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Short Communication

Monophyletic origin of brood care in damselfishes

Giacomo Bernardi *

Department of Ecology and Evolutionary Biology, University of California Santa Cruz, Santa Cruz, CA 95060, USA

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ABSTRACT

The absence of a pelagic larval stage and brood care has evolved very few times in coral reef fishes. Damselfishes, a widely represented group with more than 380 species, includes only three such species, the monotypic *Acanthochromis polyacanthus*, and the two *Altrichthys* species, *Altrichthys azurelineatus* and *Altrichthys curatus*. In a recent study, Cooper et al. provided a comprehensive phylogenetic hypothesis for the damselfish family, based on mitochondrial and nuclear sequences, with more than 100 species that included all extant genera (Cooper et al., 2009). *A. polyacanthus* and sequences from formalin-preserved tissue of *A. curatus* did not cluster together, indicating that brood care may have evolved independently twice in damselfishes. Here, we use mitochondrial and nuclear molecular markers from fresh specimens of both *Altrichthys* species. We found that *Altrichthys* and *Acanthochromis* are closest relatives, which suggests that brood care may have evolved only once in damselfishes. Due to the limited geographic range of *Altrichthys* (western Philippines) and the abundant presence of *Acanthochromis* in that region, we also suggest that brood care may have originated in that region.

1. Introduction

Most reef fishes have a bipartite lifestyle that includes a sedentary adult stage and a pelagic larval stage that typically lasts from a few days to several weeks. Indeed, the lack of a pelagic larval stage is supposed to have a number of selective disadvantages including a difficulty in dispersing over great distances and its associated risk of local extinctions (Poethke et al., 2003), and very few species of marine fishes lack a pelagic larval stage (Doherty et al., 1985; Leis, 1991).

For species that lack a pelagic larval stage (apelagic species), gene flow was found to be limited over large geographic scales in the damselfish *Acanthochromis polyacanthus* (Doherty et al., 1994; Miller-Sims et al., 2008; Bay et al., 2008), the banggai cardinalfish, *Pterapogon kauderni* (Bernardi and Vagelli, 2004; Hoffman et al., 2005), and the surfperches *Embiotoca jacksoni* and *Embiotoca lateralis* (Bernardi, 2000, 2005). *A. polyacanthus* (Planes and Doherty, 1997; van Herwerden and Doherty, 2006) and *P. kauderni* (Vagelli et al., 2009) have also been shown to experience limited gene flow over smaller geographic scales. Strong founder effects, which are expected in apelagic species, have also been described in Coral Sea populations of *A. polyacanthus* (Planes et al., 2001). Therefore, while apelagic species are rare, they represent a unique opportunity to study adult dispersal, colonization dynamics, founder effects, speciation mechanisms, and population genetics issues in general.

The care of offspring, from newly hatched larvae all the way to the recruitment stage, referred here as brood care (*sensu*-Cooper et al., 2009) provides some distinctive evolutionary advantages. Besides direct benefits, such as the provision of food when young can feed on the parents' body mucus (Robertson, 1973; Allen, 1999), and the protection of the young from predation, indirect benefits include an increased capacity for local adaptation, and the advantages that the young obtain from being directly recruited into appropriate habitats by their parents. In coral reef fishes, however, apelagic strategies have evolved only rarely (Leis, 1991).

For example, all cardinalfish (Apogonidae) males keep eggs in their mouths (mouthbrooders), yet only two species, *Pterapogon kauderni* and *P. mirifica*, prolong the care by keeping the young in their mouths until ready for recruitment (Vagelli, 1999; Vagelli pers. com.). Damselfishes (Pomacentridae) include approximately 380 species that build nests and spawn benthically (Nelson, 2006). Eggs are fanned and protected by the parents, a preadaptation that seems conducive to further brood care. Yet only three species lack a pelagic larval stage: the spiny damselfish, *A. polyacanthus*, a relatively large species that is widely distributed from Indonesia and the Philippines to the Great Barrier Reef and the Solomon Islands, and two congeneric species, *Altrichthys azurelineatus* and *Altrichthys curatus*, that live mostly sympatrically in a very restricted area of western Philippines in the Calamian islands, and for *A. curatus*, in the Cuyo Islands. Individuals of the two *Altrichthys* species live in close proximity of each other, but tend to select dif-

* Address: Department of Ecology and Evolutionary Biology, University of California Santa Cruz, 100 Shaffer Road, Santa Cruz, CA 95060, USA.
E-mail address: bernardi@biology.ucsc.edu

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ferent microhabitats, *A. curatus* prefers to associate with *Porites cylindrica* corals (Allen, 1999), while *A. azurelineatus* has less specific microhabitat preferences and is more generally distributed (Allen, 1999). In the Calamian islands, *A. azurelinatus* and *A. curatus* also live in very close proximity to *A. polyacanthus* and another damselfish, *Pomacentrus stigma* (pers. obs.).

A recent comprehensive phylogeny of the damselfish family that included all genera and more than 100 species, based on mitochondrial and nuclear markers, uncovered significant evolutionary characteristics for this group (Cooper et al., 2009). In this study, the genus *Altrichthys* was represented by formalin-preserved tissue of *A. curatus* collected in 1978 in the Cuyo Islands (USNM 00348456). Extracting DNA from formalin-preserved tissue is a technical challenge, but it is sometimes required when no other options are available. Based on mitochondrial and nuclear DNA sequences, data suggested that brood care evolved twice in damselfishes. Brood care evolved once with *A. polyacanthus*, which in this study was the sister taxon to *Hemiglyphidodon plagieometopon* (a species that does not show brood care, Hoey, pers. com.), and this clade was the sister lineage to the branch that contains the genera *Amblyglyphidodon* and *Neoglyphidodon*. Brood care evolved independently a second time with *A. curatus*, which grouped with *Pomacentrus stigma* and *Pomacentrus lepidogenys*. Thus, while the lack of a pelagic larval stage is extremely rare in coral reef fishes, it was hypothesized that such traits evolved independently at least twice in damselfishes (Cooper et al., 2009).

The goal of our study was to reassess this statement using fresh material for both *Altrichthys* species, in order to fully understand the geographic and evolutionary components of evolution of brood care in damselfishes.

2. Materials and methods

2.1. Collections and DNA samples

Four individuals of each *A. azurelineatus* and *A. curatus* were collected by hand nets in the islands of Coron (COR), Dibatuc (DIB), and Tangat (TAN), in the Calamian Archipelago, Palawan, Philippines. After collection, samples were immediately placed in 95% ethanol and stored at ambient temperature in the field, and then at 4 °C in the lab. Muscle tissue was later dissected from these samples. Total genomic DNA was prepared from 75 to 150 mg of muscle or liver tissue by proteinase K digestion in lysis buffer (10 mM Tris, 400 mM NaCl, 2 mM EDTA, 1% SDS) overnight at 55 °C. This was followed by purification using chloroform extractions and alcohol precipitation (Sambrook et al., 1989).

2.2. PCR amplification and sequencing

In order to compare our data with previously published sequences, we PCR amplified one mitochondrial marker, 16S rRNA, and two nuclear markers, RAG1 and RAG2. Primers and protocols followed Cooper et al. (2009). After purification of the PCR products, following the manufacturer's protocol (ABI, Perkin–Elmer), sequencing was performed with the primers used in the PCR amplification on an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA). In the case of the nuclear loci, all individuals were homozygous, which made the calling of alleles possible.

Additional sequences from Cooper et al. (2009) were used in our analyses. Sequences from formalin preserved *A. curatus* were limited to the 16SrRNA marker and for 232 bp of the RAG2 marker. All other sequences used here were for all three markers, 16SrRNA, RAG1, and the complete RAG2. The phylogeny presented by Cooper et al. (2009) comprises 104 species, which are mostly outside the subfamily (Pomacentrinae) that is germane to our study. We therefore decided to choose a subsample of the original dataset that is more pertinent to our question by selecting random representatives of all the major subclades that were found in topological proximity to *A. polyacanthus* and *A. curatus*. These are: *Premnas biaculeatus*, *A. polyacanthus*, *H. plagieometopon*, *Amblyglyphidodon leucogaster*, *Neoglyphidodon nigroris*, *Amblypomacentrus clarus*, *Pomacentrus amboinensis*, *Pomacentrus coelestis*, *P. lepidogenys*, *P. stigma*, and *Pomacentrus brachialis*. In the following phylogenetic analyses, the spinecheek clownfish, *P. biaculeatus*, was used as an outgroup, following Cooper et al. (2009).

2.3. Phylogenetic analyses

We used the computer program MAFFT (Katoh et al., 2002) implemented by the Geneious software package (Drummond et al., 2009) to align the DNA sequences. Phylogenetic relationships were assessed by Maximum Likelihood (ML, GARLI software, Zwickl, 2006), Maximum Parsimony (MP, PAUP* software Swofford, 2003), and Neighbor-Joining (NJ, PAUP software), methods. For Maximum Likelihood topologies, we conducted 10 independent runs in GARLI, using default settings and the automated stopping criterion, terminating the search when the ln score remained constant for 20,000 consecutive generations. The best likelihood of those runs was retained and is presented here (Fig. 1). Maximum Parsimony searches included 100 random addition replicates and TBR branch swapping with the Multrees option. Neighbor-Joining reconstructions used distances based on substitution models obtained with Modeltest (HKY + G). Statistical confidence in nodes was evaluated using 2000 non-parametric bootstrap replicates (Felsenstein, 1985) (100 replicates for Maximum Likelihood in GARLI, using the automated stopping criterion set at 10,000 generations). Topological differences were tested using a Shimodaira and Hasegawa test (Shimodaira and Hasegawa, 1999) implemented by PAUP, based on resampling of estimated log-likelihoods tests (RELL, 1000 replicates).

3. Results

3.1. Sequences

Mitochondrial 16SrRNA (484 bp) sequences, and nuclear RAG1 (900 bp) and RAG2 (766 bp) sequences were obtained from all of the individuals sampled. Out of an aligned total of 2152 bp, 312 bp were variable and 164 bp were phylogenetically informative. Sequences were deposited in GenBank (Accession Numbers HQ629877–HQ629900).

3.2. Phylogenetic relationships

Due to the technical difficulty of sequencing formalin preserved specimens, the published sequences of *A. curatus* are shorter than the sequences of other damselfishes. We therefore reconstructed phylogenetic relationships based on two datasets. The first dataset included the sequences of *A. curatus* from Cooper et al. (2009), and was therefore a truncated set of sequences. This included the full length of the 16SrRNA marker (484 bp) and a portion of the RAG2 marker (232 bp). The second dataset included the full length of sequences for the three markers, but did not include the *A. curatus* sample from Cooper et al. (2009).

Besides the presence or absence of the 2009 *A. curatus*, all three phylogenetic reconstruction methods based on either datasets resulted in topologies that were not statistically different. A phylogenetic tree based on a full dataset, but with an indicated position of the 2009 *A. curatus* individual, is presented in Fig. 1.

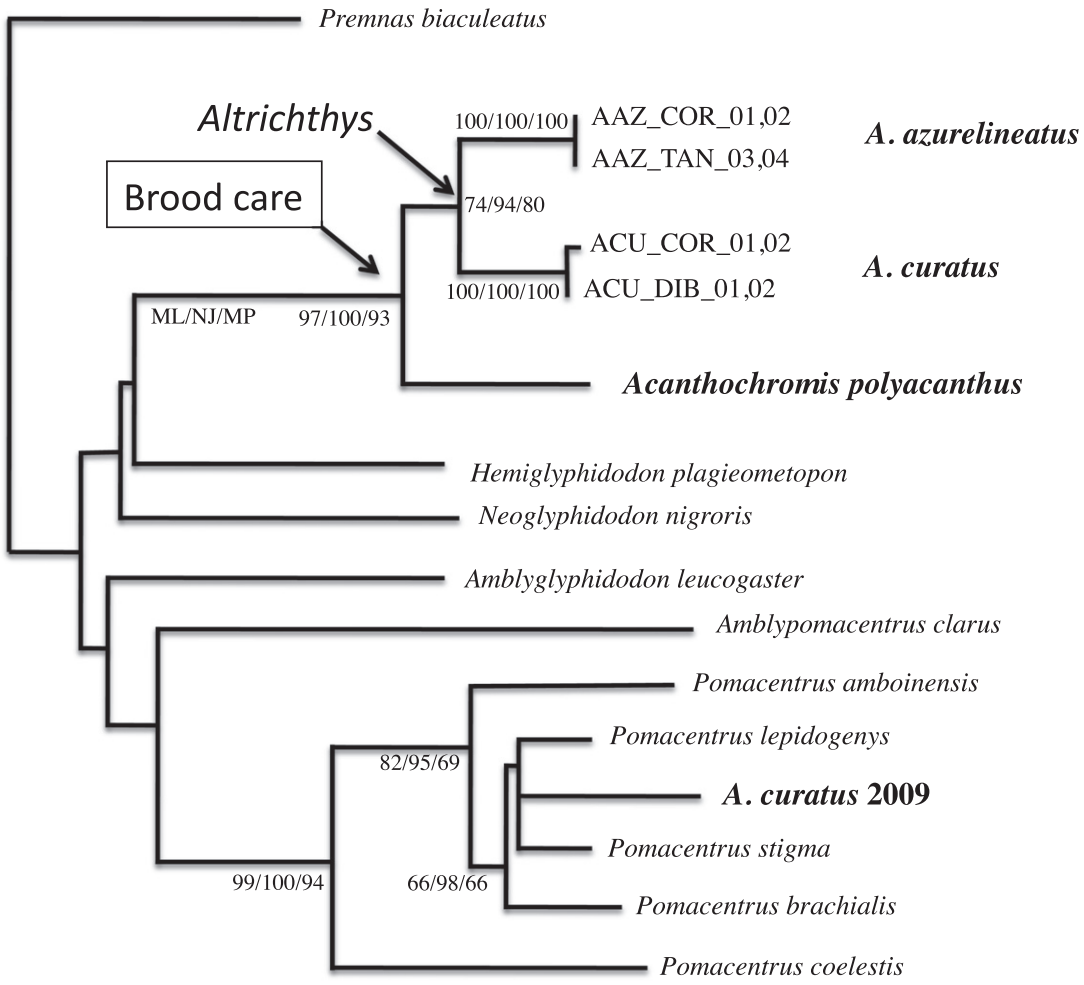

Fig. 1. Phylogenetic relationships of selected pomacentrin damselfishes based on mitochondrial 16SrRNA, and nuclear RAG1 and RAG2 sequences. Maximum likelihood reconstructions are shown, with bootstrap values for Maximum Likelihood (ML), Neighbor-Joining(NJ), and Maximum Parsimony (MP) methods shown close to the nodes. Four *Altrichthys azurelineatus* individuals (AAZ) were from Coron (COR) and Tangat (TAN) islands, four *A. curatus* (ACU) individuals were from Coron (COR) and Dibatuc (DIB) islands, Palawan, Philippines. Sequences for *A. curatus* (1999), and other damselfishes are from Cooper et al. (2009). Apelagic species, *A. azurelineatus*, *A. curatus*, and *Acanthochromis polyacanthus* are shown in bold. Their node, possibly corresponding to the evolution of brood care in damselfishes, is indicated.

The position of damselfish species included in this study, except for *Altrichthys*, was consistent with the positions presented by Cooper et al. (2009), indicating that subsampling of taxa did not affect topological positions. *A. azurelineatus* and *A. curatus* were found to be sister species, with high bootstrap support for all methods of reconstruction. These species were most closely related to *A. polyacanthus*, a relationship that was also strongly supported (93–100% bootstrap support). Forcing the new samples of *Altrichthys* in the position obtained by Cooper et al. (2009) always resulted in a significantly worse topology for both complete and truncated datasets (SH test, $p < 0.001$).

4. Discussion

The position of *Altrichthys* in Cooper et al.'s (2009) reconstruction is most likely erroneous. This is ultimately due to the difficulty in obtaining reliable sequences from formalin preserved specimens. Extraction of formalin-preserved fish specimens can occasionally be done successfully, with fish that are at least 23 years old as shown by Zhang (2010). The sample used in Cooper's study was collected in Cuyo Island in May 1978, making it a 31 year-old sample at the time of extraction. Zhang's study showed that for samples that have been preserved in formalin for 1 year, 80% of the PCR amplifications work, and 70% are actually showing the correct sequence (Zhang, 2010). These rates decline to reach a low point with the oldest 23 year-old specimens, where only 44% of the amplifications work and 31% of the sequences are correct. If one extrapolates the data presented in Zhang (2010) to a 31 year-old sample, chances of obtaining a correct sequence would be approximately 16%.

The fact that the *Altrichthys* sample presented by Cooper et al. (2009) grouped with *P. stigma* may point either towards PCR contamination, which is likely when using ancient DNA techniques and the expected success rate is so low. Alternatively, the individual used in that study may have been misidentified, although this seems unlikely. Yet, *P. stigma* lives in very close proximity to *Altrichthys*, the two species look quite alike, and it is not difficult for *P. stigma* and *A. curatus* to be caught in the same net, when one uses rotenone on a reef and many individuals are collected at once (109 specimens were collected on that day).

Brood care and apelagic life history strategies are seldom encountered in marine fishes. The data presented here indicate that such trait is likely to have evolved only once in damselfishes. The sister species *A. azurelineatus* and *A. curatus* are found in a very restricted geographic area. Work to establish the levels of genetic diversity, gene flow, and speciation processes within such a small area for these species is currently ongoing (Bernardi, unpubl.). These two species are ecologically very similar (Allen, 1999), and

further work is warranted to elucidate the mechanisms that led to their divergence.

The third apelagic damselfish, *A. polyacanthus*, is markedly different from its closest relatives, *A. azurelineatus* and *A. curatus*. *A. polyacanthus* is a larger species that lives higher in the water column, two attributes that may have played a role in its much wider range and dispersal capability. Yet, since all three apelagic damselfishes are found sympatrically, it is tempting to think that the western Philippines may have been the center of origin for brood care in damselfishes.

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