Molecular phylogeny of the subfamily Amphistichinae (Teleostei: Embiotocidae) reveals parallel divergent evolution of red pigmentation in two rapidly evolving lineages of sand-dwelling surfperch

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Pigment evolution was reconstructed in the subfamily Amphistichinae, a six-species clade of the surfperches, family Embiotocidae. Assignment was confirmed for all species within the subfamily, but low levels of differentiation were found among species within the subfamily, suggesting a recent radiation. The new phylogeny differs from previous hypotheses by the placement of the spotfin surfperch Hyperprosopon anale at the base of the subfamily, while still preserving the calico surfperch Amphistichus koelzi and the redtailed surfperch Amphistichus rhodoterus as sister species. Phenotypically, A. rhodoterus, A. koelzi and the silver surfperch Hyperprosopon ellipticum express high levels of red pigmentation. The barred surfperch, Amphistichus argenteus and the walleye surfperch Hyperprosopon argenteum express little to no red pigment, while basal H. anale expresses an intermediate amount of red pigment. Red pigmentation is proposed to have experienced parallel divergent evolution in each genus within the subfamily. [Correction added after online publication 19 July 2011: species names corrected]

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INTRODUCTION

Teleost pigmentation systems have proven to be excellent models for evolutionary analysis (Braasch et al., 2007; Miller et al., 2007). Recent work on pigmentation in the internally fertilized marine surfperches of the family Embiotocidae (DeMartini & Sikkel, 2006; Cummings, 2007) has begun to lay the foundations for a new pigmentation study system. Within the Embiotocidae, red pigmentation appears to have undergone an evolutionary divergence in the subfamily Amphistichinae, i.e. red-pigmented and unpigmented species are present in each of the two genera within the clade (Miller & Lea, 1972; Eschmeyer & Herald, 1983). The Amphistichinae therefore presents an opportunity to expand the investigation of pigment evolution into a

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new dimension, allowing the testing of evolutionary hypotheses. Although important initial steps in the evolutionary analysis of pigmentation include the measurement of ecological and behavioural variables that might interact with colouration, the primary need is to describe the recent evolutionary history of the species and the trait in question.

In this study, the colour divergence in the Amphistichinae is investigated by exploring the evolution of carotenoid-based (red and orange) pigmentation within the immediate radiation of the subfamily. The lineage comprises two genera containing three species each: *Amphistichus* [barred surperch *Amphistichus argenteus* Agassiz 1854, calico surperch *Amphistichus koelzi* (Hubbs 1933) and redtail surperch *Amphistichus rhodoterus* (Agassiz 1854)] and *Hyperprosopon* [spotfin surperch *Hyperprosopon anale* Agassiz 1861, walleye surperch *Hyperprosopon argenteum* Gibbons 1854 and silver surperch *Hyperprosopon ellipticum* (Gibbons 1854)]. In constructing the currently accepted phylogeny of the Amphistichinae, Tarp (1952) sorted species into the genera *Hyperprosopon* and *Amphistichus* according to skalation, osteological and body-shape traits (Fig. 1), some of which he himself suggested

Fig. 1. Phylogeny of the subfamily Amphistichinae based on Tarp (1952).
were ambiguous and genetically unstable. Subsequent phylogenetic analyses, based on morphology, ecology and behaviour, confirmed the partition of the six species into the two genera, although relationships within each genus remained somewhat ambiguous (DeMartini, 1969; Morris, 1982; Liem, 1986). Molecular analyses have the potential to give more rigorous insights into phylogenies. Although prior molecular studies have resolved every other clade within the family Embiotocidae at some scale (Bernardi & Bucciarelli, 1999; Bernardi, 2005, 2009), none have yet been published that include all six species within the Amphistichinae. The lack of a resolved phylogeny for this clade is unfortunate given the striking colour dichotomies between sister taxa and the potential they present for testing evolutionary hypotheses.

The analysis of colouration in vertebrates has received much attention. Formal characterization of colouration can follow a number of methodological avenues, including sensory (Endler, 1990), cytological (Mills & Patterson, 2008) and biochemical (Hudon et al., 2003; McGraw et al., 2005). Rigorous assessment of the evolutionary significance of colouration requires the identification of the agent of selection (predators v. sexual competitors or partners) and knowledge of the agent’s visual systems (Endler, 1991; Millar et al., 2006). Initial study of the evolution of colouration, however, is typically initiated by a first-principle identification of diagnosable colour traits via visual inspection. In the case of published images of the Amphistichinae, the difference in pigmentation between individuals of *A. argenteus*, which appear to lack any red colouration, and its two sister species, which both are depicted as expressing high levels of red colour, is striking (Eschmeyer & Herald, 1983). Although more subtle, the dichotomy between the sister species *H. argenteum*, which lacks red pigment, and *H. ellipticum*, which sports a bright red tail, is still apparent. What is not clear from the literature is how much the pigmentation varies within species: do some individuals of unpigmented clades express red pigment and vice versa? Characterizing within and between-population variation in traits of interest is fundamental to the use of the comparative method and is a crucial initial step in any evolutionary study (Harvey & Pagel, 1991). Therefore, characterizing fin colouration in large numbers of individuals of each species within the Amphistichinae was sought.

Biochemical analyses can greatly inform the understanding of the ecological and physiological variables that mediate colour expression (Hudon et al., 2003). Typical red pigments found in fishes include astaxanthin and tunaxanthin, both diet-limited carotenoids (Czeczuga, 1973; Maoka & Matsuno, 1985; Matsuno et al., 1985; Hudon et al., 2003). Guppies *Poecilia reticulata* Peters 1859 have also been found to express drosopterin, which they synthesize de novo (Hudon et al., 2003). Biochemical analysis of integumental pigmentation in the surfperch family has not been conducted to date. Therefore, a survey-level analysis of pigment content in the Amphistichinae was also conducted to both confirm the presence of carotenoids and assay the presence of other pigments.

To investigate the evolution of colouration in *Hyperprosopon* and *Amphistichus*, a phylogenetic analysis of the Amphistichinae was performed based on mitochondrial and nuclear DNA. In order to assess whether red pigmentation found in some members of the subfamily is an outcome of shared ancestry or convergent evolution, novel phenotypic data were also gathered and red pigmentation was traced on both the Tarp (1952) phylogeny and the new tree.
MATERIALS AND METHODS

PHENOTYPIC DATA

Colouration was scored in multiple specimens from each species. All specimens were subjected to a whole-body inspection for red and orange pigmentation but formal scoring was restricted to the fins, particularly the caudal and anal fins, where pigmentation appeared to have the highest expression in those species that expressed red pigment (Figs 2 and 3). The clear discrepancy in colour expression between species that were coloured v. those that were unpigmented allowed red and orange pigmentation to be assigned an unambiguous visual score of present v. absent. In virtually every case, fishes were scored both prior to and following death. Examination of anal-fin pigmentation of *H. ellipticum* under the dissecting scope confirmed the presence of pigment-carrying xanthophores and melanophores as well as intercellular concentrations of orange pigment (Fig. 4). Fishes were examined closely in order to distinguish true chromataphore-based pigmentation from the effects of haemorrhaging, the latter being generally negligible.

CAROTENOID ASSAY

To gain preliminary data on the biochemical nature of the pigments underlying the expression of colouration in the Amphistichinae, a carotenoid-specific pigment extraction was performed following a protocol provided by McGraw et al. (2005). The procedure was carried out on orange pigmented tissue from the anal fin of a male *H. ellipticum* and caudal fins from representatives of all six Amphistichinae species as well as a control sample from one commercially obtained gold fish *Carassius auratus* (L. 1758). The McGraw et al. (2005) procedure identifies carotenoids based on loss of colouration of tissue and gain of colouration in the solvent. To confirm the presence of carotenoids and to explore the possibility of other pigments being present in the extraction, the tertiary butyl mercaptan (TBM):hexane phasing procedure from McGraw et al. (2005) was also performed on the extracted pigment, which selectively caused carotenoids to segregate into a lens of hexane at the top of a column.

MOLECULAR DATA

Both novel and GenBank-originated sequence data were obtained from all six species within the subfamily Amphistichinae (Tables I and II). Two or more individuals were sequenced from most of the species to assess within-species molecular diversity. In addition, sequence data from GenBank were obtained for two species to use as outgroups. These outgroups were *Cymatogaster aggregata* Gibbons 1854 and *Ditrema temmincki* Bleeker 1853, which are from the sister subfamily Embiotocinae within the Embiotocidae and represent each of the two major clades within that subfamily identified by Bernardi & Bucciarelli (1999). All specimens were obtained from the wild by the authors or by correspondents by hook and line under the authority of recreational fishing licences or scientific collecting permits. Fishes from which tissues were sampled and analysed were vouchered as whole carcasses in the Scripps Institute Ichthyological Collection (Table I). Muscle tissue was collected from a 5 mm × 5 mm incision just below the left pectoral fin and stored at −80 °C.

Genomic DNA was obtained with the Puregene Genomic DNA purification kit from Gentra Systems (www.qiagen.com) using the 5–10 mg solid tissue protocol. Sequence was amplified in 30 μl reactions containing 3 μl 10× reaction buffer, 3 μl 2.5 mM deoxynucleotide triphosphates, 1.5 μl Taq polymerase, 1.5 μl MgCl₂, 1.5 μl each of the forward and reverse oligonucleotide primers and 3 μl of genomic DNA. Primers were designed from existing sequence data for the nuclear gene *Tmo-4C4* and the following mitochondrial loci: cytochrome *b*, 12S, 16S and *d-Loop* (Table II). PCR was conducted on an Eppendorf Mastercycler epgradient S (www.eppendorf.com). Run conditions varied for each locus, but some settings were common to every reaction. All reactions had an initial melting period of 96° C for 3 min, a final extension period of 72° C for 5 min and were held at 4° C when the cycling was completed. All reactions were repeated for 35 cycles. Run conditions within the cycling portion of the reaction are provided in Table II. Products were checked using a Fisher Biotech gel
Fig. 2. Three species of surperch in the genus *Amphistichus* showing among-species variation in red pigmentation: (a) *Amphistichus argenteus* (pigment absent), (b) *Amphistichus koelzi* (pigment present) and (c) *Amphistichus rhodoterus* (pigment present).

electrophoresis kit (www.fishersci.com) and viewed with a UVP BioDoc-it UV Transilluminator (www.uvp.com). Most products were purified from PCR aliquots using the illustra GFX and Gel Band Purification kit (GE Healthcare; www.gehealthcare.com). In a few cases where multiple bands were present, the band of the correct size was purified from the gel. Products were sequenced either at the Kansas State University DNA Sequencing and Genotyping Facility or at the University of Kentucky DNA Facility.
Fig. 3. Three species of surfperch in the genus *Hyperprosopon* showing red pigmentation present or absent in the caudal fin: (a) *Hyperprosopon anale* (pigment present), (b) *Hyperprosopon argenteum* (pigment absent) and (c) *Hyperprosopon ellipticum* (pigment present).

**ANALYSIS**

Sequences were viewed and aligned using ClustalX (www.clustal.org) and imported into Mesquite (www.mesquiteproject.org) for initial analysis and conversion to NEXUS files. Two data sets were analysed. One data set included the cytochrome *b* gene only, with most species being represented by multiple sequences. A second data set included only one individual per species, but included all loci. The effect of partitioning the data was explored according to locus and for protein-coding genes, by codon position. Exploratory analyses were performed independently for each partition using maximum parsimony. For the final parsimony
Fig. 4. Polymorphism in anal fin colouration in *Hyperprosopon ellipticum*. (a) Anal fin from male showing black marking. (b) Anal fin from a second male showing orange marking. (c) Anal fin from female showing side-by-side orange and black markings. (d) Microscopic image of orange-black boundary from individual in (c) showing melanophore and xanthophores as well as intercellular orange pigment. (e) Microscopic image of boundary of orange spot from (b) showing wash of orange pigment very clearly distinguishable black melanophores.

analysis, all data partitions were combined with 2000 bootstrap replicates. Maximum-likelihood analysis for both data sets was analysed in PAUP* (http://paup.csit.fsu.edu/). A model of molecular evolution was chosen using jModeltest 1.0 (Posada & Crandall, 1998; http://darwin.uvigo.es/software/jmodeltest.html/) for the AIC and Bayesian information criteria. Maximum likelihood was run for each model type. For final ML analysis, bootstrapping was performed over 2000 replicates.

Underparameterized models perform poorly using Bayesian analysis, so the analysis errs on the side of overparameterization (Lemmon & Moriarty, 2004). Consequently, the data were divided into a total of nine partitions (three codon positions for cytochrome *b* and Tmo-4 and one partition each for 12S, 16S and the d-Loop). A model was selected for each partition using MrModelTest (Nylander, 2004). The final analysis utilized the combined data with
Table I. Species, museum accession number and GenBank numbers for all sequences obtained for phylogenetic analysis in six species of Amphistichinae

<table>
<thead>
<tr>
<th>Locus</th>
<th>Species</th>
<th>Mus. Acc. #*</th>
<th>Cyt b†</th>
<th>12S</th>
<th>16S</th>
<th>d-Loop</th>
<th>Tmo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amphistichus argenteus</td>
<td>SIO-08-178</td>
<td>JN125234 JN125222 JN125228 JN125216 JN125210</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. koelzi</td>
<td>SIO-08-174</td>
<td>JN125237 JN125223 JN125229 JN125217 JN125211</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A. rhodoterus</td>
<td>SIO-08-176</td>
<td>JN125240 JN125224 JN125230 JN125218 JN125212</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hyperprosopon anale</td>
<td>SIO-08-177</td>
<td>JN125245 JN125225 JN125231 JN125219 JN125213</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H. argenteum</td>
<td>SIO-08-176</td>
<td>JN125250 JN125226 JN125232 JN125220 JN125214</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H. ellipticum</td>
<td>SIO-08-176</td>
<td>JN125246 JN125227 JN125233 JN125221 JN125215</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cymatogaster aggregata</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AP009128 (complete mt genome)</td>
</tr>
<tr>
<td></td>
<td>Ditrema temmincki</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Accession number refers to catalogue number for whole specimen. All numbered specimens are located at the Scripps Institute Ichthyological Collection.

Table II. Primer sequences used to amplify sequences for phylogenetic analysis in six species of Amphistichinae

<table>
<thead>
<tr>
<th>Locus</th>
<th>Name</th>
<th>Size</th>
<th>Sequence</th>
<th>Run conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyt b</td>
<td>SPF12</td>
<td>1103</td>
<td>TAA CCA GGA CCA ATG GCT TG GAG CTA CTA ATG CAT TGT CA</td>
<td>96° C-15 s, 52° C-30 s, 72° C-90 s</td>
</tr>
<tr>
<td></td>
<td>SPR12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12S</td>
<td>SPF6</td>
<td>898</td>
<td>CTA AAG CAT AAC ACT GAA GA GGT GAC TTC TCA GTG TAA GG</td>
<td>96° C-15 s, 50° C-30 s, 72° C-75 s</td>
</tr>
<tr>
<td></td>
<td>SPR6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S</td>
<td>SPF5</td>
<td>401</td>
<td>CTG CCC TGT GAC CAT GAG TTT ATC GTT GAA CAA ACG AAC CCT</td>
<td>96° C-15 s, 57° C-30 s, 72° C-75 s</td>
</tr>
<tr>
<td></td>
<td>SPR5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Loop</td>
<td>SPF8</td>
<td>538</td>
<td>TCA AAG AAG GGG GAT TTT AAC C</td>
<td>96° C-15 s, 48° C-30 s, 72° C-90 s</td>
</tr>
<tr>
<td></td>
<td>SPR8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tmo</td>
<td>SPF3</td>
<td>386</td>
<td>ACG AGT CTT TGA AAA CGA CTC</td>
<td>96° C-15 s, 50° C-30 s, 72° C-45 s</td>
</tr>
<tr>
<td></td>
<td>SPR3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

models chosen according to the AIC. The data were run in MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001; http://mrbayes.csit.fsu.edu/) for 10 million generations from random starting trees with one cold and three heated Markov chains. The final majority-rule consensus tree was constructed from 7500 trees sampled every 1000 generations after the first 2·5 million generations were discarded as burn-in. Convergence was evaluated in the online programme AWTY (Are we there yet?; Wilgenbusch et al., 2004; Nylander et al., 2008).

RESULTS

PHENOTYPIC DATA

Morphological variation was assessed in a total of 397 fishes (n = 7–222, mean sample size per species = 66·2) from the six species. Red pigmentation was clearly
Table III. Presence of red pigmentation in the six species of Amphistichinae

<table>
<thead>
<tr>
<th>Species</th>
<th>Locality</th>
<th>n</th>
<th>Red pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphistichus argenteus</td>
<td>Aptos, CA, U.S.A.</td>
<td>22</td>
<td>Absent</td>
</tr>
<tr>
<td>A. koelzi</td>
<td>Aptos, CA, U.S.A.</td>
<td>30</td>
<td>Present</td>
</tr>
<tr>
<td>A. rhodoterus</td>
<td>Coastal OR, U.S.A.</td>
<td>222</td>
<td>Present</td>
</tr>
<tr>
<td>Hyperprosopon anale</td>
<td>Pacifica, CA, U.S.A.</td>
<td>13</td>
<td>Present caudally</td>
</tr>
<tr>
<td>H. argenteum</td>
<td>Aptos, CA and Coastal OR, U.S.A.</td>
<td>40</td>
<td>Absent</td>
</tr>
<tr>
<td>H. ellipticum</td>
<td>Yachats, OR, U.S.A.</td>
<td>76</td>
<td>Present caudally; orange splash on anal fin in 13 fish (17%)</td>
</tr>
</tbody>
</table>

Present in all A. rhodoterus, A. koelzi, H. ellipticum and H. anale examined, but was wholly lacking in every individual of A. argenteus and H. argenteum (Figs 2 and 3 and Table III). The four species expressing red pigment exhibited a tendency to express more intense pigmentation after death, but the change was particularly striking in H. ellipticum and H. anale. In both living and dead specimens, H. ellipticum was found to be highly polymorphic for red pigmentation on the anal fin, but all individuals expressed red pigmentation in the tail (Table III). The pigmentation was pale in life but extremely bright in dead individuals. Hyperprosopon anale was also found to have a red-pigmented tail that was brighter in death than in living individuals. Intriguingly, A. koelzi, which exhibit dispersed gold spots in an erratic barring pattern as adults, were found to be strongly barred as juveniles, rendering them nearly identical to adult A. rhodoterus and A. argenteus.

CAROTENOID ASSAY

The initial extraction caused a complete leaching of colour from the surfperches tissue [Fig. 5(a), (b)], suggesting that carotenoids were the causative pigment (McGraw et al., 2005). The solvent showed pale but visible colouration for two species, A. rhodoterus and H. ellipticum. After the phase step was performed, the colouration was found to be confined to the upper TBM:hexane phase as predicted for pigment composed of 100% carotenoids [Fig. 5(c)].

MOLECULAR DATA

Sequence data were obtained at the cytochrome b locus from multiple individuals in representative species within each putative genus. Within Amphistichus, three individuals were sequenced from each species and sequence was obtained from a fourth A. argenteus from GenBank. Within Hyperprosopon, one H. argenteum was sequenced and data were obtained for a second individual from GenBank. Four H. anale were sequenced, and four H. ellipticum expressing variation in anal-fin pigmentation were also sequenced. Two of the sequenced H. ellipticum expressed orange anal fins, one expressed black pigment on the anal fin, and the fourth expressed no pigment on the anal fin. Sequence sizes for each locus are given in Table II.
Fig. 5. Results of carotenoid assays. (a) Anal-fin tissue from male *Hyperprosopon ellipticum* before leaching, showing orange pigment. (b) Same tissue after leaching, showing complete extraction of pigment, an indicator that all pigment was carotenoid based. (c) Another, confirmatory test of carotenoid origin of colouration: yellow pigment is wholly segregated into the upper phase in the two *H. ellipticum* test tubes, indicating that pigment was wholly carotenoid-based. Also shown is the assay from *Carassius auratus*.

Ambiguous alignments were removed and sequences were concatenated for analysis. Of the total 3326 bp, 804 bp were variable and 351 bp were parsimony informative. Uncorrected sequence divergence was relatively low within the Amphistichinae between species, ranging from 2.1 to 6.9% (Table IV). Within species, however, variation for cytochrome *b* was much lower (0.0–0.6%) than average between-species distances (4.6–9.0%).
<table>
<thead>
<tr>
<th></th>
<th>A. argenteus</th>
<th>A. koelzi</th>
<th>A. rhodoterus</th>
<th>H. anale</th>
<th>H. argenteum</th>
<th>H. ellipticum</th>
<th>C. aggregata</th>
<th>D. temmincki</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. argenteus</td>
<td></td>
<td>0.029</td>
<td>0.024</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. koelzi</td>
<td>0.029</td>
<td></td>
<td>0.062</td>
<td>0.067</td>
<td>0.044</td>
<td></td>
<td></td>
<td>0.115</td>
</tr>
<tr>
<td>A. rhodoterus</td>
<td>0.062</td>
<td>0.069</td>
<td></td>
<td></td>
<td>0.106</td>
<td>0.120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. anale</td>
<td>0.067</td>
<td>0.040</td>
<td>0.041</td>
<td>0.108</td>
<td>0.114</td>
<td>0.114</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. argenteum</td>
<td>0.044</td>
<td>0.106</td>
<td>0.106</td>
<td>0.114</td>
<td>0.117</td>
<td>0.117</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. ellipticum</td>
<td>0.106</td>
<td>0.120</td>
<td>0.114</td>
<td>0.117</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. aggregata</td>
<td>0.114</td>
<td>0.114</td>
<td>0.114</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>D. temmincki</td>
<td>0.115</td>
<td>0.117</td>
<td>0.117</td>
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</tr>
</tbody>
</table>
PHYLOGENETIC ANALYSIS

The cytochrome \( b \) topology demonstrates that within-group variation appears to be much smaller than between-group variation [Fig. 6(a)]. All Bayesian runs converged very early on, and posterior probabilities for all clades were high. Inclusion of additional loci in the Bayesian analysis resulted in a change in topology, with \( H. \) argenteum and \( H. \) ellipticum being monophyletic in the full data set but not for cytochrome \( b \) only [Fig. 6(b)]. Model selection for the whole data set using the AIC in jModeltest selected the GTR+G model, while the Bayesian information criterion chose a TIM2+G model. The model selected in ML analysis had little effect on topology, but did affect support values. Bootstrap support for all parsimony and maximum-likelihood (ML) analyses were low and variable for the relationships of the three species within the genus Amphistichus and the monophyly of \( H. \) ellipticum and \( H. \) argenteum. The optimal tree in both analyses for the full data set, however, was identical to that recovered with Bayesian analysis.

Overall, bootstrap support was considerably lower than the posterior probabilities. Experimentation with partitioning strategies reveals that increasing the amount of partitioning tends to result in higher posterior probabilities, indicating that the high level of difference in support values between Bayesian and likelihood approaches is a result of partitioning (which was not done in the ML analysis). Regardless of the partitioning strategy used, however, all analyses of all data sets recovered \( H. \) anale as the sister taxon to all other species in the family Amphistichinae and the monophyly of the genus Amphistichus. A statistical test of monophyly using a Shimodaira–Hasegawa test found no significant difference in likelihoods between a tree with monophyletic Hyperprosopon and the optimal tree with \( H. \) anale sister to the rest of the Amphistichinae (\( P = 0.26 \)). Consequently, the evidence for the monophyly of the group sister to \( H. \) anale is ambiguous in the maximum-likelihood analysis. Even so, the Bayesian posterior probabilities, which can be interpreted as statistical tests for monophyly, strongly support the sister status of \( H. \) anale to the rest of the Amphistichinae in the complete data set.

DISCUSSION

The data support Tarp’s (1952) hypothesis of monophyly of the Amphistichinae and the genus Amphistichus. The genus Hyperprosopon appears to be polyphyletic due to the basal location of \( H. \) anale relative to all the other species. Because this result was strongly supported only in the Bayesian analysis, the monophyly of Hyperprosopon cannot be rejected with the data at hand. Nonetheless, the strong Bayesian result suggests that additional data may provide more robust support for a determination of polyphyly for Hyperprosopon. In accordance with Tarp’s (1952) suggestion, \( H. \) ellipticum and \( H. \) argenteum also appear to be sister species in the Bayesian analysis of the complete data set. Bootstrap values in the ML and parsimony trees, as well as for the reduced cytochrome \( b \) phylogeny in the Bayesian analysis, however, produced conflicting results. The Bayesian phylogeny recovered high support for the basal assignment of \( A. \) argenteus within the genus Amphistichus, as had been assigned by Tarp (1952) based on a general perception of shared body shape in \( A. \) koelzi and \( A. \) rhodoterus. Support for this node, however, is low for the
Fig. 6. (a) Majority-rule consensus phylogeny for the partitioned Bayesian analysis of the Amphistichinae for cytochrome b only. Posterior probabilities are indicated ‘(**)’ for all fully supported nodes. Bootstrap support from maximum-likelihood (using the AIC model) and maximum-parsimony analyses are also included below the posterior probabilities. (b) Majority-rule consensus phylogeny for partitioned Bayesian analysis across all loci. **, posterior probabilities for fully supported nodes.
ML and maximum-parsimony (MP) analyses and for the posterior probabilities for cytochrome \( b \) only.

**Morphological Evolution**

The key character which Tarp (1952) found informative to the division between *Hyperprosopon* and *Amphistichus* was the retention of the ancestral anal sheath in *Amphistichus*. In the phylogeny presented here, independent loss of the anal sheath would have had to have occurred at least twice: once in the lineage of *H. anale* following its divergence from the other species, and again in the divergence of the remaining *Hyperprosopon*. Tarp (1952), however, also cited numerous examples of reductive evolution in the *Hyperprosopon* species, particularly in *H. anale*. Such a loss has occurred elsewhere in the family, suggesting that the trait may not be reliable. Another trait that Tarp (1952) considered was the presence or absence of the fraenum, which is absent in all *Hyperprosopon* but present in *A. argenteus* and polymorphic in *A. koelzi*. Clearly convergent loss has occurred in the system under either phylogeny, and Tarp (1952) himself called into question the utility of this trait. Precedence is therefore given here to the new molecular data in establishing the phylogeny of the Amphistichinae.

Phylogenetic hypotheses subsequent to Tarp (1952) based on feeding morphology (DeMartini, 1969) and multivariate phonetic analyses (Morris, 1982) did not significantly diverge from the Tarp (1952) model. DeMartini (1969) provides an account of relationships within the subfamily and concludes that *H. argenteum* and *H. anale* are the most ‘highly specialized’ forms in the lineage. Morris (1982) produced conflicting results for *H. anale* but assigned the taxon to a basal spot within *Hyperprosopon*, whereas *A. rhodoterus* was assigned a basal position within *Amphistichus*.

The new phylogeny recovered in this study raises interesting questions regarding the evolution of the sandy shore-adapted surfperches. It now seems the case that, counter to Tarp’s (1952) hypothesis, the subfamily arose from stock more closely allied with *Hyperprosopon* than with the large, gold-barred forms represented in *Amphistichus*. In general terms, an evolutionary scenario can be constructed revolving around adaptation to sandy beaches. Given the overall low sequence divergence in the lineage, it seems likely that the subfamily represents a rapid radiation of forms into a new landscape, which in this case may be sandy beaches in the eastern Pacific Ocean. Adaptive radiation is an outcome of diversification in phenotypic, behavioural and life-history traits (Balz, 1983; Schluter, 2000). *Amphistichus argenteus*, *A. koelzi* and *A. rhodoterus*, which can now be considered to belong to the most derived group in the subfamily, all feed primarily on decapods and amphipods in the surf zone (Carlisle, 1960; DeMartini, 1969; Bennett & Wydoski, 1977; Love, 1996). In contrast, the basal *H. anale* as well as *H. argenteum* can be found in the surf zone but do not feed there. *Hyperprosopon argenteum* forages nocturnally in deeper water, primarily on small fishes and zooplankton (DeMartini, 1969; Love, 1996). Diel patterns in foraging behaviours of *H. anale* have not been characterized, but the diet consists mainly of zooplankton (DeMartini, 1969; Barry et al., 1996). Furthermore, *H. anale* was the only member of the Amphistichinae found to be associated with drift algal beds in northern California (Allen & Pondella, 2006), again suggesting a pelagic affinity. *Hyperprosopon ellipticum*, however, is closely associated with the surf zone (Wydoski & Bennett, 1973) and has essentially the same
diet preferences as *A. argenteus*, *A. koelzi* and *A. rhodoterus*, namely, decapods and amphipods (DeMartini, 1969; Morris, 1982; Love, 1996; M.F.W., unpubl. data). It would therefore appear that the Amphistichinid lineage records the evolution of a group of surf-adapted forms from a planktivore ancestor.

Barring patterns in fishes at early developmental stages have been found to be phylogenetically informative in centrarchids (Mabee, 1995). The strong barring in juvenile *A. koelzi*, which was found to be basal to the other members of the genus, suggests that a gold-barred form was ancestral to that clade. The apparent absence of any barring from *H. anale* is exceptional with regard to the Embiotocidae, most species of which have some kind of lateral barring pattern. Barring is known to play a role in signalling during courtship in the Embiotocidae (Cummings, 2007) and determining whether the lack of barring in *H. anale* is ancestral or derived merits further study.

When viewed in light of the revised phylogeny, the phenotypic data presented here strongly suggest that a red-pigmented form was ancestral to all the Amphistichinae. A convergent loss of red pigmentation can therefore be inferred in *A. argenteus* and *H. argenteum*. Given the low expression of pigmentation in *H. anale*, a corresponding evolutionary trend towards high pigment expression may have occurred in *A. koelzi*, *A. rhodoterus* and *H. ellipticum*. In *H. ellipticum* and *H. anale*, the enhancement of red pigmentation in the caudal fin after death suggests a gain-of-function mutation that actively suppresses pigmentation in life.

The mechanisms behind the evolutionary phenotypic divergence within the subfamily await study. One guidepost might be the patterns of geographic sympathy and ecological overlap among the six species. *Hyperprosopon argenteum* and *H. ellipticum* are sympatric and appear to overlap in microhabitat use in some part of their diel and seasonal cycles, given that they were frequently collected in the same localities in a single day. *Amphistichus rhodoterus* is also closely sympatric with the two previously named species and has a feeding ecology most similar to *H. ellipticum*. *Amphistichus argenteum* and *A. koelzi* are sympatric over much of their range; are frequently collected at the same time, locality and depth and share essentially the same feeding ecology. Given these patterns, it may be possible to test hypotheses based on established evolutionary models such as character displacement (Schluter & McPhail, 1992; Barluenga et al., 2006) to gain insights into the evolution of red pigment in surfperches.

The polymorphic expression of anal fin colouration in *H. ellipticum* is difficult to interpret, but may provide a snapshot of either a transitional stage in the evolutionary loss of pigmentation or its ongoing maintenance through some mechanism (e.g. frequency- or density-dependence) of pigmentation in a region of the body crucial to courtship and reproduction. Chemical assays reported here suggest that red pigmentation in the anal fin is wholly composed of carotenoid pigments. Because carotenoids are sequestered from food items, it is possible that variation in feeding habits can contribute to the observed variation in pigmentation within *H. ellipticum*. In contrast, the appearance of melanophore-based black markings on the anal fin, either alongside or instead of the orange markings (Fig. 4), cannot be attributed to diet alone. The four-way polymorphism (clear v. black v. orange v. black-and-orange) may be the outcome of complex evolutionary interactions such as has been found in *P. reticulata* (Millar et al., 2006) and lizards (Sinervo & Lively, 1996; Leal & Fleishman, 2004). In any case, *H. ellipticum* provides a fascinating potential
for studying the contemporary evolution of pigmentation in an internally fertilized fish.

The status of *H. anale* should now be re-examined with additional data. If this taxon is sister to the rest of the Amphisticinae as the Bayesian analysis suggests, one solution would be to resurrect the monotypic genus *Hypocriticthys*, to which *A. anale* belonged before being assigned to *Hyperprosopon* by Tarp (1952). Alternatively, given the overall low sequence divergence within the subfamily and the potential for one of the other *Hyperprosopon* species to be sister to *Amphistichus*, conflating all Amphistichinae into a single genus may be the most sensible revision.

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