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Genetic structure of *Polycera alabe* and *P. atra* (Mollusca: Opistobranchia: Nudibranchia) in the Pacific coast of North America

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**Abstract.**—*Polycera alabe* and *Polycera atra* are closely related opisthobranch sea slugs found in coastal habitats along the eastern Pacific. Both species are extremely variable in external coloration and some of this variation appears to be correlated with geographic range. To determine the phylogenetic relationships and genetic structure of *P. alabe* and *P. atra* molecular phylogenies were generated using two genes: H3 (nuclear) and 16S (mitochondrial). Sequence data indicate that populations of *P. atra* are genetically homogeneous and lack geographic structure along the range of the species. In contrast, *Polycera alabe* consists of three previously unrecognized, distinct clades with overlapping ranges. The northernmost clade of *P. alabe* is sister to *P. atra*, thus the current definition of *P. alabe* constitutes a paraphyletic assemblage. The southernmost clade presents morphological differences in the radula compared to the other two clades. These data suggest that *P. alabe* is most likely a species complex.

The opisthobranch mollusks *Polycera alabe* Collier & Farmer, 1964 and *Polycera atra* MacFarland, 1905 (family Polyceridae Alder & Hancock, 1845) are benthic marine invertebrates found in coastal habitats along the eastern Pacific. Anecdotal evidence suggests that El Niño Southern Oscillation (ENSO) plays significant roles in range expansions and contractions of both species (Kerstitch and Bertsch 2007), which otherwise have allopatric ranges, with the boundary at Punta Eugenia and Isla Cedros, Mexico (Angulo Campillo 2003, 2005). The known geographic range of *P. atra* spans from Cape Arago, Oregon (Goddard 1984) to La Paz, Mexico (Angulo Campillo 2003, 2005). However, the northernmost record of *P. atra* in Oregon was during the intense El Niño of 1982–1983 (Goddard 1984), and reports of this species south of Punta Eugenia are very rare (Angulo Campillo 2003, 2005). *Polycera alabe* is found from the Channel Islands in Southern California to Panama (Behrens and Hermosillo 2005), with an isolated record from northern Chile (Schrödl 2003), but the only record of *P. alabe* north of Punta Eugenia, in Southern California, was during the strong El Niño of 1997–1998 (Engle and Richards 2001).

Both *P. atra* and *P. alabe* are extremely variable in external coloration. Behrens and Hermosillo (2005) recognized four distinct color forms in *P. alabe* that they classified as variations A–D, each with slightly different geographic ranges. Variation A is found throughout the Gulf of California, the Pacific coast of Baja California and Southern California; variation B is found in the Pacific coast of Mexico; variation C is present from Mexico to Costa Rica and Panama; and variation D is only found in Central America. Based on these data, Behrens and Hermosillo (2005) suggested the possibility of the existence of several species. Animals of *P. atra* are also found to vary in coloration from...
very dark to light; MacFarland (1966) indicated that southern animals tend to be lighter, suggesting some geographic variation. However, both our observations and unpublished data (H. Bertsch, pers. comm.) indicate a possible relationship between coloration and diet. Both species feed commonly on bryozoans of the genus *Bugula* and *Membranipora* (McDonald 1983), and *P. atra* collected on *Membranipora* are consistently lighter than those found on *Bugula*.

The objective of this paper is to examine the genetic structure of *P. alabe* and *P. atra* in the Pacific coast of North America, including specimens from northern California to the southern Mexican state of Guerrero. A mitochondrial (16S) and a nuclear (H3) gene were sequenced.

### Material and Methods

**Source of Specimens**

Eight specimens of *P. alabe*, five specimens of *P. atra*, and one specimen of *Polycera hedgpethi* Marcus, 1964 were sequenced (Table 1). Specimens were obtained from the collections of the Natural History Museum of Los Angeles County (LACM) and the Cal Poly Pomona research collections (CPIC). Sequences for one specimen of *Polycera cf. capensis* Quoy & Gaimard, 1824 deposited at the California Academy of Sciences (CASIZ) were obtained from GenBank.

**Morphological Examination**

Four specimens of *Polycera alabe* were dissected, and the radula of each was removed. Surrounding tissue on the radula was removed manually and then placed in low concentration [0.1] sodium hydroxide for approximately 24 hours to remove any remaining tissue. The radula was then dried, mounted with electrically conductive double-sided adhesive tape, and sputter coated with gold/palladium alloy for examination with a scanning electron microscope (SEM) Hitachi S-3000N at the Natural History Museum of Los Angeles County.

### Table 1. List of specimens used in the study; including locality, voucher number, and GenBank Accession numbers. Specimens for which the radula was studied are marked with an asterisk (*).

<table>
<thead>
<tr>
<th>Species</th>
<th>Locality</th>
<th>Date</th>
<th>Voucher</th>
<th>16S</th>
<th>H3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. cf. capensis</em></td>
<td>South Africa</td>
<td>-</td>
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<td>HM162503</td>
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<td>KF425292</td>
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<tr>
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<td>KF425277</td>
<td>KF425291</td>
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<tr>
<td><em>P. atra</em></td>
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<td>CPIC 00425</td>
<td>KF425276</td>
<td>KF425290</td>
</tr>
<tr>
<td><em>P. atra</em></td>
<td>Santa Barbara, CA</td>
<td>18 Aug 2010</td>
<td>CPIC 00807</td>
<td>KF425275</td>
<td>KF425289</td>
</tr>
<tr>
<td><em>P. atra</em></td>
<td>Long Beach, CA</td>
<td>17 Jul 2010</td>
<td>CPIC 00808</td>
<td>-</td>
<td>KF425288</td>
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<tr>
<td><em>P. atra</em></td>
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<td>-</td>
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<tr>
<td><em>P. alabe</em></td>
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<td>KF425272</td>
<td>KF425284</td>
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<tr>
<td><em>P. alabe</em></td>
<td>Sonora, MX</td>
<td>Nov 1975</td>
<td>LACM 140736</td>
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<td><em>P. alabe</em></td>
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<td>KF425286</td>
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<tr>
<td><em>P. alabe</em></td>
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<tr>
<td><em>P. alabe</em></td>
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<td>15 Feb 2008</td>
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<tr>
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<td>18 Feb 2008</td>
<td>LACM 174953</td>
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<td><em>P. alabe</em></td>
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<td>KF425280</td>
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<tr>
<td><em>P. alabe</em></td>
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<td>18 Mar 2004</td>
<td>CPIC 00809</td>
<td>KF425269</td>
<td>KF425281</td>
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</table>
DNA Extraction

DNA extraction was performed using either a hot Chelex® protocol or the DNeasy Blood and Tissue Kit (Qiagen). Approximately 1–3 mg of tissue was taken from the foot or tail of the animals and cut into fine pieces for extraction for each of the two protocols. For the Chelex® extraction, the tissue was rinsed and rehydrated using 1.0 mL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for 15–20 minutes. A 10% (w/v) Chelex® 100 (100–200 mesh, sodium form, Bio-Rad) was prepared using TE buffer. After rehydration, the mixture was then centrifuged, 975.00 mL of the supernatant was removed, and 175.00 mL of the Chelex® solution was added. Samples were then heated in a 56°C water bath for 20 minutes, heated in a 100°C heating block for 8 minutes, and the supernatant was used for PCR. The DNeasy® protocol supplied by the manufacturer was followed, with some modifications. The elution step was modified such that the first elution was collected using 100.00 μL of Buffer AE and was allowed to incubate at room temperature for 5 minutes. In a new test tube, a second elution step was conducted using 200.00 μL of Buffer AE and was also allowed to incubate at room temperature for 5 minutes. The first elution was used for PCR. Such a variety of extraction protocols was implemented because not all tissue samples could be amplified when using the same extraction method. The use of the DNeasy® extraction protocol was only used as an alternative to the Chelex® extraction technique when it became apparent that the DNA could not be successfully extracted using that technique.

Primers

Universal H3 (Colgan et al. 1998) and 16S primers (Palumbi et al. 1996) were used to amplify the regions of interest for all specimens (Table 2). Internal primers for 16S designed for another group of opisthobranchs (Ornelas-Gatdula et al. 2011) were used resulting in additional partial sequences for some specimens. Multiple attempts to obtain CO1 sequences using different primers (LCO1490/HCO2198, developed by Folmer et al. 1994 and NAF-COI/NAR-COI, developed by Ornelas-Gatdula et al. 2011) were unsuccessful.

PCR Amplification and Sequencing

The master mix was prepared using 34.75 mL H2O, 5.00 mL of either Buffer B (ExACTGene, Fisher Scientific) or Taq Buffer, 5.00 mL 25 mM MgCl2, 1.00 mL 20mM dNTPs, 1.00 mL 10mM primer 1, 1.00 mL 10mM primer 2, 0.25 mL 5 mg/mL Taq, and

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’–3’</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HexAF (F)</td>
<td>ATG GCT CGT ACC AAG CAG ACG GC</td>
<td>Colgan et al. (1998)</td>
</tr>
<tr>
<td>HexAR (R)</td>
<td>ATA TCC TTG GGC ATG ATG GTG AC</td>
<td>Colgan et al. (1998)</td>
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<tr>
<td>16S rRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16Sar-L (F)</td>
<td>CGC CTG TTT ATC AAA AAC AT</td>
<td>Palumbi et al. (1996)</td>
</tr>
<tr>
<td>16Sbr-H (R)</td>
<td>CCG GTC TGA ACT CAG ATC ACG T</td>
<td>Palumbi et al. (1996)</td>
</tr>
<tr>
<td>16Sar-FAP (F)</td>
<td>AAA GAC GAG AAG ACC CTT AGA GTT TT</td>
<td>Ornelas-Gatdula et al. 2011</td>
</tr>
<tr>
<td>16Sbr-FAP (R)</td>
<td>AAA ACT CTA AGG GTC TTC TCG TCT TT</td>
<td>Ornelas-Gatdula et al. 2011</td>
</tr>
</tbody>
</table>
2.00 mL extracted DNA. Some master mixes included 2 mL 20mg/ml bovine serum albumin (Fermentas) and therefore 32.75 mL of H₂O in order to maximize enzymatic activity. Reaction conditions for H3 and 16S rRNA were as follows: an initial denaturation for 2 min at 94°C, 35 cycles of 1) denaturation for 30 sec at 94°C, 2) annealing for 30 sec at 50°C, and 3) elongation for 1 min at 72°C, and a final elongation for 7 min at 72°C. PCR products yielding bands of appropriate size (approximately 375 bp for H3 and 195 bp for the 16S fragments) were purified using the Montage PCR Cleanup Kit (Millipore). Cleaned PCR samples were quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). PCR products were diluted to 6.0 ng/mL, and a sample of each primer was diluted to 2.0 pmol/mL. These samples were sequenced at the City of Hope DNA Sequencing Laboratory (Duarte, CA) using chemistry type BigDye V1.1 for fragments less than 500 bp.

**Phylogenetic Analyses**

Sequences for each gene were assembled and edited using Geneious Pro 4.7.4 (Biomatters Ltd.). Geneious was also used to extract the consensus sequence between the primer regions and to construct the alignment for each gene using the default parameters. The sequences were not trimmed after alignment. A total of 395 bp for H3, and 495 bp for 16S were used for the phylogenetic analyses. Two other species of *Polycera*, *P. cf. capensis* and *P. hedgpethi*, were selected as outgroups. Because of the lack of comprehensive phylogenies for *Polycera*, outgroup choice was based on available sequences and specimens. An analysis was conducted for both genes concatenated. To assess whether H3 and 16S have significantly conflicting signals, the incongruence length difference (ILD) test (Mickevich and Farris 1981, Farris et al. 1994), implemented in PAUP*4.0 (Swofford 2002) as the partition homogeneity test (Swofford 2002), was conducted for all genes combined. The levels of saturation for each gene and for the first and second versus third codon positions of H3 were investigated using the substitution saturation test developed by Xia et al. (2003) and Xia and Lemey (2009) implemented in the program DAMBE (Xia and Xie 2001). The Akaike information criterion (Akaike 1974) was executed in jModelTest (Posada 2008) to determine the best-fit model of evolution. Maximum likelihood analyses were conducted using GARLI v0.96b8 (Zwickl 2006). Model parameters determined from jModelTest were entered into GARLI and the default parameters were used at 100 replicates each. Robustness of each clade was assessed by bootstrap support (Felsenstein 1985) based on 2000 replicates with heuristic search, TBR branch-swapping algorithm, multrees option, and 100 random additions. Bayesian analyses were executed in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001), partitioned by gene (unlinked). The Markov chain Monte Carlo analysis was run with two runs of six chains for five million generations, with sampling every 1000 generations. Convergence of runs was assessed using Tracer 1.5 (Rambaut and Drummond 2009). The default 25% burn-in was applied before constructing majority-rule consensus tree/s. Diagnostic nucleotides for each clade were identified visually in the alignments after collapsing identical haplotypes using the program Collapse 1.2 (Posada 2004).

**Results**

**Phylogenetic Analyses**

The phylogenetic analyses produced a resolved and well-supported phylogenetic hypothesis for the specimens examined (Figure 1). There is very little genetic diversity and no phylogenetic structure in *P. atra*. All specimens examined from San Francisco...
Bay, California to La Paz, Mexico are identical for H3 and almost identical for 16S (only one nucleotide substitution was found in a specimen from Monterey Bay, California). The clade containing all the specimens identified as *P. atra* is well supported (posterior probability = 1). In contrast, *P. alabe* exhibits a large degree of variation in both genes, providing a strong phylogenetic signal. The phylogenetic analyses have recovered three distinct and well-supported clades including specimens identified as *P. alabe*. One of the clades (named the Northern Clade herein) includes specimens collected in Sonora and La Paz, Mexico (both in the Gulf of California) and Jalisco, Mexico. This clade is well supported (posterior probability = 0.95) and it is sister to *P. atra*. Within this Northern Clade three specimens from the Gulf of California form a well-supported subclade (posterior probability = 0.97). The clade containing the Northern Clade of *P. alabe* and *P. atra* is also well supported (posterior probability = 0.98). The two other clades of *P. alabe* are called Southern Clade 1 and 2 herein and they appear to be sister, but this is weakly supported (posterior probability = 0.8). Southern Clade 1 includes specimens collected in Sonora and Jalisco and is well supported (posterior probability = 0.96), whereas Southern Clade 2 includes animals collected in southern Mexico (Colima and Guerrero) and is also well supported (posterior probability = 0.97). The ranges of the Northern Clade and Southern Clade 1 overlap in the central Gulf of California (Figure 2). However, the specimen of Southern Clade 1 from Sonora was collected during the El Niño of 1977 and it could be the result of unusual oceanographic conditions. Based
on the information available, it appears that \textit{P. atra} + the Northern Clade and the Southern Clade 2 are completely allopatric.

\textbf{Morphological Analyses}

A morphological examination of representative specimens from the three \textit{P. alabe} clades indicates some differences in radular morphology. All radulae examined have a hook-shaped innermost lateral tooth in each half-row, with a short, triangular cusp and a secondary cusp mid-length (Figure 3). The innermost tooth is followed by a larger tooth with a similar morphology. However, the number and morphology of the outer lateral teeth is different among clades. Northern Clade and Southern Clade 1 exhibit identical radula morphology, with 2–3 vestigial outer teeth (Figure 3A-D). However, specimens of Southern Clade 2 consistently have 7–8 large outer teeth (Figure 3E-F).

\textbf{Discussion}

The sequence data and phylogenetic analyses presented here show that populations of \textit{P. atra} from a broad geographic range (San Francisco Bay, California to La Paz, Mexico) are genetically very similar for 16S and H3. The 16S gene is variable enough to distinguish among species of opisthobranchs and is even informative in population genetics studies (Krug et al. 2012). The lack of geographic structure in \textit{P. atra} suggests rampant gene flow across the range of the species. Alternatively, this pattern might have been caused by rapid postglacial range expansion of \textit{P. atra} from refugia at the southern range of the species. This is a well-documented explanation for the lack of genetic structure in several other species of marine invertebrates along the Pacific Northwest (e.g., Marko 1998, Hellberg et al. 2001, Edmands and Harrison 2003). Although the northern range limit of \textit{P. atra} is well south of the last glacial maximum, colder ocean temperatures during the Pleistocene in California (Bemis et al. 2002) might have restricted the range of this species, which could have rapidly expanded to the north during the
transition to the Holocene (see Jacobs et al. 2004). The specimen of *P. atra* from La Paz, Mexico (confirmed with both genetic markers) was collected in April 1974 during a strong La Niña, suggesting that this species has a broad dispersal potential and that water temperature may be an important factor limiting its range.

The same genetic markers demonstrate a completely different picture for *P. alabe*. In this case we have identified three well-supported clades with several molecular synapomorphies in the 16S and H3 genes. Southern Clade 2 is also supported by radula morphological differences, indicating that it is likely a distinct species. The other two clades could also possibly constitute different species based on their genetic synapomorphies. One of the clades of *P. alabe* (Northern Clade) is sister to *P. atra*, thus

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**Fig. 3.** Radula SEM photographs for representatives of each clade of *P. alabe* and *P. atra*. All photographs taken with the same orientation (distal above, proximal below). A, *P. atra* (CPIC 00808). B, *P. alabe* Southern Clade 1 (LACM 76-5). C, *P. alabe* Northern Clade (LACM 140736). D, *P. alabe* Northern Clade (LACM 174953). E-F, *P. alabe* Southern Clade 2 (LACM 140739).
the current definition of *P. alabe* constitutes a paraphyletic assemblage. However, there is no available genetic information available for populations from the Pacific coast of Central and South America – the known range of *P. alabe* includes Costa Rica, Panama, and Chile – and the sample size and sequence data are relatively small. Therefore, we do not think it is appropriate to suggest taxonomic changes at this point.

Another problem is to elucidate which of the two clades found in the Gulf of California (Northern Clade or Southern Clade) is the original *P. alabe*, since the range of both clades includes the type locality of *P. alabe* (Isla de Cedros, Baja California). These taxonomic issues should be addressed in a comprehensive study including specimens from the entire range of *P. alabe* and detailed morphological examinations including the reproductive anatomy of all the species involved.

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**Literature Cited**


