" and "barnacle" morphotypes are superbly camouflaged to match their respective habitats. Murphy (1978) proposed that *L. digitalis* ranged mostly from the Aleutian Islands, Alaska to Point Conception, California, becoming considerably rarer to the south into southern California. In contrast, *L. austrodigitalis* ranged continuously from the northern Pacific coast portions of Baja California Sur, Mexico to Point Conception, California, including a more northern population on Monterey Peninsula in Central California (Murphy 1978). Valentine (1966) observed that Monterey Bay is 2–3°C warmer than the waters to the north and south and that some warmer-water organisms are found there.

After comparing limpets from 10 locations (Fig. 1) between Corona Del Mar, CA (33.59°N) and Yachats State Park, Oregon (44.30°N), Murphy (1978) reported allozyme differences attributed to the differing distributions of *L. digitalis* versus *L. austrodigitalis*. The rock and barnacle microhabitats at each site were sampled equally. Murphy


**Fig. 1** A map of the California coastline showing the location of collection sites from the current study as well as those from Murphy's studies (1978, 1983). Current collection sites are *underlined* while Murphy's collection sites are marked with an *asterisk*. Those sites sampled in both studies are *underlined* and marked with an *asterisk*. From south to north: Laguna Beach (*LAG*), Point Mugu (*MUG*), Gaviota Beach (*GAV*), Cayucos (*CAY*), San Simeon (*SSM*), Mill Creek (*MIL*), Monterey Peninsula (*MON*), Santa Cruz (*SCZ*), Pigeon Point (*PPT*), and Bodega Bay (*BOD*)

reported statistically significant differences in allele frequencies at the leucine amino peptidase (LAP, EC 3.4.11.1) and glucose phosphate isomerase (GPI as "PGI", EC 5.3.1.9) loci between the three northernmost populations (Yachats, OR; Bodega Head, CA; Santa Cruz, CA) and the two southernmost populations (Point Mugu, CA and Corona Del Mar, CA). Further, he reported that the LAP locus could be used by itself as a diagnostic species marker with 98.8% probability of assigning an individual to the correct species. No evidence for hybridization was found in the zone of sympatry, which spanned from Gaviota State Beach, CA to Santa Cruz, CA (Fig. 1). Judging from the fact that Murphy's proposal has been largely ignored, these earlier findings were not enough to convince limpet taxonomists and biologists working on these limpets that *L. digitalis* was actually the northern counterpart of a sibling (or cryptic) species duo (Abbott and Haderlie 1980; Ricketts et al. 1985; Lindberg et al. 1987, 1998; Hahn and Denny 1989b; Lindberg and Pearse 1990; Hobday 1995; Denny and Blanchette 2000; Denny 2000). Only one gene locus showed strong differences between southern and northern populations and the subtle shell differences noted by Murphy have been considered by some to reflect mere intraspecific geographic variation in a species already known to be extremely plastic. As a result, the newer (southern) sibling species, *L. austrodigitalis*, has rarely been recognized in subsequent literature.

In addition to the south versus north allozyme differences, Murphy (1983) reported that in the Monterey Peninsula portion of the zone of sympatry, *L. digitalis* was found in higher frequency in the barnacle microhabitat while *L. austrodigitalis* was found in higher frequency in the rock microhabitat. The LAP allele signature was used to distinguish between siblings and was considered by Murphy to be more reliable than any morphological distinctions he was able to detect. In the Monterey Peninsula population, 72% of individuals genetically determined to be *L. austrodigitalis* were from the rock microhabitat and 65% of individuals genetically determined to be *L. digitalis* were from the barnacle microhabitat.

In the current study, we used seven polymorphic allozyme loci to investigate both allopatric and sympatric populations of *L. digitalis* and the proposed *L. austrodigitalis* by using horizontal starch gel electrophoresis, a well-established technique that has been used extensively in the examination of cryptic species (Ward and Janson 1985; Staub et al. 1990; Matsuoka and Hatanaka 1991; Crawford et al. 1996; Manchenko and Kulikova 1996) as well as in taxonomic studies (Ayala 1983; Bullini 1983; Thorpe 1983). By sampling more allozyme loci, we generated additional data supporting the hypothesis that *L. austrodigitalis* and *L. digitalis* are distinct species and attempted to characterize current species boundaries. We also investigated multiple sympatric populations for evidence of ecological partitioning between rock and barnacle microhabitats.

## Materials and methods

### Collection of samples

Limpet populations were sampled from six Californian intertidal sites (Table 1; Fig. 1). Collections were made between January 1998 and October 1999 from Shaw's Cove in Laguna Beach, Orange County (southern site); Point Pinos on Monterey Peninsula, Monterey County (central site); and Horseshoe Cove in Bodega Bay, Sonoma County (northern site). Three additional sites (San Simeon, San Luis Obispo County; Santa Cruz, Santa Cruz County; and Pigeon Point, San Mateo County) were sampled within the reported zone of sympatry (Murphy 1978) in 2001 and 2002 to better assess the current distribution of each species. Limpets identified as *L. digitalis* were chosen based on the species' physical descriptions (Light et al. 1975) and only specimens larger than 5 mm were collected because it is easy to misidentify juvenile *Lottia* species. There was no a priori knowledge of whether collected individuals were *L. digitalis* or *L. austrodigitalis* because Murphy reported that the two proposed species were nearly morphologically indistinguishable. Limpets were collected from both the gooseneck barnacle and rock microhabitats at each site. Collection sites were sampled two to three times within a 21-month period, 50–70 limpets in total being taken from each microhabitat. Limpets were placed into dry zip lock bags and packed in dry ice immediately upon collection and then stored in a −80°C freezer.

**Table 1** Sample sizes (*n*) from populations of *L. austrodigitalis* and *L. digitalis* (combined) from each microhabitat type at six different locations

| Site | Latitude (°N) | Longitude (°W) | Rock (*n*) | Barnacle (*n*) |
|---|---|---|---|---|
| Laguna Beach (Shaw's Cove) | 33.55 | 117.80 | 154 | 116 |
| San Simeon (Piedras Blancas) | 35.40 | 121.17 | 47 | 48 |
| Monterey Pen (Point Pinos) | 36.64 | 121.93 | 83 | 86 |
| Santa Cruz (Point Santa Cruz) | 36.95 | 122.05 | 49 | 34 |
| Pigeon Point (North) | 37.15 | 122.35 | 48 | 46 |
| Bodega Bay (Horseshoe Cove) | 38.32 | 123.08 | 126 | 110 |

The posterior body tissue was utilized for electrophoresis and the heads and surrounding tissue were removed and stored at either −80 or −20°C or were transferred to 95% ethanol. These vouchers have been maintained in the lab of DJE and, upon completion of follow-up DNA studies, will eventually be deposited in an appropriate museum for potential future analysis.

## Protein electrophoresis

In initial screening of loci, approximately 26 enzyme systems were tested for activity. Eighteen of the enzyme systems were not pursued further due to lack of detected activity and two of them were excluded because southern and northern populations were monomorphic for the same allele. The analysis included six enzyme systems (Table 2) and these allowed scoring of seven polymorphic loci (although MDH-2 was nearly fixed for a single allele). The enzyme assay recipes (Table 3) were modified from Shaw and Prasad (1969) and Brewer (1970) as practiced in the lab of Dr. Dennis Hedgecock, Bodega Marine Lab, University of California, Davis and DJE at the University of California, Santa Cruz and Friday Harbor Marine Laboratories, University of Washington.

Posterior body tissue from the limpets was homogenized in gel buffer (76 mM TRIS and 5 mM citric acid, pH 8.65) using a sharpened glass stir rod as a pestle and a pinch of white quartz sand to create a rough grinding surface. The homogenate was absorbed onto 3 × 11 mm wicks made of Whatman #1 filter paper for insertion into horizontal starch gels. All samples were run on 12.5% (w:v) gels made with "Starchart" hydrolyzed potato starch and a Tris–citric acid gel buffer (Table 2). Gels were scored by designating the most frequent band in one arbitrarily selected population as the "100" allele and then measuring in mm the distance from each band to the 100 allele band. We refer to our six localities as populations and further divide these populations into two microhabitats using the terms "rock sample" and "barnacle sample". Bands that migrated further than the 100 allele were assigned distances >100 while bands that migrated less than the 100 allele were assigned distances <100. Allelic

**Table 2** Six polymorphic enzyme systems and their corresponding gel and electrode buffers

| Enzyme full name | Enzyme abbreviation | Enzyme commission number | Gel/electrode buffer |
|---|---|---|---|
| Aspartate Amino Transferase | AAT (GOT) | 2.6.1.1 | REG |
| Esterase (nonspecific) | EST | 3.1.1.- | REG |
| Glucose Phosphate Isomerase | GPI | 5.3.1.9 | REG |
| Leucine Amino Peptidase | LAP | 3.4.11.1 | JRP |
| Malate Dehydrogenase | MDH | 1.1.1.37 | REG |
| Peptidase Leucyl-Glycyl-Glycine | PEP-LGG | 3.4.11.- | REG |

REG gel buffer: 76 mM Tris and 5 mM citric acid, pH 8.65. REG bridge buffer: 300 mM boric acid and 60 mM NaOH, pH 8.1. JRP gel buffer: 9 mM Tris, 3.3 mM citric acid, and 1 mM EDTA, pH 7.0. JRP bridge buffer: 135 mM Tris, 39 mM citric acid, and 1 mM EDTA

**Table 3** Stain recipes for six polymorphic enzyme systems

| Enzyme | Recipe |
|---|---|
| AAT | AAT substrate solution: 80 mg of α-ketoglutaric acid, 2.7 g of aspartic acid, 14 g of dibasic sodium phosphate, 10 g of PVP, and 1 g of sodium EDTA dissolved into 1 l of deionized water. 100 ml of substrate solution was added to 55 mg of Fast Garnet GBC or Fast Blue BB |
| EST | 100 ml 0.1 M phosphate buffer pH 6.5, 1.5 ml 1% α-naphthyl acetate, 60 mg Fast Garnet GBC. Soak gel for 15–20 min in a 0.5 M boric acid solution prior to staining |
| GPI | Agar overlay: 20 ml 0.15 M TRIS–HCl buffer pH 8.0, 50 mg fructose-6-phosphate, 10 mg NAD, 10 mg MTT, 5 mg PMS, 0.5 ml 1 M $MgCl_2$ solution, 20 units glucose-6-phosphate dehydrogenase was added to 30 ml of hot (65°C) 1% agarose solution, and then poured immediately over the gel slice(s) |
| LAP | 50 ml 0.2 M maleic anhydride, 0.2 M NaOH solution, 10 ml 0.2 M NaOH, and 40 ml deionized water (final pH, 5.0), 70 mg L-leucine-β-napthyl amide HCl, 30 mg Black K salt. Soak gel for 15–20 min in a 0.5 M boric acid solution prior to staining |
| MDH | 100 ml 0.05 M TRIS–HCl buffer pH 8.6, 50 mg L-malic acid, 30 mg NAD, 20 mg MTT, 1 mg PMS |
| PEP-LGG | Agar overlay: 20 ml 0.1 M phosphate buffer pH 7.5, 20 mg leucyl-glycyl-glycine, 10 mg peroxidase, 5 mg snake venom *(Crotalus adamanteus)*, 10 mg *o*-Dianisidine, 0.5 ml 0.1 M manganese chloride solution. This stain solution was added to 30 ml of hot (65°C) 1% agarose solution, and then poured immediately over the gel slice(s) |

*MTT* (3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazoliumbromide: Thiazolyl blue); *NAD* nicotinamide adenine dinucleotide; *NADP* nicotinamide adenine dinucleotide phosphate; *NADH* nicotinamide adenine dinucleotide, reduced form; *PMS* phenazine methosulfate; *PVP* polyvinylpyrolidone

data were analyzed with the software Genetic Data Analysis v. 1.0 (d16c) (Lewis and Zaykin 2001).

## Results

### Southern versus northern populations

Pronounced allele frequency differences were observed between the Laguna Beach (southern) and Bodega Bay (northern) populations at five enzyme loci (Table 4). The Laguna Beach and Bodega Bay populations (both rock and barnacle samples) were nearly fixed for either "southern" or "northern" alleles, respectively, at the MDH-1 locus and allele frequencies were significantly different ($P < 0.001$; Fisher's Exact test for this and other tests below). Allele frequencies were also significantly different at the PEP-LGG ($P < 0.001$), LAP-2 ($P < 0.001$), EST ($P < 0.001$), and AAT-2 ($P < 0.002$) loci. However, differences at the GPI locus were not significant ($P = 0.088$ for rock morph; $P = 0.099$ for barnacle morph) and the MDH-2 locus was nearly fixed for the same allele in all populations. The genetic distance (Nei 1978) between the Laguna Beach and Bodega Bay populations, using all seven polymorphic loci, was 0.519 and the genetic identity (Nei 1978) was 0.595.

### Zone of sympatry

Based on Murphy's (1978) report over two decades earlier that Monterey Peninsula was the zone of sympatry, Monterey Peninsula populations were tested at each of the six polymorphic allozyme loci. The Monterey Peninsula populations showed a mixture of northern and southern alleles and significant allele frequency differences were found between the rock and barnacle microhabitats at the MDH-1, EST, and AAT-2 loci (Fisher's Exact test: $P < 0.05$). No significant allele frequency differences were found between microhabitats in the Laguna Beach population. Interestingly, in Bodega Bay the GPI and LAP loci showed significant allele frequency differences between microhabitats (Fisher's Exact test: $P < 0.05$).

Three additional sites between Laguna Beach and Bodega Bay were sampled and only the MDH-1 and PEP-LGG loci were analyzed to determine where *L. digitalis* populations overlap with those of *L. austrodigitalis*. Using additional populations from San Simeon, Santa Cruz, and Pigeon Point (Fig. 1), the expected count (based on HWE probabilities assuming a single panmictic population) of rare alleles appearing in homozygous individuals were compared to the observed count at both the MDH-1 and PEP-LGG loci. Observed counts deviated drastically from expected counts (Table 5). For example, the expected number of homozygous individuals expressing rare alleles at both the MDH-1 and PEP-LGG loci in the Monterey Peninsula barnacle sample was 0.14 of 26 total animals (less than one animal) and the actual observed count was seven animals. The observed count of rare homozygous alleles occurring at both loci greatly exceeded the expected count at all four sites between Laguna Beach and Bodega Bay. There were no individuals with homozygous rare alleles in the extreme southern and northern populations. These data support the hypothesis that two species exist at the four sampled presumably sympatric sites.

### Rock versus barnacle microhabitat

Using diagnostic MDH-1 alleles as a marker for species identification in sympatric populations, *L. austrodigitalis* was found in higher frequency in the rock microhabitat while *L. digitalis* was found in higher frequency in the barnacle microhabitat (Table 6). In addition, the relative percentage of *L. digitalis* gradually increased to the north (Table 6). For example, in the Monterey Peninsula population, *L. austrodigitalis* comprised 93% of the rock sample and 68% of the barnacle sample. A little further north on a south-facing Santa Cruz shore, *L. austrodigitalis* comprised 60 and 59% of the rock and barnacle samples respectively. Approximately 30 miles north of Santa Cruz, at the more exposed Pigeon Point (north side), there was a drastic change in species composition between the rock and barnacle microhabitat; *L. austrodigitalis* made up 55% of the rock sample and only 12% of the barnacle sample. Further north at Bodega Bay, *L. digitalis* dominated both microhabitats.

### Hardy Weinberg

In the Laguna Beach, Monterey Peninsula, and Bodega Bay populations, in which all six allozyme loci were analyzed; HW disaccord was seen in a large proportion of the loci (Exact Tests; Table 7). In Laguna Beach (southernmost site), there was HW disaccord at one out of six loci in the rock sample and at three out of six loci in the barnacle sample. In Monterey Peninsula (proposed zone of sympatry), there was HW disaccord at five out of six loci in both the rock and barnacle samples. In Bodega Bay (northernmost site), there was HW disaccord at five out of six loci in the rock sample and at three out of six loci in the barnacle sample. All of the loci that were in HW disaccord showed a deficit of heterozygotes across populations. The MDH-1 and PEP-LGG loci were analyzed in three additional populations between Laguna Beach and Bodega Bay. The alleles at the MDH-1 locus were in HW disaccord in all of the population samples except the Laguna Beach rock sample and the Bodega Bay barnacle sample (two extremes). Likewise, the alleles at the PEP-LGG locus were also in HW disaccord for all except the Laguna Beach rock sample.

**Table 4** Allele frequencies of seven loci examined from populations of *L. austrodigitalis* and *L. digitalis*

| Locus allele | Laguna Beach | | San Simeon | | Monterey Pen | | Santa Cruz | | Pigeon Point | | Bodega Bay | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R | B | R | B | R | B | R | B | R | B | R | B |
| AAT-2 | (42) | (36) | | | (26) | (32) | | | | | (29) | (32) |
| 96 | – | 0.01 | | | 0.08 | 0.03 | | | | | 0.02 | 0.11 |
| 98 | – | – | | | 0.08 | 0.08 | | | | | 0.02 | 0.02 |
| 100 | 0.99 | 0.96 | | | 0.85 | 0.72 | | | | | 0.83 | 0.75 |
| 102 | – | – | | | – | 0.09 | | | | | 0.07 | – |
| 103 | – | 0.03 | | | – | 0.02 | | | | | 0.03 | 0.06 |
| 106 | – | – | | | – | 0.06 | | | | | 0.03 | 0.06 |
| 110 | 0.01 | – | | | – | – | | | | | – | – |
| EST | (34) | (22) | | | (20) | (20) | | | | | (27) | (22) |
| 96 | 0.01 | – | | | 0.03 | 0.08 | | | | | 0.09 | 0.02 |
| 98 | 0.13 | 0.07 | | | 0.15 | 0.30 | | | | | 0.48 | 0.66 |
| 100 | 0.81 | 0.93 | | | 0.68 | 0.63 | | | | | 0.35 | 0.32 |
| 102 | 0.04 | – | | | 0.13 | – | | | | | 0.07 | – |
| GPI | (54) | (46) | | | (25) | (24) | | | | | (49) | (50) |
| 90 | 0.03 | 0.04 | | | – | – | | | | | 0.02 | 0.02 |
| 95 | 0.01 | – | | | 0.02 | 0.02 | | | | | 0.05 | 0.03 |
| 98 | 0.20 | 0.15 | | | 0.18 | 0.40 | | | | | 0.32 | 0.16 |
| 100 | 0.74 | 0.77 | | | 0.62 | 0.42 | | | | | 0.57 | 0.70 |
| 102 | 0.02 | 0.02 | | | 0.18 | 0.17 | | | | | 0.02 | 0.09 |
| 105 | – | – | | | – | – | | | | | – | – |
| 110 | – | 0.01 | | | – | – | | | | | 0.01 | – |
| LAP-2 | (41) | (37) | | | (16) | (11) | | | | | (32) | (39) |
| 96 | 0.12 | 0.07 | | | 0.19 | 0.14 | | | | | 0.03 | – |
| 98 | 0.34 | 0.32 | | | 0.28 | 0.32 | | | | | 0.02 | – |
| 100 | 0.54 | 0.55 | | | 0.34 | 0.50 | | | | | 0.13 | – |
| 102 | – | 0.05 | | | 0.13 | 0.09 | | | | | 0.30 | 0.50 |
| 104 | – | – | | | 0.06 | – | | | | | 0.53 | 0.50 |
| MDH-1 | (114) | (87) | (40) | (45) | (54) | (75) | (46) | (32) | (46) | (43) | (91) | (88) |
| 100 | 1.00 | 0.99 | 0.92 | 0.72 | 0.93 | 0.65 | 0.60 | 0.54 | 0.55 | 0.12 | 0.04 | – |
| 104 | – | – | – | – | – | 0.05 | – | 0.08 | – | – | – | – |
| 110 | – | 0.01 | 0.08 | 0.28 | 0.07 | 0.30 | 0.40 | 0.38 | 0.45 | 0.88 | 0.96 | 1.00 |
| MDH-2 | (136) | (94) | | | (53) | (75) | | | | | (102) | (89) |
| 98 | 0.01 | – | | | – | – | | | | | 0.03 | – |
| 100 | 0.99 | 0.95 | | | 0.99 | 0.99 | | | | | 0.96 | 0.99 |
| 102 | – | 0.05 | | | – | 0.01 | | | | | – | 0.01 |
| 104 | – | – | | | 0.01 | – | | | | | – | – |
| PEP-LGG | (28) | (26) | (35) | (23) | (28) | (26) | (11) | (10) | (17) | (17) | (31) | (23) |
| 95 | – | – | – | – | – | – | – | – | – | 0.06 | – | – |
| 97 | – | – | 0.07 | 0.41 | 0.13 | 0.23 | 0.32 | 0.70 | 0.32 | 0.68 | 1.00 | 0.91 |
| 100 | 0.93 | 0.88 | 0.84 | 0.59 | 0.76 | 0.62 | 0.41 | 0.10 | 0.50 | 0.21 | – | 0.02 |
| 102 | 0.05 | 0.10 | 0.09 | – | 0.11 | 0.15 | 0.27 | 0.20 | 0.18 | 0.06 | – | 0.04 |
| 106 | 0.02 | 0.02 | – | – | – | – | – | – | – | – | – | – |
| 110 | – | – | – | – | – | – | – | – | – | – | – | 0.02 |

For each of the six sample sites, "R" designates the rock microhabitat and "B" designates the barnacle bed microhabitat. The number of individuals scored for each locus is shown in parenthesis for each population

**Table 5** Expected versus observed counts of rare alleles co-occurring in homozygous individuals when MDH-1 and PEP-LGG loci are grouped. Sample size indicated the number of limpets sampled at both the MDH-1 and PEP-LGG loci. Rare alleles did not co-occur in the extreme northern and southern populations (Bodega Bay and Laguna Beach)

| Population | Microhabitat | Sample size | Expected | Observed |
|---|---|---|---|---|
| Laguna Beach | Rock | 28 | – | – |
| | Barnacle | 11 | – | – |
| San Simeon | Rock | 28 | 0.00 | 2.00 |
| | Barnacle | 20 | 0.26 | 5.00 |
| Monterey Peninsula | Rock | 27 | 0.00 | 2.00 |
| | Barnacle | 26 | 0.14 | 7.00 |
| Santa Cruz | Rock | 9 | 0.13 | 2.00 |
| | Barnacle | 8 | 0.00 | 0.00 |
| Pigeon Point | Rock | 16 | 0.34 | 6.00 |
| | Barnacle | 17 | 0.02 | 0.00 |
| Bodega Bay | Rock | 25 | – | – |
| | Barnacle | 10 | – | – |

### Hybridization

There was no evidence of hybridization between *L. digitalis* and the proposed *L. austrodigitalis.* Across all six of the populations, none of the sampled individuals were heterozygous for the southern and northern allele at both the MDH-1 and PEP-LGG loci. There were of course individuals that were heterozygous for the southern and northern allele at one of the two loci, but the frequency of these heterozygotes was actually well below Hardy Weinberg expectations.

## Discussion

### Evidence for separate species

The current data support the hypothesis that *L. austrodigitalis* and *L. digitalis* are distinct species. Given their striking similar morphology and ecology it is reasonable to suspect that they are sibling species with a parapatric pattern of distribution but the data presented herein does not address whether they are sister species within *Lottia*. Allele frequency data from five allozyme loci, AAT-2, EST, LAP-2, MDH-1, and PEP-LGG showed significant differences between the Laguna Beach and Bodega Bay populations. If one assumed there was but a single species, then the occurrence of individuals with rare alleles in homozygous combinations was significantly higher than expected based on HWE probabilities for both the MDH-1 and PEP-LGG loci. For instance, the chance that one individual would be homozygous for a rare allele at both the MDH-1 and PEP-LGG loci is approximately 1 in 1,000 in the Monterey Peninsula barnacle population and yet out of 28 limpets scored for both loci, 10 of them were homozygous for the rare allele at both loci. In the extreme southern and northern populations (Laguna Beach and Bodega Bay), no sampled individual was homozygous for rare alleles at both the MDH-1 and PEP-LGG loci. The remaining four sites in between generally were observed to have high counts of individuals homozygous for a rare allele at the MDH-1 and PEP-LGG loci. For these sites, only the Santa Cruz barnacle population and the Pigeon Point barnacle population, which are furthest north, lacked such individuals.

**Table 6** Proportions of *L. austrodigitalis* (*a*) versus *L. digitalis* (*d*) within each microhabitat at six locations

| Population | Species | Rock | Barnacle |
|---|---|---|---|
| Laguna Beach (201) | *a* | 1.00 | 1.00 |
| | *d* | 0.00 | 0.00 |
| San Simeon (85) | *a* | 0.92 | 0.72 |
| | *d* | 0.08 | 0.28 |
| Monterey Peninsula (129) | *a* | 0.93 | 0.68 |
| | *d* | 0.07 | 0.32 |
| Santa Cruz (78) | *a* | 0.60 | 0.59 |
| | *d* | 0.40 | 0.41 |
| Pigeon Point (89) | *a* | 0.55 | 0.12 |
| | *d* | 0.45 | 0.88 |
| Bodega Bay (180) | *a* | 0.05 | 0.00 |
| | *d* | 0.95 | 1.00 |

Species were identified using MDH-1 alleles. Sample size (*n*) at each location is shown in parenthesis and the number of animals sampled from each microhabitat varies

The estimated genetic distance (Nei 1978) between Laguna Beach and Bodega Bay may be slightly inflated because the seven allozyme loci used to calculate this value were polymorphic, although MDH-2 was nearly monomorphic (95–99% fixed for a single allele). The genetic distance between the Laguna Beach and Bodega Bay populations (0.519) falls within the range of "separate species" as reported by Weber et al. (1997) in a study comparing several similar species of *Helcion* limpets where genetic distance ranged from 0.351 to 0.610. In addition, a study of sibling species in the marine snail genus *Cerithium* reported a genetic distance of 0.444 between a lagoon

**Table 7** Exact tests for Hardy Weinberg proportions from populations of *L. austrodigitalis* and *L. digitalis*. Individual *P* values are shown for the rock and barnacle microhabitats from each of the six sample sites. *P* values denoted with an asterisk indicate that the genotype frequencies are significantly different than Hardy Weinberg proportions

| Population | Microhabitat | AAT-2 | EST | GPI | LAP-2 | MDH-1 | PEP-LGG |
|---|---|---|---|---|---|---|---|
| Laguna Beach | Rock | 1.000 | 0.275 | 0.006* | 0.377 | 1.000 | 0.109 |
| | Barnacle | 0.014* | 1.000 | 0.564 | 0.291 | 0.006* | 0.004* |
| San Simeon | Rock | | | | | <0.001* | <0.001* |
| | Barnacle | | | | | <0.001* | <0.001* |
| Monterey Peninsula | Rock | <0.001* | 0.008* | 0.056* | 0.062 | <0.001* | <0.001* |
| | Barnacle | <0.001* | <0.001* | 0.056* | 0.267 | <0.001* | <0.001* |
| Santa Cruz | Rock | | | | | <0.001* | <0.001* |
| | Barnacle | | | | | <0.001* | 0.001* |
| Pigeon Point | Rock | | | | | <0.001* | <0.001* |
| | Barnacle | | | | | 0.008* | <0.001* |
| Bodega Bay | Rock | <0.001* | 0.215 | 0.003* | <0.001* | 0.023* | 0.015* |
| | Barnacle | 0.003* | 0.139 | <0.001* | 0.195 | 1.000 | <0.001* |

species and a parapatric open-sea species (Boisselier-Dubayle and Gofas 1999). As a final point of reference amongst limpets, *Lottia gigantea* was recently reported as a sister taxon to the clade that includes *Lottia limatula* (Simison 2000), and the reported genetic distance between *L. gigantea* and *L. limatula* is 1.006 (Sly 1984). Genetic distances between sympatric populations are not reported here because a comparison of samples that contain various mixtures of two species would not be appropriate.

Could the allele frequency differences between southern and northern populations simply be a result of local adaptation to different environments rather than evidence for two separate species? In marine invertebrates, there has been an observed correlation between allele frequency and temperature and salinity gradients. The frequency of certain LAP alleles has been correlated with salinity gradients in mussel populations (Koehn et al. 1980; Hilbish and Koehn 1985) as well as with temperature in a bryozoan (Schopf and Gooch 1971). The frequencies of certain AAT alleles were also correlated with temperature in a bryozoan (Schopf 1974) and a marine snail (Johannesson et al. 1995). If there is a single locally adapted species, we might expect there to be allele frequency differences between southern and northern populations, perhaps as a result of different selection pressures. If locally adapted populations come together, and there is genetic exchange, one would expect to find individuals that are heterozygous for the northern and southern allele in Hardy Weinberg proportions. Expectations are different if there is more than one species. If there were two species that were not hybridizing in sympatry, one would expect to find few individuals that were heterozygous for the southern and northern allele. For the two loci that showed nearly fixed allele differences in the southern vs. northern populations, MDH-1 and PEP-LGG, there were very few individuals that were heterozygous for the northern and southern allele at one of the two loci (well below HW proportions) and there were no individuals from any of the six populations that were heterozygous for the southern and northern allele at both loci.

## Explanations for heterozygote deficiency

Although not statistically significant with the given sample sizes, Murphy (1978) reported a deficiency in heterozygosity in each of his 10 sample populations of *L. digitalis* and *L. austrodigitalis* and Gresham and Tracey (1975) reported that observed heterozygosity was, on average, half of the expected value for three populations of *L. digitalis* in Bodega Head. Due to high polymorphism at 23 enzyme loci, Gresham and Tracey (1975) attributed this deficiency to the Wahlund effect (the inclusion of two or more genetically distinct subpopulations into a single sample). Other potential causes for heterozygote deficiency are inbreeding and strong selection against heterozygotes. Limpets have a planktonic larval stage lasting at least 5 days (Kessel 1964; Proctor 1968) and possibly up to 3 weeks if proper settlement sites are unavailable (Karp 1970). Given such dispersal potential, one might expect gene flow between local populations to be fairly high. In addition, the degree of polymorphism in the loci that were sampled was high, which suggests that inbreeding is an unlikely cause of the observed heterozygote deficiency. Selection against heterozygotes is also unlikely if there is a single species because we found individuals that were homozygous for different alleles. In order to become homozygous for rare alleles, there would need to be heterozygotes that survive and reproduce. Therefore, the observed heterozygote deficiencies are more likely the result of either the Wahlund effect or the existence of more than one species.

If one considers that there are two distinct species, then there is an additional explanation for the observed heterozygote deficiency based on one or more founder events and subsequent population "bottlenecks" during the expansion process. Murphy (1978) proposed that, during the Pleistocene

or earlier climatic fluctuations, the warmer water species, *L. austrodigitalis* may have extended its range northward during a warming trend and then contracted its range southward during a subsequent cooling trend. During the range contraction, *L. austrodigitalis* may have left behind a northern founder population in a warm water embayment. This northern founder population may have given rise to a new cold-water species, *L. digitalis*, via allopatric speciation. In contrast to this view, we prefer to merely postulate that a parental species, whose temperature preferences are unknown, was fragmented into the present two daughter species due to range expansions and subsequent contractions. Reduced heterozygosity at high latitudes has been observed in a number of populations believed to be founded recently following postglacial warming (Hellberg 1994; Merila et al. 1996; Shaffer and McKnight 1996; Marko 1998, 2004; Hellberg et al. 2001; Jacobs et al. 2004).

## Microhabitat partitioning

In agreement with Murphy's (1983) findings, *L. austrodigitalis* was found in higher frequency in the rock microhabitat and *L. digitalis* was found in higher frequency in the barnacle microhabitat in the four sympatric populations. The MDH-1 locus was used to identify individuals to species whereas Murphy (1983) used the LAP locus. The most extreme examples of microhabitat differences in species composition were found near the end points in the zone of sympatry. In a similar study of the gastropod sibling species pair *Nucella emarginata* and *N. ostrina*, it was reported that in the zone of sympatry, each species was found in a different microhabitat; individuals of *N. emarginata* were found in relatively shallow, quiet-water environments, while individuals of *N. ostrina* were found on shores exposed to slightly greater wave action (Marko 1998).

Species of temperate molluscan provinces tend to submerge toward the lower latitude ends of their ranges (Valentine 1961) and many marine species that occur in shallow water or even intertidally, in the cooler portions of their range, are found progressively deeper near the warmer limits of their distribution (Fields et al. 1993). At all of the sample sites, the gooseneck barnacle microhabitat was found lower in the intertidal zone than the vertical rock microhabitat. The observed microhabitat partitioning in sympatric populations might be a result of *L. digitalis* not surviving in the upper of its two normal microhabitats at lower latitudes where air temperatures are warmer. It is also possible that *L. digitalis* has displaced *L. austrodigitalis* from the barnacle microhabitat through interspecific competition for food or space. Biotic factors, such as competition, tend to influence vertical zonation patterns in the lower intertidal and abiotic factors, such as desiccation, tend to influence vertical zonation patterns in the high intertidal (Ricketts et al. 1985). Further studies might investigate the hypothesis of interspecific competition causing ecological displacement or resource partitioning by utilizing a series of competition experiments in the zone of sympatry. One might test whether the presence or absence of one species affects the proportion of the other species in each microhabitat.

## Current data versus data reported in 1983

After comparing the proportion of *L. austrodigitalis* to *L. digitalis* in each of the six sample sites in the current study (MDH-1 alleles used for species ID) to proportions reported from 14 sample sites in a prior study (LAP alleles used for species ID; Murphy 1983), it appears that there has been a northern range expansion for *L. austrodigitalis* in the last two decades at the expense of *L. digitalis*. There were no *L. austrodigitalis* present in Santa Cruz, Pigeon Point, or Bodega Bay in 1977 (Murphy 1983), but in the current study, relatively high percentages of *L. austrodigitalis* were found in Santa Cruz (60% rock; 49% barnacle) and Pigeon Point (55% rock; 12% barnacle). In Bodega Bay, two out of 92 rock morphs were homozygous for the MDH-1 southern allele but none of the barnacle morphs displayed the MDH-1 southern allele. Comparing 1977 species ratios to current species ratios in the Monterey Peninsula, *L. austrodigitalis* comprised 66% versus the current 93% of the rock population and comprised 29% versus the current 64% of the barnacle population (Table 8). In the prior study, Monterey Peninsula was found to be the northernmost limit for *L. austrodigitalis*, but current data show that Bodega Bay may now be the northernmost limit. Sampling more locations north of Bodega Bay would confirm the northern limit of *L. austrodigitalis*. This drastic change in species composition over the last two decades shows quite a prominent range expansion for *L. austrodigitalis* in a relatively short period of time.

A study comparing 1931–1933 intertidal surveys in Pacific Grove, CA to re-sampled surveys at the same location in 1993–1996 (Sagarin et al. 1999) documented changes in the abundance of 46 invertebrate species over

**Table 8** Comparison of *L. austrodigitalis* and *L. digitalis* proportions within each microhabitat in Monterey Peninsula from the current study versus Murphy (1983)

| Microhabitat | Current data 1998–1999 | | Murphy data 1977 | |
|---|---|---|---|---|
| | *austrodigitalis* | *digitalis* | *austrodigitalis* | *digitalis* |
| Rock | 0.93 | 0.07 | 0.66 | 0.34 |
| Barnacle | 0.64 | 0.32 | 0.29 | 0.71 |

MDH-1 alleles were used to identify individuals to species in the current study and LAP alleles were used in the prior study (Murphy 1983)

60 years. The abundance of most southern species (10 out of 11) increased while most of the northern species abundances decreased and cosmopolitan species showed no clear trend. These marked shifts in species ranges were attributed to the effects of ocean climate change on sea surface temperatures, which were reported to increase significantly (avg. 0.79°C, max. 1.26°C) during the period of 1920–1995. One might expect *L. austrodigitalis* to increase in proportion to *L. digitalis* in Santa Cruz, Pigeon Point and even Bodega Bay in the last two decades because, as Sagarin's study reveals, temperature shifts will usually favor species more closely adapted to the new thermal regime (Fields et al. 1993).

We believe that this limpet species complex will continue to be a good system for tracking ongoing northern shifts by southern species that might also be an indicator for anthropomorphic-related global warming trends. In contrast to more difficult to interpret tallies of lower-latitude species expanding their range to higher latitudes, these limpet species are among the most common and accessible marine animal species throughout California and beyond, and are likely to remain so. We propose that continuing to track the history of a shifting transition zone will provide a useful index climate change.

## Recognizing cryptic species

The recognition of cryptic (including sibling) species is important for many reasons. Knowlton (1993) stated that the increase in biodiversity from discoveries of cryptic species is often four-fold or greater in comprehensive studies of a single region. She also stated that as a rough estimate, one could expect the number of marine species to increase by an order of magnitude if cryptic species are considered. In evaluating the ecology and behavior of marine species, incorrect conclusions can be reached if one fails to recognize cryptic species that have different competitive abilities, different relationships with symbionts or predators, and different responses to environmental factors. Cryptic species have been used as environmental indicators and some have been shown to respond differently to environmental pollutants (Linke-Gamenick et al. 2000a, b; Warwick and Robinson 2000; Rocha-Olivares et al. 2004). For example, three cryptic species of polychaete worms exhibited diverse responses to the toxin, fluorenthene (Linke-Gamenick et al. 2000a, b) and it was recently shown that co-occurring cryptic species of harpacticoid copepods exhibit unique toxic responses to heavy metals (Rocha-Olivares et al. 2004). Viewing these cryptic species as a single species in a bioassay experiment would yield confusing results and misleading conclusions. The management of marine fisheries and marine conservation efforts might also be jeopardized if cryptic species with different ecological traits are not recognized. Geller (1999) revealed that a decline in the native mussel, *M. trossulus*, was masked by a cryptic species invasion of *M. galloprovincialis*. We cannot protect species of whose existence we are ignorant.

Limpets referred to as *L. digitalis* have been the subject of many studies covering a diversity of subject matter including limpet phylogenetics (Lindberg 1986; Ponder and Lindberg 1996), interspecific competition (Haven 1973), limpet predation (Frank 1982; Lindberg et al. 1987; Marsh 1987; Hahn and Denny 1989b), community dynamics (Farrell 1988; Wootton 1993), human impact (Lindberg et al. 1998), parasite infection (Ching and Grosholz 1988), habitat partitioning (Hahn and Denny 1989a), effects of grazing on microflora (Nicotri 1977; Harley 2003), temperature and desiccation resistance (Collins 1977; Roland and Ring 1977), respiratory response to temperature variation (Doran and Mckenzie 1973), spawning, larval development and settlement (Koppen et al. 1996; Shanks 1998), gamete structure (Hodgson and Chia 1993), homing behavior and seasonal migration (Breen 1972), growth factors (Giesel 1969), shell polymorphism and body size variation (Giesel 1970; Hartwick 1981; Byers 1989; Lindberg and Pearse 1990; Hobday 1995), evolution of shell shape (Denny 2000), and factors affecting orientation and movement (Miller 1969). Moreover, the mitochondrial genome of *L. digitalis* was recently sequenced (Simison et al. 2006), and a congener's (*L. gigantea*) nuclear genome sequence was approved in 2005 as among the first lophotrochozoan (and molluscan) genomes to be sequenced and is currently underway. This extensive list of studies involving "*L. digitalis*" demonstrates how critical it is that we recognize the existence of *L. austrodigitalis* and *L. digitalis* as co-occurring cryptic species.

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