Journal of Shellfish Research, Vol. 20, No. 1, 453–457, 2001.

A MOLECULAR ASSAY IDENTIFIES MORPHOLOGICAL CHARACTERS USEFUL FOR DISTINGUISHING THE SIBLING SPECIES *LITTORINA SCUTULATA* AND *L. PLENA*

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ABSTRACT Sibling species *Littorina scutulata* and *L. plena* are difficult to distinguish in the field. Here we present a new molecular tool and use it to evaluate the discrete and quantitative morphological characters that have been proposed as diagnostic. We collected 385 snails of both species from 11 sites in Washington state and used restriction enzyme digestion of a PCR-amplified, 480 bp fragment of the mitochondrial cytochrome b gene to distinguish the species. This new molecular assay produces species-specific restriction fragment patterns that correspond with identification of males by penis morphology. To evaluate the usefulness of morphological characters, we scored three discrete shell characters (presence of basal band, presence of basal ridge, and size of checker pattern) as well as tentacle coloration. The four discrete characters differ significantly between the two species, though none is completely diagnostic. Tentacle coloration is the most reliable character and may be combined with the shell characters for successful identification. The two species also differ significantly in overall size and in three out of five size-independent shell shape measurements, with *L. scutulata* having larger, taller-spired shells with narrower apertures. However, shell shape does not separate the species well because of intraspecific variation, and it is unlikely to be useful for species identification. Further analysis suggests that at least some of this intraspecific variation is genetic rather than environmental. The distributions of the two species overlap broadly in Washington, though only *L. plena* was found in exposed outer coast habitats, contrary to previous work.

KEY WORDS: *Littorina scutulata*, *L. plena*, species identification, sibling species, molecular systematics

INTRODUCTION

The taxonomic history of the *Littorina scutulata* species complex, a group of sympatric intertidal prosobranch gastropods in the Northeastern Pacific, has been complicated by morphological similarity across species and variation within species. Currently two sibling species are recognized, *L. scutulata* (*sensu stricto*) Gould 1849 and *L. plena* Gould 1849, which are distinguished on the basis of reproductive characters, including penis, pallial oviduct, and egg capsule morphology (Murray 1979, Mastro et al. 1982, Reid 1996). These characters, however, are difficult to use for the non-destructive field identification that is necessary for many ecological studies. Reproductive characters cannot be used for juveniles, and we have found the dissection necessary for examining pallial oviduct morphology to be difficult, especially in small specimens.

Other diagnostic morphological characters have been proposed. Three discrete shell characters have been described: a pale basal band (Murray 1982, Reid 1996, Chow 1987, Rugh 1997) and a narrow basal ridge (Rugh 1997), both found more often in *L. plena*, and the pattern of checkers on many shells, which tend to be smaller in *L. plena* than in *L. scutulata* (Reid 1996, Rugh 1997). Rugh (1997) was able to use these three shell characters alone to correctly identify 17 male specimens of both species from southern California. Reid (1996) described differences in tentacle coloration: *L. scutulata* individuals tend to have "transverse black bands and flecks," while *L. plena* tend to have a "broad, unbroken black stripe with transverse flecks, or all black." Murray (1982) described a set of discriminant functions of four quantitative shell measurements that correctly classified 96% of specimens. Further principal component analysis by Murray (1982) showed *L. scutulata* shells to be generally taller with narrower spire angles and shorter aperture openings relative to shell height. Chow (1987) combined three quantitative shell measurements with number of whorls, presence of a basal band, and presence of tesselation in another discriminant function analysis. This analysis correctly classified 92% of specimens, but only when using snails from one habitat; combining specimens from different habitats introduced too much intraspecific variation to allow correct classification. Chow (1987) also found *L. scutulata* shells to be larger, narrower, and less likely to have a basal band, which agreed with past work. Other characters used with varying success to distinguish these species include spiral sculpture on the shell and radular characters (Reid 1996, Mastro et al. 1982).

Mastro et al. (1982) found eight polymorphic allozyme loci at which the two species differ in their allele frequencies. However, none of these loci was diagnostic. No other molecular studies to date have identified a reliable molecular character at a polymorphic locus that distinguishes the two species.

The previous studies of morphological differences used specimens that were positively identified using reproductive characters, thus excluding pre-reproductive animals. Here we present a molecular technique for identifying individuals of all ages using mitochondrial DNA. We use this tool to evaluate the reliability of characters that can be observed on intact animals: the three discrete shell characters described above, tentacle coloration, and quantitative shell shape differences.

MATERIALS AND METHODS

We collected 385 snails of both species from 11 areas around Puget Sound and the outer coast of Washington state in January 1998 (see Fig. 1) and kept them alive until DNA extraction. Animals of all sizes, including juveniles, from within a randomly chosen, small (~1 m^2) area of rocky shore habitat were collected. Individuals anesthetized in 7% $MgCl_2$ seawater solution were

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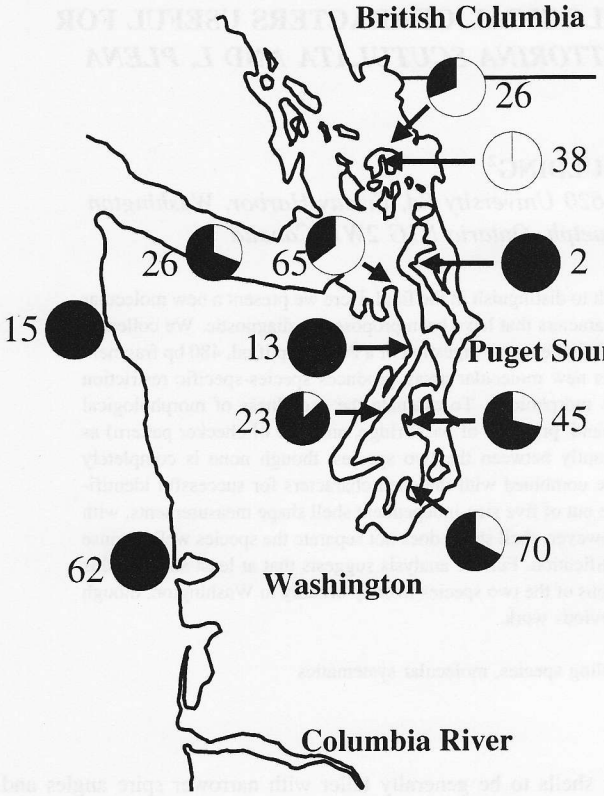

Figure 1. Map of western Washington state showing collection sites in Puget Sound and on the outer coast. Pie diagrams show relative abundance of *L. plena* (dark) and *L. scutulata* (light) with the total sample size for each site.

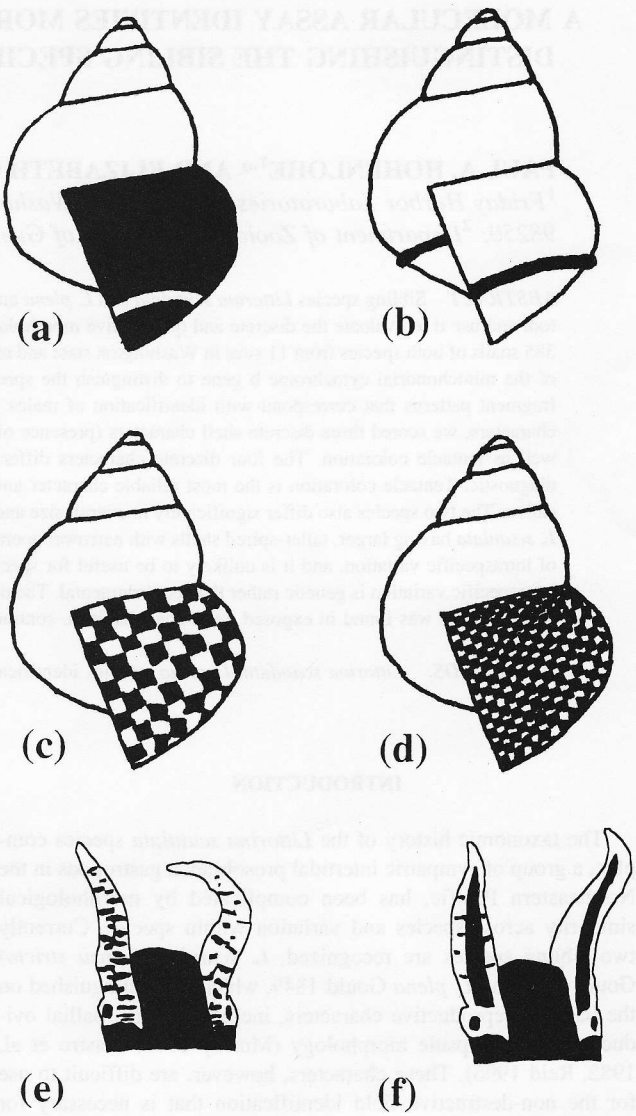

Figure 2. Discrete characters. (a) A pale basal band and (b) basal ridge are found more often in *L. plena*. (c) A pattern of large checkers typifies *L. scutulata*, while (d) small checkers are found more often in *L. plena*. (e) Two tentacle coloration patterns found in *L. scutulata*: transverse bands (left) and bands and spots (right). (f) Two tentacle patterns found in *L. plena*: a broad central stripe with bands (left) and without bands (right).

scored for three discrete shell characters (Fig. 2a–d): basal band present or absent, basal ridge present or absent, and checkers large, small, or absent. Large checkers lie in five to 12 spiral rows, depending on shell size, while small checkers number more than 10 spiral rows. Tentacle coloration was scored in one of seven categories: dark transverse bands, spots and bands, spots, dark central stripe, central stripe with bands, no color, or all dark (Fig. 2e–f; see also Reid 1996). In all further analyses, the first three categories were grouped as "transverse bands," and the last four were grouped as "central stripe." Individuals' sex was also recorded and males scored as *L. plena* or *L. scutulata* penis type. *L. scutulata* penes are relatively short with a terminal bifurcation, while *L. plena* penes are longer, often coiled, with a bifurcation near the base (Reid 1996).

Animals were then sacrificed for molecular analysis. Using the extraction protocol and PCR primers described in Kyle and Boulding (1998), a 480 bp fragment of the mitochondrial cytochrome-b gene was amplified for each individual. These were then digested for two hours using the restriction enzyme *Alu* I and the digests run on a 2% agarose gel. To predict restriction sites for the two species, we examined 36 *L. plena* and 18 *L. scutulata* haplotype sequences from Kyle and Boulding (2000) (Genbank accession nos. AF077238–AF077291). Identification of these sequences by Kyle and Boulding (2000), however, depended on a single *L. plena* sequence from Reid et al. (1996) (Genbank accession no. U46815), so our analysis also functioned to confirm the identification of those sequences. We expected fragments of 161, 233, and 86 bp for *L. scutulata* and 109, 15, and 356 bp for *L. plena*.

Eight quantitative shell measurements were taken from each individual using a dissecting microscope connected to computer imaging software (Fig. 3). Since six of these measurements are lengths, they were combined into three non-dimensional ratios to remove the effect of overall shell size as follows: Relative aperture height = Aperture height / Shell height; Whorl ratio = Whorl n–2 / Whorl n–1; and Aperture shape = Short axis / Long axis. Statistical tests and discriminant function analysis used these three ratios as well as spire angle and aperture angle (Fig. 3).

In an attempt to improve differentiation of the two species based on shell morphology, further discriminant function analyses were done using different sets of variables. In the first, size was included specifically by adding shell height to the three ratios and two angles, providing six variables for discriminant analysis. In the second revision, the original six linear measurements were not combined into ratios as above, but rather normalized by the

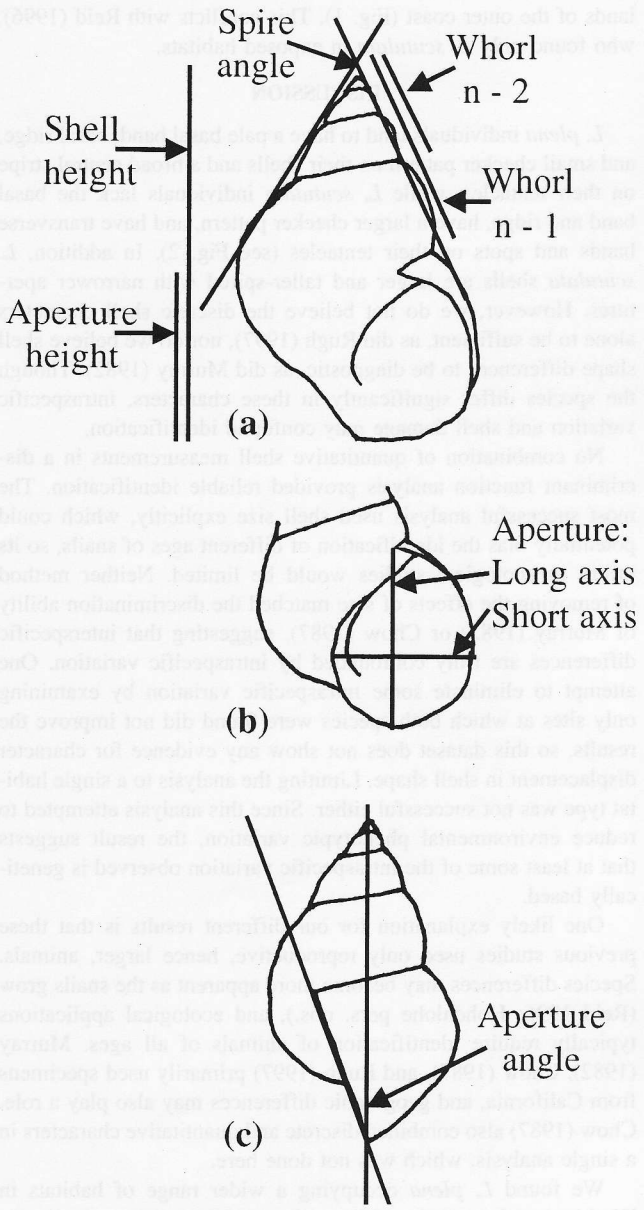

Figure 3. Quantitative shell measurements. Shells were viewed through a dissecting scope and video camera connected to a computer and measurements were taken using imaging software. The two angles were measured in degrees and the six lengths in mm.

method of Clarke et al. (1999). The effects of size were removed from each linear measurement by dividing the geometric mean of each specimen's measurements:

$$\text{geometric mean} = 10\text{^}[(\log_{10}(x_1) + \log_{10}(x_2) + \ldots + \log_{10}(x_6))/6]$$

where x_1 through x_6 are the original linear measurements. This provided two angles and six normalized linear measurements for discriminant analysis.

The third attempt to improve the discrimination used only the specimens from six sites at which both species were found (see Fig. 1) and analyzed the three ratios and two angles as above. Finally, another analysis was performed on data limited to three sites of similar habitat following Chow (1987). These three sites are the three southern Puget Sound sites shown in Figure 1. All are protected shores at which both species were found.

RESULTS

Restriction enzyme digestion with *Alu* I produced two discrete fragment length patterns as expected: one pattern with three closely spaced bands (*L. scutulata*) and another with two widely spaced bands (*L. plena*; the 15 bp fragments typically migrated off the gel). These corresponded precisely with identification of males by penis morphology (n = 48 *L. scutulata* and 71 *L. plena*). This supports the identification of sequences in Kyle and Boulding (2000) and suggests that these restriction sites are consistent across haplotypes within each species. The following analyses are based on these 119 males as well as females identified using only restriction digest and males identified by penis morphology alone. Sample sizes vary because of damage to some shells during DNA extraction.

The frequency of each of the four discrete characters differs significantly between the species (Table 1) consistent with Rugh (1997) and Reid (1996). The differences remain significant following Bonferroni correction of the original p-values from four separate contingency table analyses (Rice 1989). However, no single character completely separates the two species. The shell characters were often not visible because of shell erosion from wave action or fungal or other epiphytic growth, creating a potential bias toward identification as *L. scutulata* from the first two characters. Many undamaged shells also lack any checker pattern. Tentacle coloration, because it was always scorable in live animals, was the most reliable discrete character.

Following Bonferroni correction of the original p-values from six separate two-tailed *t*-tests (Rice 1989), the species differ significantly in four shell measures (Table 2). *L. scutulata* shells are significantly larger than *L. plena* shells. Of the five size-independent shell measures, the two species differed significantly in spire angle, whorl ratio, and aperture shape. These results are consistent with those of Murray (1982) and Chow (1987), confirming that *L. scutulata* shells are larger, narrower, and taller-spired with narrower apertures. Relative aperture height, though not significantly different, also follows the trend found by Murray (1982). Three of these shell measures, shell height, whorl ratio, and aperture shape, remain significant when considering only the six sites at which both species were found (Table 2). The difference is lost for spire angle. However, the difference in relative aperture height reverses and becomes statistically significant when only these sites are considered.

Combining specimens from all the collection sites, these five

TABLE 1.

Species differences for four discrete characters. Species were identified by restriction enzyme digest and penis morphology. Data given are number of specimens (percentage) in each category. The p-values are Bonferroni corrections (Rice 1989) of separate χ^2-tests.

Character	State	*L. scutulata*	*L. plena*	p-value
Basal band	present	18 (12.4)	124 (51.7)	<0.001
	absent	127 (87.6)	116 (48.3)	
Basal ridge	present	23 (15.9)	149 (62.1)	<0.001
	absent	122 (84.1)	91 (37.9)	
Checker pattern	large	122 (84.1)	25 (10.4)	<0.001
	small	5 (12.4)	93 (38.8)	
	absent	18 (12.4)	122 (50.8)	
Tentacle color	transverse bands	130 (89.7)	10 (4.2)	<0.001
	central stripe	15 (10.3)	230 (95.8)	

TABLE 2.

Quantitative shell measurements. Numbers given are mean (standard deviation), and the p-values are Bonferroni corrections (Rice 1989) of separate two-tailed *t*-tests. For each measure, the first row includes specimens from all sites, and the second row includes only specimens from the 6 sites at which both species were found.

Measurement all sites: 6 sites:	*L. scutulata* n = 142 n = 104	*L. plena* n = 210 n = 146	p-value
Shell height (mm)	8.1 (1.6)	6.3 (1.3)	<0.001
6 sites	7.4 (1.2)	6.3 (1.2)	<0.001
Spire angle (deg.)	54.2 (4.0)	55.5 (5.8)	0.045
6 sites	54.4 (4.1)	54.5 (5.5)	>0.5
Aperture angle (deg.)	24.0 (2.4)	23.8 (2.1)	>0.5
6 sites	23.9 (2.4)	23.9 (2.2)	>0.5
Relative ap. Height	0.516 (0.038)	0.519 (0.039)	>0.5
6 sites	0.528 (0.034)	0.513 (0.039)	0.01
Whorl ratio	0.595 (0.163)	0.555 (0.045)	<0.001
6 sites	0.607 (0.188)	0.568 (0.002)	0.045
Aperture shape	0.703 (0.038)	0.726 (0.037)	<0.001
6 sites	0.697 (0.040)	0.721 (0.037)	<0.001

shell measures were used in a discriminant function analysis without much success: the function correctly classified only 69% of *L. scutulata* and 66% of *L. plena* specimens. This seems to be the result of overlapping intraspecific variation for all of the characters.

Attempts to improve the discriminant function analysis were marginally successful. Because *L. scutulata* shells were significantly larger, including shell height improved posterior classification to 76% for *L. scutulata* and 81% for *L. plena*. Normalizing the linear measurements by the geometric mean (Clarke et al. 1999) improved classification of *L. scutulata* to 82%, but reduced successful classification of *L. plena* to 62%. Limiting the analysis to the six sites where both species were found made no improvement over the original dataset: 69% for *L. scutulata* and 68% for *L. plena*. Finally, restricting the analysis to the three protected Puget Sound sites only slightly improved classification: 66% for *L. scutulata* and 74% for *L. plena*. These results are summarized in Table 3.

The geographic ranges of these two species overlap significantly in Washington, although *L. scutulata* is found only in moderately exposed to sheltered areas of Puget Sound while *L. plena* is found from sheltered sites in Puget Sound to the exposed headlands of the outer coast (Fig. 1). This conflicts with Reid (1996), who found only *L. scutulata* in exposed habitats.

TABLE 3.

Varying success of discriminant function analyses of quantitative shell measurements. Correct posterior classification percentages are given along with total sample size of specimens for each species.

Variable combination	L. scutulata	L. plena
2 angles, 3 ratios	69 (n = 142)	66 (n = 210)
2 angles, 3 ratios, shell height	76 (n = 142)	81 (n = 210)
2 angles, 6 linear measurements normalized by geometric mean	82 (n = 142)	62 (n = 210)
2 angles, 3 ratios from sites with both species	69 (n = 104)	68 (n = 146)
2 angles, 3 ratios from protected habitats only	66 (n = 38)	74 (n = 99)

DISCUSSION

L. plena individuals tend to have a pale basal band, basal ridge, and small checker pattern on their shells and a broad central stripe on their tentacles, while *L. scutulata* individuals lack the basal band and ridge, have a larger checker pattern, and have transverse bands and spots on their tentacles (see Fig. 2). In addition, *L. scutulata* shells are larger and taller-spired with narrower apertures. However, we do not believe the discrete shell characters alone to be sufficient, as did Rugh (1997), nor do we believe shell shape differences to be diagnostic, as did Murray (1982). Though the species differ significantly in these characters, intraspecific variation and shell damage may confound identification.

No combination of quantitative shell measurements in a discriminant function analysis provided reliable identification. The most successful analysis used shell size explicitly, which could potentially bias the identification of different ages of snails, so its utility in ecological studies would be limited. Neither method of removing the effects of size matched the discrimination ability of Murray (1982) or Chow (1987), suggesting that interspecific differences are truly confounded by intraspecific variation. One attempt to eliminate some intraspecific variation by examining only sites at which both species were found did not improve the results, so this dataset does not show any evidence for character displacement in shell shape. Limiting the analysis to a single habitat type was not successful either. Since this analysis attempted to reduce environmental phenotypic variation, the result suggests that at least some of the intraspecific variation observed is genetically based.

One likely explanation for our different results is that these previous studies used only reproductive, hence larger, animals. Species differences may become more apparent as the snails grow (Reid 1996; Hohenlohe pers. obs.), and ecological applications typically require identification of animals of all ages. Murray (1982), Chow (1987), and Rugh (1997) primarily used specimens from California, and geographic differences may also play a role. Chow (1987) also combined discrete and quantitative characters in a single analysis, which was not done here.

We found *L. plena* occupying a wider range of habitats in Washington, from sheltered Puget Sound sites to the exposed outer coast. In contrast, *L. scutulata* was found only on sheltered to moderately exposed shores. This result conflicts with some previous work (Reid 1996) but is consistent with other data on species distributions (Hohenlohe 2000). This discrepancy is investigated further in Hohenlohe (2000).

These species can be distinguished non-destructively by combining the characters discussed here. Male penis morphology can be easily examined by holding the snail upside down, underwater, under a dissecting microscope. For females and non-reproductive males, tentacle coloration is the most reliable character and can be combined with shell characters on undamaged specimens. For positive identification of all ages and both sexes, restriction enzyme digestion of cytochrome b with *Alu* I is straightforward and reliable and provides a diagnostic character independent of morphology.

ACKNOWLEDGMENTS

This work was supported by a NSF Graduate Fellowship to P.A.H. and an NSERC Research Grant to E.G.B. Thanks to A. Tie for assistance with molecular work and to D.G. Reid and A.J. Kohn for helpful comments.

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