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MOLECULAR MARKERS USED TO ANALYZE SPECIES-SPECIFIC STATUS IN ABALONES WITH AMBIGUOUS MORPHOLOGY

S. A. MARÍN,[1] P. A. HAYE,[1,2]* S. MARCHANT[1] AND F. M. WINKLER[1,2]
[1]*Departamento de Biología Marina, Facultad de Ciencias del Mar, Universidad Católica del Norte;*
[2]*Centro de Estudios Avanzados en Zonas Áridas (CEAZA), Larrondo 1281. Coquimbo, Chile*

ABSTRACT Pacific abalone (*Haliotis discus hannai*) and Californian red abalone (*Haliotis rufescens*) are easily distinguished by their shell and epipodial characteristics. A few individuals of *H. rufescens* were discovered to have epipodial coloration similar to that of *H. discus hannai* suggesting that they may have been hybrids of the two species, because hybridization of these species has been informally reported from local hatcheries. The present study uses molecular genetic methods to determine whether the rarely colored variants represented hybrids between the two species. Two hundred RAPD primers were tested with PCR on two DNA pools of eight individuals of each species. Primers that showed different amplification profiles between species were tested on 27 randomly sampled individuals of each species to check the existence of polymorphisms. Two primers differentiate both species. Primer 356 amplified an approximately 450 bp and 460 bp specifics-DNA fragments in *H. discus hannai* that were not present in red abalone, whereas primer 368 amplified a 750 bp, 850 bp, 860 bp, and 1,190 bp specific-DNA fragments in *H. rufescens* that were not present in *H. discus hannai*. The amplification pattern of the DNA of individuals with ambiguous morphology for both primers was characteristic of red abalone. The results strongly suggested that the specimens with unusual epipodial coloration were not hybrids, but rather, phenotypic variants of *H. rufescens*. Future studies should focus on the cause of the variation of epipodium color in *H. rufescens*, which could be either genetic polymorphism or phenotypic plasticity.

KEY WORDS: abalone, hybridization, *Haliotis rufescens*, *Haliotis discus hannai*, DNA testing, RAPD, culture, Chile

INTRODUCTION

Abalones are gastropod molluscs belonging to the genus *Haliotis* (Mollusca: Gastropoda). These molluscs have a dorsally flattened bowl-shaped shell that protects most of the body, and a large ventral foot only slightly smaller than the shell. A major feature of the exterior morphology is the epipodium, a mantle extension around the edge of the foot. The epipodium varies in its structural detail, coloration, and texture among species and is one way to distinguish between the different species of abalone (Anderson 2003).

Two species of abalone have been introduced to Chile for culture purposes, the Pacific abalone *Haliotis discus hannai* Ino 1953 and the Californian red abalone, *H. rufescens* Swainson 1822. These two species are easily differentiated based on their shell and epipodium morphology. The shell of the Pacific abalone is normally green and variably rugose with brown and white epipodia. In contrast, the shell of the California abalone cultured in Chile has a finely lined shell with variable coverage of red and turquoise color and black, smooth epipodia.

Ten individuals obtained from the Center for Abalone Production of the Universidad Católica del Norte (CAP-UCN) in 2004 had the typical shell and epipodial morphology of *H. rufescens*, but the coloration of the epipodia were similar to that of *H. discus hannai*. Two possible hypotheses were considered: (1) hybridization had occurred between Pacific and Californian abalone and (2) phenotypic variation within *H. rufescens* caused the unusual coloration of the epipodia. The latter might be explained by environmental adaptation and/or genetic polymorphism.

Several authors have reported the existence of hybrids between abalone species (Owen et al. 1971, Leighton & Lewis 1982, Brown 1995, Freeman 2001, Zhao et al. 2004, Ibarra et al. 2005), and hybridization between *H. rufescens* and *H. discus hannai* has been informally noted by commercial abalone growers in Chile (A. Zuñiga, pers. comm.). On the other hand, morphological variation produced by genetic factors has been described for various bivalve molluscs, particularly with regard to shell color (Chanley 1961, Innes & Haley 1977, Mitton 1977, Newkirk 1980, Adamkewicz & Castagna 1988, Wada & Komaru 1990, Winkler et al. 2001), abalones (Kobayashi et al. 2004) and other gastropods (Boulding & Hay 1993, Rolán et al. 2004). Morphological variability produced by phenotypic plasticity has also been reported for gastropod shells (Cole 1975, Wullschleger & Jokela 2002, Véliz et al. 2001, Smith & Ruiz 2004). However, we have found no published information on the variability of epipodial coloration within abalone species.

The use of molecular markers has proved to be a useful tool for complex taxonomic identification where morphological characteristics are ambiguous or cryptic (e.g., Douek et al. 2002, Westheide et al. 2003, Miura et al. 2005, Park et al. 2005). The randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique is a relatively simple and cheap method capable of differentiating taxa without the need to know their genomes (Welsh & McClelland 1990, Williams et al. 1990). RAPDs are dominant markers that result from the use of short (10 bases long) primers (synthetic oligonucleotides) of random sequence that can amplify multiple segments of genomic DNA by PCR. The number of segments depends on the number of sites of the genome recognized by a particular primer. The main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amounts of DNA without the need of a molecular characterization of the genome of the taxa under study. Species in which such markers were used include oyster genera *Crassostrea, Saccostrea,* and *Striostrea* (Klinbunga et al. 2000), amphipods

*Corresponding author. E-mail: phaye@ucn.cl

(*Gammarus*: Costa et al. 2004) and the tropical abalones *Haliotis asinina, H. ovina* and *H. varia* (Klinbunga et al. 2004).

The goal of the present study was to obtain species-specific RAPD molecular markers that could distinguish between *H. discus hannai* and *H. rufescens* and verify if individuals with ambiguous morphology found at CAP-UCN represent hybrids or intraspecific phenotypic variability.

MATERIALS AND METHODS

A total of 27 individuals of each species (*H. discus hannai* and *H. rufescens*) were obtained for this study from the CAP-UCN. About 2 cm^3 of muscle tissue dissected from the foot of each sacrificed specimen was kept in a cryogenic tube at –20°C until DNA was extracted. The 10 DNA samples of the abalones with ambiguous morphology were obtained from the same tank of *H. rufescens* hatchery broodstock (from CAP-UCN) and were given to the Laboratory of Molecular Diversity when they were already dead.

DNA extraction was carried out using a QIAamp Mini Kit (Qiagen Inc.). DNA extractions were verified using the PCR reaction with the HCO-LCO universal primers (Folmer et al. 1994) for the Cytochrome Oxidase I mitochondrial gene. The presence and intensity of amplification (visualized in an agarose electrophoresis) was used as DNA quality criterion. Clear and intense amplicons (bands) in the gel were considered to reflect that the DNA used was of high quality and quantity. The extracted DNA was stored at –20°C until further use.

To analyze a large number of individuals in each PCR reaction, two pools of DNA were prepared for each species, each pool made up of equal aliquots of DNA from eight different individuals of the species. Consequently, with two PCR reactions per species, 16 individuals of each species would be screened. Each DNA pool was assayed using 200 RAPD primers (RAPD primers 300–500, University of British Columbia) in independent reactions (each primer and DNA sample in an independent reaction). The PCR reactions were carried out in a final volume of 10 uL, which contained 1X PCR buffer (200 mM Tris-HCL, pH 8.4, 500 mM KCL), 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.2 µM of each RAPD primer, 1.45 U Taq DNA polymerase (Invitrogen) and approximately 5 ng/µL of genomic DNA. In all the PCRs a negative and positive control were included, to verify the absence of contamination in the samples and the success of PCRs respectively.

Amplification was carried out in a PTC100-MJ Research thermal cycler, with an amplification program starting with 2 min at 94°C, followed by 40 cycles consisting of 1 min at 94°C, 1 min at 42°C, and 2 min at 72°C, followed by a final extension of 5 min at 72°C. The PCR products (amplicons) were separated in a 6% denaturing Polyacrylamide gel containing 19:1 acrylamide: bis-acrylamide and 5 M urea. Electrophoresis was conducted using a SequiGen sequencing gel electrophoresis system (BIO-RAD laboratories, Hercules, CA). Runs were performed at 60–100 Watts and 55°C for 7–24 h depending on the sizes of the amplicons. Amplicons were visualized on the gel using the Promega (Madison, WI) silver-staining protocol (following manufacturer's instructions) and a 200 base pair (bp) DNA ladder as a marker to estimate amplicon size. The primers that produced good amplification (clearly defined bands that differentiated between the two abalone species) were used in the next amplification step.

We screened the primers that simultaneously showed different and consistent amplification results between species. The potentially useful primers were used to amplify by PCR the DNA of each of the 27 individuals of *H. discus hannai* and *H. rufescens* (i.e., primers were used to open the DNA pools). At this stage, primers were considered as informative and reliable for identification and differentiation between *H. discus hannai* and *H. rufescens* when they produced a specific amplicon in all (27) individuals of one species and none from the other species. These markers were then used to identify the specific status of the 10 abalone with ambiguous morphology found at the CAP-UCN.

RESULTS

Of the 200 primers assayed with the pools of DNA from both species, 40 were potentially useful for differentiating between the two abalone species. Four of the 40 primers found produced amplicons exclusive for *H. discus hannai* and 31 for *H. rufescens*; whereas five primers simultaneously produced different size amplicons unique for each species. The five primers that simultaneously produced amplicons of different sizes and four other primers that produced good band intensities, difference in molecular weight between amplicons of the two species, and repeatability among assays were chosen and tested in 27 individuals of each species.

All primers that produced amplicons of different sizes in both species showed intraspecific polymorphisms when they were tested with the 27 individuals of each abalone species, disallowing unequivocal differentiation between the two species. However, two primers produced specific amplicons on the 27 individuals; primer 356 (5′ GCG GCC CTC T 3′) for *H. discus hannai* and primer 368 (5′ ACT TGT GCG G 3′) for *H. rufescens*.

The PCR banding pattern (fingerprint) obtained with primer 368 shows an amplicon of approximately 700 bp that is present in most of the individuals of *H. discus hannai*, and in some of *H. rufescens* (Fig. 1, band A). Even though it was not a specific amplicon for either of the two species, it was much more frequent and intense in *H. discus hannai*. With this same primer, four amplicons specific for *H. rufescens* were found. These were of approximately 750 bp, 850 bp, 860 bp, and 1,190 bp, which are present in all individuals of *H. rufescens*, and in none of *H. discus hannai* (Fig. 1, bands B-E), therefore making unequivocal identification of the two species possible using primer 368. The banding patterns obtained from assaying the DNAs of the 10 individuals with ambiguous morphology with primer 368 showed the amplicons of approximately 750 bp and 850 bp unique to *H rufescens,* in some of the 10 individuals. The 750 bp amplicon is only present in two specimens with ambiguous morphology and the 850 bp amplicon in three individuals (Fig. 1).

The banding pattern produced with primer 356 shows an amplicon of approximately 990 bp present in the 27 individuals of *H. rufescens* and in nine of *H. discus hannai* (Fig. 2, band A). This amplicon, therefore, was not specific for either of the two species, although it was more frequent and intense in *H. rufescens*. The amplicons of approximately 450 bp and 460 bp, in contrast, were present in all the individuals of *H. discus hannai* and none of the *H. rufescens* (Fig. 2, bands B and C). Consequently, these two amplicons allow unequivocal species

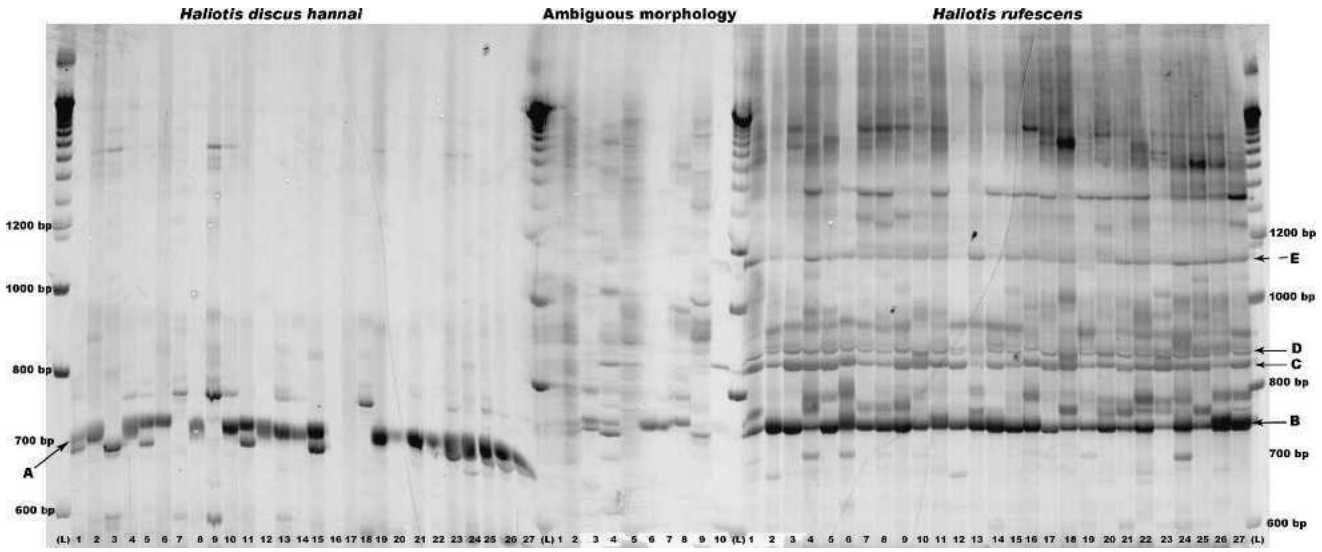

Figure 1. RAPD banding pattern obtained with primer 368. Left side of the gel, 27 individuals of *H. discus hannai*; middle of the gel, 10 abalone individuals with ambiguous morphology; right side of the gel, 27 individuals of *H. rufescens*. (L) = 200 bp molecular weight ladder. A = amplicon of 700 bp; B = amplicon of 750 bp; C = amplicon of 850 bp; D = amplicon of 860 bp; E = amplicon of 1,190 bp. All amplicon sizes are approximate.

identification. The RAPD banding pattern of the 10 individuals with ambiguous morphology with primer 356 did not show discriminatory amplicons unique to *H. discus hannai*, whereas all the individuals with ambiguous morphology showed the 990 bp amplicon, frequent and more intense in *H. rufescens*.

DISCUSSION

Our results strongly suggest that the 10 specimens with ambiguous morphology found at the CAP-UCN correspond to *H. rufescens* and that there is phenotypic variability within this species. The banding patterns from the RAPD-PCRs carried out on the DNA of these specimens show greater similarities with the banding patterns of *H. rufescens*. Some of the DNA of the individuals with ambiguous morphology show amplicons unique to *H. rufescens* (750 bp and 850 bp with primer 368), whereas the amplicons unique to *H. discus hannai* (450 bp and 460 bp with primer 356) were not detected in any of them. The specific amplicons of both species would be expected to appear in the banding pattern of the individuals with ambiguous

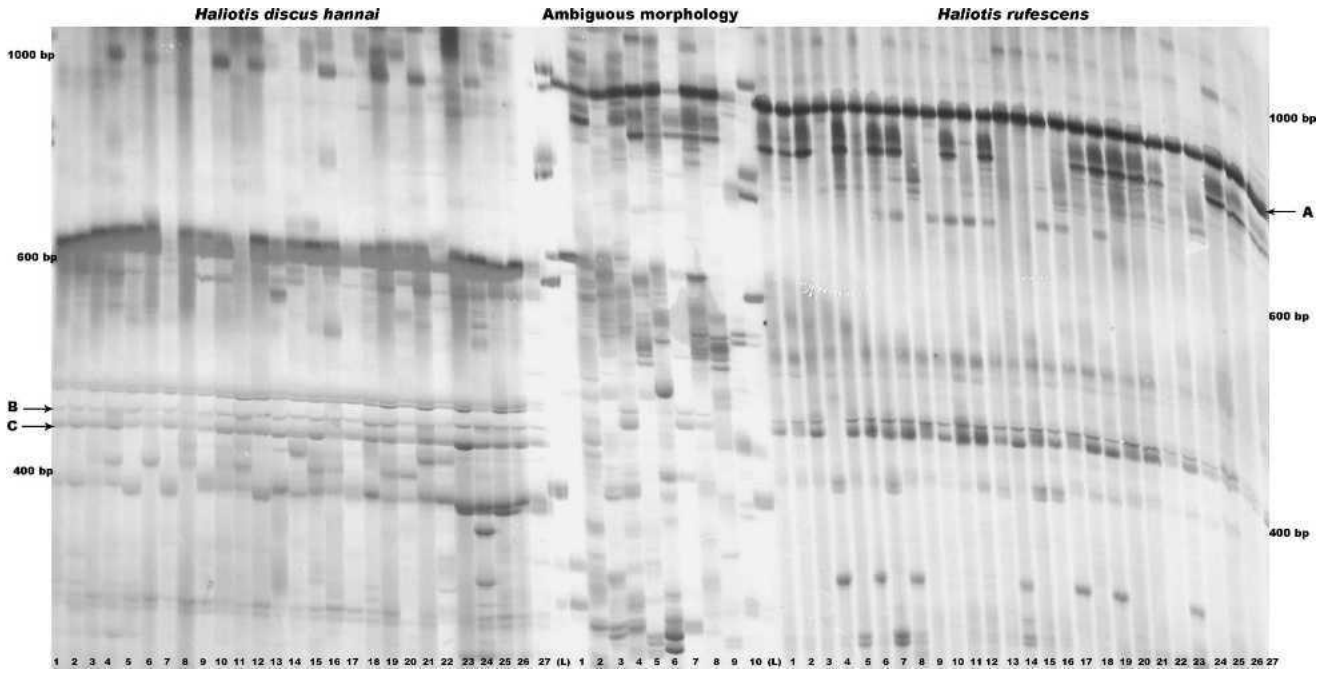

Figure 2. RAPD banding pattern obtained with primer 356. Left side of the gel, 27 individuals of *H. discus hannai*; middle of the gel, 10 abalone individuals with ambiguous morphology; right side of the gel, 27 individuals of *H. rufescens*. (L) = 200 bp molecular weight ladder. A = amplicon of 990 bp; B = amplicon of 460 bp; C = amplicon of 450 bp. All amplicon sizes are approximate.

morphology if the specimens in question had been hybrids. Although the fingerprint is not always consistent, the whole fingerprint of 10 specimens with ambiguous morphology is visibly more consistent with the fingerprint of *H. rufescens* than *H. discuss hannai* (Fig. 1 and Fig. 2). The inconsistency in the fingerprint of the specimens with ambiguous morphology could be caused by the inadequate maintenance of tissue samples that produces poor and degraded DNA. These results emphasize the importance of good maintenance of tissues that will be used for molecular work. Unfortunately we did not have more or fresh tissues of specimens with ambiguous morphology to perform the study.

The results show that RAPD-PCR is a simple and efficient method to identify and differentiate between *H. discus hannai* and *H. rufescens*. The banding patterns obtained from some RAPD-PCR show specific amplicons for molecular differentiation.

The RAPD markers tested herein, allowed us to reject the hypothesis that the 10 individuals with ambiguous morphology were hybrids between the two species of abalone. The banding patterns produced with the DNA of the 10 individuals with ambiguous morphology did not simultaneously carry the amplicons obtained with primers 368 and 356, for *H. rufescens* and *H. discus hannai* respectively. Other molecular genetic tools may allow better discrimination of hybrids and could be used to corroborate our findings, including microsatellites (Ibarra et al. 2005), sequencing (Spies et al. 2006) and allozymes (Brown 1995, Ibarra et al. 2005).

Variability in the size and morphology of gastropod shells can be influenced both genetically and environmentally (Boulding & Hay 1993, Trussell & Etter 2001, Jordanes et al. 2003, Rolán et al. 2004). Phenotypic plasticity represents the potential of an organism to alter its phenotype caused by environmental stimuli (Levins 1968, Adler & Harvell 1990). Genetic polymorphism is caused by the presence of two or more alleles at a given locus and depending on the allele combination, can be expressed as different phenotypes. Because the 10 abalone individuals showing ambiguous morphology were obtained from a single breeding population of *H. rufescens* (even though their parentage is unknown) and cultured under the same hatchery conditions (i.e., same food and environmental conditions) it seems improbable that the morphological variability found was a manifestation of phenotypic plasticity, and is more likely ascribed to genetic polymorphism. The present study represents the first published report on intraspecific variability in the color of the epipodium of *H. rufescens* (or any other abalone species). Other experiments therefore could be carried out in which crosses are made between abalones to confirm the hypothesis that the color variation represents intraspecific polymorphism (if more individuals with the trait are found). The crosses would allow verification of the stability of a character throughout the ontogenetic development and possible patterns of character segregation. Additionally, molecular markers associated with the trait could be developed.

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