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Evolution of the suborder Blennioidei: phylogeny and phylogeography of a shallow water fish clade.

A dissertation submitted in partial satisfaction of the

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in

Marine Biology

by

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2009

The dissertation of Hsiu-Chin Lin is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2009

DEDICATION

This work is dedicated to my family who are not sure why I have to be far away from home but always have faith in me nonetheless.

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Chapter 2, in full, is currently being prepared for submission for publication. The dissertation author is the primary investigator and Philip Hastings who provided the morphological data is the co-author of this paper. Chapter 3, in full, is a reprint of the material as it appears in Molecular Ecology 18: 2476-2488. The dissertation author is the primary investigator. Carlos Sánchez-Ortiz who assisted in field collection in the Gulf of California and Philip Hastings who provided critical ideas and instructions are co-authors of this paper. Chapter 4, in full, was recently submitted to Copeia. The dissertation author is the primary investigator and Grantly Galland, who described the morphological characters of the new species, is the co-author.

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ABSTRACT OF THE DISSERTATION

Evolution of the suborder Blennioidei: phylogeny and phylogeography of a shallow water fish clade.

by

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Doctor of Philosophy in Marine Biology

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The perciform suborder Blennioidei comprises six families, 151 genera and 883 species. In Chapter 1, I analyzed the phylogenetic relationships of 160 blennioids and ten gobiesocids as the outgroup with one mitochondrial and four nuclear DNA markers. According to the consensus of Bayesian, Maximum Likelihood, and Maximum Parsimony analyses, the monophylies of the six families are supported except for the Labrisomidae and Chaenopsidae. The globally distributed families Tripterygiidae and Blenniidae, are the basal clades branched out of the blennioids. Relationships of the remaining families are partially resolved with the two temperate lineages, the tribe Cryptotremini (Labrisomidae) and the family Clinidae, positioned at the base. Our data

suggest that the three *Labrisomus* subgenera *Brockius*, *Gobioclinus*, and *Labrisomus* should be recognized as separate genera. The recent expansion of the Chaenopsidae with the inclusion of *Neoclinus*, *Mccoskerichthys*, and *Stathmonotus* is not supported. Relationships of the mostly New World family Chaenopsidae are further investigated with additional 145 morphological characters in Chapter 2. The phylogeny based on the combined molecular and morphological dataset supports the monophyly of the subfamily Chaenopsinae and all included genera, but partially agrees with the formerly hypothesized inter-generic relationships based on morphology. Four major clades were identified. The genus *Coralliozetus* is sister to the remaining three clades which have unresolved relationships. Chaenopsids show higher-than-average genetic variation, therefore are ideal models for studying evolutionary mechanisms. In Chapter 3, a Gulf of California endemic chaenopsid *Acanthemblemaria crockeri* is shown to have a species-level divergence with subsequent differentiation based on two mitochondrial markers (COI and d-loop). The geographical boundary between the northern "Gulf" and southern "Cape" species roughly corresponds to a hypothetical Pliocene seaway north of the La Paz. Surprisingly, this pattern is not congruent with the well-recognized northern melanic and southern red color morphs. Another speciation event is confirmed from the *A. hancocki* species group. In Chapter 4, two mitochondrial and one nuclear intron markers were used to discover a new species endemic to the Gulf of California. *Acanthemblemaria hastingsi* sp. nov. is separated from its sister species *A. macrospilus*, by the Sinaloan Gap and differ in head and dorsal-fin color pattern.

INTRODUCTION

There is a long history of interest in understanding the evolutionary relatedness among organisms (Cracraft and Donoghue, 2004). Diverse approaches have been applied to reconstructing phylogenetic relationships. These have included insights gained from morphological similarities, anatomical homologies, protein electrophoresis similarities, and most recently the rapidly growing efforts of direct sequencing of DNA. Despite the method used, the ultimate goal is to estimate a reasonably well-supported hypothesis of relationships and to utilize it as architecture in studying evolutionary processes and mechanisms. For instance, the evolutionary origins and history of anatomical, behavioral, and physiological traits can be hypothesized through character mapping on phylogenetic trees (reviewed by Avise, 2004). Also, knowledge of evolutionary relationships are essential to identify historical events that may have led to speciation, and present-day biogeographical patterns.

With the advance of molecular techniques (Avise, 2004), algorithms and models (e.g. Kingman, 2000; Huelsenbeck et al., 2001; Giribet, 2005) and analytical methods (e.g. Huelsenbeck and Ronquist, 2001; Excoffier and Heckel, 2006; Goloboff et al., 2008), it is now possible to construct robust hypotheses of relationships at deep nodes as well as at the tips of clades. This dissertation applies modern molecular techniques to reconstruct the phylogeny (inter-specific) and phylogeography (intra-specific) of blennioid fishes. In the phylogeny section, former hypotheses based on morphological characters and limited molecular data are also revisited. The phylogeography section

provides insights into the speciation mechanisms of shallow water reef fishes in the Tropical Eastern Pacific (TEP) and the Gulf of California in particular.

The suborder Blennioidei comprises six families, 151 genera and 883 species (Hastings and Springer, 2009b). This perciform fish lineage is characterized by small body size, coastal distributions, and usually strong association with benthic habitats (Patzner et al., 2009). Three included families, Tripterygiidae, Blenniidae, and Clinidae have circumglobal distributions with the last one restricted to temperate waters. The remaining three families Labrisomidae, Chaenopsidae, and Dactyloscopidae are New World-restricted with a few exceptions (Hastings, 2009). Because of their often high abundance in many coastal ichthyofaunas (Paulin and Roberts, 1992; Thomson and Gilligan, 2002; Griffiths, 2003), blennioids are convenient models in the study of ecology and behavior. However, further development of these studies, especially those focused on evolution, is hindered by the uncertain phylogenetic relationships of blennioids. This is mainly due to the diverse morphological features of blennies, difficulty in studying such a large, speciose group, and conflicting evidence of their phylogenetic relationships (Fricke, 2009; Hastings and Springer, 2009b; Hastings and Springer, 2009a). Among the six families, it is generally agreed that the Tripterygiidae and Blenniidae are the basal two clades, a hypothesis recently supported by several features of the dorsal gill-arch muscles (Springer and Orrell, 2004). However, a consensus has not been reached regarding relationships among the Clinidae, Labrisomidae and Chaenopsidae partially due to the controversial monophyly of the Labrisomidae. The relationships of the Dactyloscopidae, the most recent major lineage to be added to the Blennioidei (Springer, 1993), are also uncertain. The dactyloscopids were placed as another unresolved lineage with the

Clinidae, Labrisomidae and Chaenopsidae by Springer and Orrell (2004). In this dissertation, the phylogenetic relationships of the major blennioid clades with special emphases on the Labrisomidae and Chaenopsidae are analyzed with multiple molecular markers and the broadest taxon sampling yet. Morphological characters of chaenopsids are also included in the analysis to provide a total evidence analysis of relationships within this lineage.

This dissertation also explores the patterns of population differentiation and ultimately speciation in two blennioids from the Tropical Eastern Pacific. The main problems of studying evolutionary mechanisms of population differentiation and speciation are identifying the isolation barriers that restrict gene flow and the evolutionary forces that enforce reduced gene flow and ultimately lead to speciation (Coyne and Orr, 2004). Although absolute geographical barriers in marine ecosystems are rare compared to terrestrial ecosystems, reduced gene flow and significant diversification of marine populations are not rare. In addition, there is increasing evidence showing that absolute geographical barriers are unnecessary to build and enforce phylogenetic breaks (Irwin, 2002). Ecological traits (e.g., dispersal period, nearshore development of larva, habitat specialization, color morphs), and intra- and inter- species interactions (e.g., competition, predation, mate choice) are also potent evolutionary forces that may initiate speciation (Irwin, 2002; Maan et al., 2004; Bernardi, 2005; Rocha et al., 2005; Langerhans et al., 2007; Mank, 2007).

The Tropical Eastern Pacific includes a semi-enclosed marginal sea, the Gulf of California, that is an ideal setting to study evolutionary processes because of its high biodiversity and environment heterogeneity. Within the Gulf, oceanographic features

such as water temperature and circulation patterns (Pegau et al., 2002; Marinone, 2003; Alvarez Borrego, 2006), and paleogeography (Murphy and Aguirre-Léon, 2002) have been proposed as plausible evolutionary mechanisms leading the evolution of the Gulf's biota. For instance, genetic studies on marine fishes have implicated the Pleistocene mid-peninsula seaway (about 1 MYA) (Grismer, 2000; Riddle et al., 2000; Murphy and Aguirre-Léon, 2002) as a factor in the population-level differentiation between the upper Gulf (northern Gulf) and lower Gulf (central and southern Gulf) (Riginos, 2005). However, the patterns and underlying processes leading to genetic differentiation of marine organisms in the Gulf are not fully understood. Earlier genetic sampling of Gulf species is typically inadequate to reveal biogeographic regions within the Gulf or does not fully cover the distributional range of most species (Riginos and Nachman, 2001; Riginos, 2005).

This dissertation studies the phylogeography of two TEP *Acanthemblemaria* species (Family Chaenopsidae) with previously reported intra-specific morphological variation. They provide convenient models for studying population differentiation and the underlying evolutionary mechanisms leading to divergence. *Acanthemblemaria* species have several life-history traits that potentially lead to reduced gene flow and increased possibilities of genetic divergence. (Riginos and Victor, 2001; Carreras-Carbonell et al., 2007). These include demersal eggs (Hastings, 1988), short pelagic larval duration (Almany and Baldwin, 1996), near-shore development of larvae (Brogan, 1994), and limited adult dispersal ability. The first study focuses on *Acanthemblemaria crockeri*, the Browncheek Blenny, that is endemic and abundant in the Gulf of California (Thomson et al., 2000). Two color morphs have been reported, the northern "Gulf" morph and

southern “Cape” morph with a limited area of sympatry (Stephens, 1963; Lindquist, 1980). However, the evolutionary status of these two morphs and their distributions remain obscure. Another *Acanthemblemaria* species, *A. macrospilus*, the Barnacle Blenny, occurs in both the Cortez Province (Gulf of California) and the Mexican Province (coastal Mexico south of the Gulf) of the TEP. These areas are separated by the “Sinaloan Gap”, a stretch of open water and a coast line largely devoid of rocky substrate (Hastings, 2000). Based on coloration, Hastings and Robertson (1998) recognized individuals from the two regions as the northern “Cortez morph” and southern “Mexican morph”. This study evaluates the evolutionary status of the morphs of these species using both molecular and morphological features.

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CHAPTER 1

Phylogeny of the Suborder Blennioidei (Teleostei: Perciformes)

Abstract

The perciform suborder Blennioidei comprises six families, 151 genera and 883 species. In this study, we explored the phylogenetic relationships with the broadest taxon sampling to date (160 blennioids and 10 gobiesocids as the outgroup) and multiple molecular markers (one mitochondrial and four nuclear DNA). According to the consensus of Bayesian Inference, Maximum Likelihood, and Maximum Parsimony analyses, the monophylies of four of the six families are supported (Tripterygiidae, Blenniidae, Clinidae, and Dactyloscopidae). The circumglobal families Tripterygiidae and Blenniidae are the basal clades of the blennioids. Relationships of the remaining four families (clinioids *s.l.*) are partially resolved with the early branching of two temperate lineages, a representative of the labrisomid tribe Cryptotremini and the family Clinidae. The remaining clinioids *s.l.* mostly occur in the Neotropical region. Bayesian analysis provides resolution of a chaenopsid *Neoclinus*-*Mccoskerichthys* clade sister to two labrisomid clades, one labrisomid species *Dialommus macrocephalus*, and one clade comprising the Chaenopsinae sister to a Dactyloscopidae-Stathmonotidae clade. The unresolved relationships of these Neotropical clinioid *s.l.* clades and short branch lengths to their most recent common ancestor suggest a rapid radiation of these fishes in the New World.

Introduction

The perciform suborder Blennioidei comprises 6 families, 151 genera and 883 species (Hastings and Springer, 2009b). The blennioids are small coastal fishes usually closely associated with their benthic habitats. Even though small, blennioids can be dominant in rocky reef ichthyofaunas (Paulin and Roberts, 1992; Thomson and Gilligan, 2002; Griffiths, 2003) and thus provide convenient models in studying ecology and behavior (Patzner et al., 2009). The included families Tripterygiidae, Blenniidae and Clinidae have circumglobal distributions but the last one is primarily from temperate waters. Instead, the other three families Labrisomidae, Chaenopsidae, and Dactyloscopidae are mostly restricted to the New World (Hastings, 2009). The breakup of the Tethys Sea (Rosenblatt, 1963), formation of Central America Isthmus (Hastings, 2000), and climate warming during the Pliocene (Stepien et al., 1997) have been suggested as important historical events shaping their current distribution patterns.

There is a long history of the classification of the Blennioidei (reviewed in Springer, 1968; 1993; Rosenblatt, 1984; Hastings and Springer, 2009b). Members of the currently recognized blennioid families Tripterygiidae, Blenniidae, Labrisomidae, Clinidae and Chaenopsdiae are persistently included but extra families have been added depending on the definition of "true" blennies (Regan, 1912; Jordan, 1923; Hubbs, 1952; Gosline, 1968; Springer, 1993). A widely accepted concept of the monophyletic Blennioidei as the "tropical" blenny families (=Blenniicae sensu Hubbs, 1952) was revisited with morphological characters and formalized to include the above five families with the addition of Dactyloscopidae by Springer (1993). The Blennioidei (sensu Springer 1993) shares several unique morphological features including presences of a

bean-shaped pelvis, a reduced branchial apparatus, proximal pectoral-fin radials longer than wide, unbranched pectoral-fin rays, relatively simple caudal fin morphology, 0-2 spines and simple segmented rays in the anal-fin, and no neural spine on the first vertebra (Johnson, 1993; Springer, 1993).

Inter-family relationships have remained unresolved because of conflicting morphological evidence (recently reviewed by Hastings and Springer, 2009b). It is generally agreed that the Tripterygiidae and Blenniidae are serial sister groups to the remaining blennioids, this is supported by several features of the dorsal gill-arch muscles (Springer and Orrell, 2004). However, a consensus has not been reached regarding relationships among the Clinidae, Labrisomidae and Chaenopsidae, partially due to the controversial monophyly of the Labrisomidae. The labrisomids include generalized blennioids that do not fall in other relatively well-defined families and synapomorphies have not been identified (Springer, 1993). This family has long been considered as the closest sister group to the Clinidae (Hubbs, 1952). The relationships of the Dactyloscopidae, the most recent major lineage to be added to the Blennioidei (Springer, 1993), have only been evaluated with dorsal gill-arch anatomy (Springer and Orrell, 2004). It was placed as another unresolved lineage with the Clinidae, Labrisomidae and Chaenopsidae.

Two molecular studies intended to resolve the phylogenetic relationships among the blennioid families by Stepien and colleagues. In one study using 40 allozyme loci and one blenniid as the outgroup, the monophyly of Clinidae was supported and it was nested within the Labrisomidae (Stepien et al., 1993). This Clinidae-Labrisomidae clade was sister to two *Neoclinus* species (included in the Chaenopsidae by Hastings and Springer,

1994), two chaenopsins, and one triplefin (Stepien et al., 1993). In the same paper, relationships were also hypothesized based on sequence data of seven species for a non-functional 281 base-pair long Internal Transcribed Spacer 1 (ITS-1) of ribosomal DNA. However, inadequate taxon sampling with short and highly variable sequences can easily bias phylogenetic results by long-branch attraction (Bergsten, 2005) and substitution saturation (Xia et al., 2003) likely in ITS-1 sequence, making results from this study questionable. Taxon sampling was greatly improved (45 blennioids) in a subsequent study based on a 400 base-pair long mitochondrial 12SrDNA (Stepien et al., 1997). With 14 notothenioids and zoarcoids as the outgroup, the single dactyloscopid species included in the study was sister to the remaining blennioids in the consensus of the three most parsimonious trees (Stepien et al., 1997). Unlike the formerly hypothesized topology based on allozyme data (Stepien et al., 1993), the Clinidae was sister to a Labrisomidae-Chaenopsidae clade, and as a group the Tripterygiidae and the Blenniidae were its serial sister groups.

In the past decade, the field of phylogenetics has entered a new era of estimating species relationships through extensive taxon sampling with multi-locus data (recently reviewed by Degnan and Rosenberg, 2009). This achievement is collectively the result of the development of molecular techniques, universal primers (e.g. Sorenson et al., 1999; Chen et al., 2008), fast tree-searching algorithms (e.g. Huelsenbeck et al., 2001; Giribet, 2005), free analytical software (e.g. Huelsenbeck and Ronquist, 2001; Goloboff et al., 2008), and user-friendly guides (Lemey et al., 2009). This study takes advantage of these developments in reanalyzing the phylogeny of blennioids based on significantly broader taxon sampling and substantially more genetic information. In addition, the systematics

of blennioids based on morphological characters were recently reviewed, providing convenient references: Blenniidae by Hastings and Springer (2009a), Tripterygiidae by Fricke (2009), and Labrisomidae, Clinidae, Chaenopsidae, and Dactyloscopidae by Hastings and Springer (2009b).

In this study, we use one mitochondrial and four nuclear markers from170 species in constructing the blennioid phylogenetic relationships. This includes representative blennioid taxa of 19 triplefins, 48 blenniids, 36 labrisomids, 14 clinids, 38 chaenopsids, and five dactyloscopids. Following recent studies on higher-level relationships of fishes (Chen et al., 2003; Simmons and Miya, 2004; Springer and Orrell, 2004; Dettai and Lecointre, 2005; Miya et al., 2005), ten gobiesocids are also included as the outgroup (Hastings and Springer, 2009b).

Materials and methods

Taxon sampling

Molecular data for 170 terminal taxa were collected to reconstruct the phylogenetic relationships of the suborder Blennioidei. Table 1 details the included species, collection localities and deposition of voucher specimens. The taxon sampling included all six blennioid families: Tripterygiidae, Blenniidae, Labrisomidae, Chaenopsidae, Clinidae and Dactyloscopidae and one outgroup family Gobiesocidae. Currently recognized subfamilies and tribes of these six families following recent reviews (Fricke, 2009; Hastings and Springer, 2009b; Hastings and Springer, 2009a) were sampled with representative species where available (Table 2). Tissue samples were from the Marine Vertebrate Collection at Scripps Institution of Oceanography, University of

Kansas Natural History Museum, Biodiversity Research Museum at Academia Sinica, Taiwan and Australian Museum.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from muscle tissue with a Qiagen (Chatsworth, CA) QIAquick Tissue Kit following the manufacturer's instructions. DNA sequences of one mitochondrial DNA marker, Cytochrome C Oxidase I (COI), and four nuclear markers, TMO-4C4, RAG1, Rhodopsin and Histone H3, were used to reconstruct the phylogenetic relationships. In addition to the primers used in our other work on chaenopsid phylogeny (Chapter 2), six new primers were designed for amplifying PCR products across this broad taxon sampling: two extended inside primers from TMO-F3 and TMO-R3 for TMO-4C4, TMO-F4 5'-GGTGAAGTGGTTCTGCAACA-3' and TMO-R4 5'-GCYGTGTACTCNGGRATRGT-3'; two gobiesocid-specific inside primers for RAG1, Rag-GoF 5'-TTCCTCGATCATTTAGTTTCCA-3' and Rag-GoR 5'-GAAGGGCTTGGAGGAAACTC-3'; two blennioid-specific inside primers for Rhodopsin, Rhod-BleF 5'-CGTCACCCTCGAACACAAGAA-3' and Rhod-BleR 5'-GTTGTAGATGGAGGAACTCTT-3'. The PCR was performed on a Mastercycler EP Gradient S (Eppendorf, Hamburg, Germany) with the following conditions: 94°C for one minute for initial denaturing, 35 cycles of 94°C for 30 sec, 52-56°C for 45 seconds, and 72°C for 45 sec, follow by 72°C for 5 minutes as the final extension. Resulting amplicons were purified with Exonuclease I (20U/μl, New England Biolabs) and Shrimp Alkaline Phosphatase (1U/μl, Roche) in order to remove single-stranded DNA and unincorporated dNTPs. Sequencing was done in both directions with the amplification primers and

DYEnamicTM ET dye terminator sequencing kit on an automated MegaBACE^{TM} 500 DNA sequencer (Amersham Biosciences Corp., Piscataway, NJ).

DNA Sequence alignment and analysis

Sequences were assembled and edited with Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI), then aligned with CLUSTAL X (Thompson et al., 1997) and adjusted by eye in MacClade 4.07 (Maddison and Maddison, 1997). Nucleotide sequences were checked on NCBI database (http://www.ncbi.nlm.nih.gov/) for possible gaps and translated for possible stop codons as an indication of pseudogenes. Prior to phylogeny reconstruction, substitution saturation tests were performed in DAMBE 5.1.1 (Xia et al., 2003; Xia and Lemey, 2009) on first and second codons and third codon of each gene. Substitution number, including transition and transversion versus pairwise TN93 (Tamura and Nei, 1993) sequence distance plot were used to explore the degree of saturation present in the dataset. A plateau is expected with increasing distance if saturation has been reached. In addition, by comparing the Iss (Index of Substitution Saturation) with Iss.c (critical Iss value), datasets with significant larger Iss values than Iss.c, indicative of severe substitution saturation (Xia et al., 2003) were discarded for subsequent phylogenetic analysis. The Akaike Information Criterion (AIC) (Akaike, 1974) implemented in MrModeltest v2.2 (Nylander, 2004) was used to select the best-fit evolutionary model for each marker. The General Time Reversible model (GTR+I+G) (Tavaré, 1986) was selected as the best-fit nucleotide substitution model by AIC for all the genetic markers.

Phylogenetic analysis

Because model-based tree methods are more accurate compared to parsimony methods when the phylogeny contains long branches or complex evolutionary histories (Swofford et al., 1996), we used Bayesian Inference (BI) as the main method and compared the results with those from both Maximum Likelihood (ML) and Maximum Parsimony (MP).

Bayesian Metropolis coupled Markov chain Monte Carlo (MCMC) estimation of phylogeny was carried out using the parallel version of MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001) on a Quad-Core MacPro with 8 simultaneous processors. Bayesian Inference of phylogeny is based on a quantity called the posterior probability distribution of trees, which is the probability of a tree conditioned on the observations. For BI analyses, best-fit evolutionary models selected by MrModeltest were applied to each genetic marker with a partitioned mixed-model where the optimal molecular evolution model was applied to each of the five data partitions and model parameter values were "unlinked" among partitions (Ronquist and Huelsenbeck, 2003). Two simulated independent runs were performed starting from different random trees. Each run comprised four chains (one cold and three heated) and was sampled every 1000 generations. The total generation number was determined by the average standard deviation of split frequencies between the two independent runs while approached to zero and Effective Sample Size (ESS) larger than 200 in Tracer v1.4 (Rambaut and Drummond, 2007). In all analyses, the sampled parameter values from Bayesian MCMC were evaluated in Tracer and the first 20% of generations from each run were discarded as burnin. The convergence of topologies after burnin was tested within and between runs

by the AWTY (Are We There Yet) system (Wilgenbusch et al., 2004; Nylander et al., 2008). Samples from the stationary phase of the two runs were then pooled to produce one 50% majority rule consensus tree for each analysis.

Due to the unfeasible computation time (independent runs had not reached convergence after 80 hours of 6 million generations) for our complete dataset (170 taxa, 3,369bp), we reduced the data matrix by removing several blenniid, triplefin, chaenopsin and gobiesocid taxa when multiple congeneric species were available. The computation time for this reduced dataset of 128 taxa had a practical running time of 98 hours and the two independent runs reached convergence within 10 million MCMC generations. This Bayesian-based topology with reduced dataset should represent the same topology as the complete dataset at the family level of blennioids and intra-family level of the Labrisomidae, Clinidae, and Dactyloscopidae. Phylogenetic relationships within the Tripterygiidae and Blenniidae were analyzed separately using gobiesocids and triplefins as respective outgroups with the same settings as described above.

Maximum likelihood tree searching was executed through RAxML on the complete dataset with data partitioned by genes and 1000 non-parametric bootstrapping on Cipres Portal v1.14 (Stamatakis, 2006; Stamatakis et al., 2008). Parsimony analyses of the complete dataset were performed in TNT v1.0 (Goloboff et al., 2008) using sectorial searches and tree fusing algorithms. The initial level was set to 60 and gaps were treated as missing data. The global optimum was required to be found at least twenty times. Support of nodes was assessed using jackknifing with 36% character deletion (Farris et al., 1996) and 1000 replicates with the same search options as specified above.

Results

Sequence analysis

The dataset comprises 160 blennioid and 10 gobiesocid terminal taxa with 3,559 bp including 570bp in COI, 421bp in TMO-4C4, 1,503bp in RAG1, 737bp in Rhodopsin and 328bp in Histone H3. The alignment of COI, Rhodopsin and Histone H3 was unambiguous, but there were several indels observed especially in TMO-4C4 and RAG1 of the Tripterygiidae. Among these five molecular markers, only the nucleotide substitutions of the third codon position of COI showed a plateau with the increase of TN93 distance (data not shown), and the observed Iss value (0.727) was significantly higher than the Iss.c value (0.321, $p<0.005$). Therefore the third codon of COI was discarded from the matrix and the remaining 3,369bp were used for subsequent phylogenetic analyses. Among the 3,369bp, the alignment comprised 1,464 variable sites, of which 1,219 were parsimony informative. After reducing the data matrix to 128 taxa, the alignment comprised 1,415 variable sites, of which 1,165 were parsimony informative.

Phylogenetic relationships

For the complete dataset, the score of the best ML trees found was -60257.41. One hundred and sixty equally parsimonious trees of 11,573 steps were returned with the MP analysis. Both ML and MP generated topologies based on the complete dataset showed similar relationships with BI based on the reduced dataset at the family-, subfamily-, and tribe-levels (Fig 1-4). The relationships of major blennioid clades based on Bayesian Inference are shown in Fig 1. The concatenated molecular data strongly

supported the monophylies of five major blennioid clades: Tripterygiidae (Bayesian posterior probability/ML bootstrap value/MP jackknifing value = 100/97/96), Blenniidae (100/99/100), Clinidae (100/92/10), Chaenopsinae (100/100/100) and Dactyloscopidae (100/100/99). With the gobiesocids as a monophyletic outgroup (100/100/100), the Tripterygiidae, Blenniidae, *Calliclinus geniguttatus* (currently a labrisomid), and Clinidae were serial sister groups to the remaining blennioids which had only partially resolved relationships.

There were few disagreements of relationships within the Tripterygiidae among ML and MP based on the complete dataset and BI based on the dataset including this family and the gobiesocid taxa (Fig 2). In general, BI and ML had congruent results and the node support values were low. The monophylies of tribes Tripterygiini and Norfolkiini were not supported. The two Helcogrammini *Helcogramma* species were well grouped together and sister to the Trianectini *Ruanoho whero*.

Similarly, relationships within the Blenniidae showed few disagreements among analytical methods (Fig 3). The monophylies of genera including more than one sampled species were well supported except that *Alticus saliens* was nested within the *Andamia* and *Atrosalaris fuscus* was nested within *Salarias* (Fig 3). The monophylies of the four tribes Parablennini, Salariini, Nemophini, and Omobranchini were all supported except the Salariini was not supported by MP. The two Blenniinae tribes Nemophini and Omobranchini were grouped together and as a group were sister to the Salariinae tribe Parablennini. This whole group was sister to the other Salariinae tribe Salariini. *Rhabdoblennius nitidens* from the Salariini-*Rhabdoblennius* group was nested within the Salariini-*Salarias* group.

Relationships among the remaining four families were partially resolved and few disagreements were observed especially between MP, and BI and ML (Fig 1 and 4). In the Clinidae, the two tribes Myxodini and Clinini were both monophyletic and sister to each other (Fig 4). BI and ML provided more resolution than MP within the Labrisomidae. All three methods grouped *Alloclinus* and *Auchenionchus* together, although with limited support. This group was sister to all other labrisomid taxa (except *Calliclinus*) and the family Clinidae based on BI and ML. The genus *Labrisomus* was divided into two clades. One clade including the subgenera *Brockius* and *Gobioclinus* was sister to the tribe Starksini though with limited support by BI and ML. The second clade including the subgenus *Labrisomus* was well grouped with *Malacoctenus* and as a group was sister to the tribe Paraclinini. In the Chaenopsidae, the *Neoclinus*-*Mccoskerichthys* clade branched out following the *Alloclinus*-*Auchenionchus* clade with limited support from BI and ML. The Dactyloscopidae was sister to one chaenopsid genus *Stathmonotus* and as a group was sister to the Chaenopsinae.

Discussion

In attempting to resolve the phylogenetic relationships within the suborder Blennioidei, this study includes multiple molecular markers and the broadest taxon sampling to date. This includes representatives of all six families, with special emphasis on major clades of the family Labrisomidae. This family is likely non-monophyletic (Springer, 1993) and its relationships with the Clinidae and Chaenopsidae are unresolved based on both morphological and molecular data (Stepien et al., 1993; Stepien et al., 1997; Springer and Orrell, 2004). Although the relationships among these three families

and also the Dactyloscopidae are still not fully resolved (Fig 1-4), this study provides new insights into the evolution of blennioids that are discussed below.

Inter-family relationships of the blennioids

With gobiesocids as the outgroup, this study supports monophyly of four of the six currently recognized blennioid families, the Tripterygiidae, Blenniidae, Clinidae and Dactyloscopidae. Each of these is also well-supported by morphological synapomorphies (Springer, 1993). The Tripterygiidae and Blenniidae are serial sistergroups to the remainder of the blennioids (Fig 1). The remaining four families, as a group referred to herein as the clinioids *s.l.*, have complicated relationships, consistent with earlier studies based on morphological characters (Springer and Orrell, 2004), allozyme data (Stepien et al., 1993), and mitochondrial 12SrDNA sequence data (Stepien et al., 1997). The complication is mainly contributed by the paraphyly of the families Chaenopsidae and Labrisomidae (Fig 1 and 4). One species of labrisomid of the tribe Cryptotremini, *Calliclinus geniguttatus*, and the monophyletic family Clinidae are the first two lineages branching out of the clinioids *s.l.*. The remaining taxa have partially resolved relationships, but with very short branch lengths to their most recent common ancestors (Fig 4), suggesting a possible rapid radiation (Walsh et al., 1999). Bayesian Inference and Maximum Likelihood analyses support another two cryptotremins *Alloclinus holderi* and *Auchenionchus microcirrhis*, and two chaenopsids *Neoclinus blanchardi* and *Mccoskerichthys sandae* as serial sister clades to the remaining taxa (Fig 4). These are followed by two lineages that include most of the labrisomid species, a labrisomid *Dialommus macrocephalus*, and a newly identified clade that includes the monophyletic

Chaenopsinae sister to a clade comprising the Dactyloscopidae and the chaenopsid genus *Stathmonotus* (Fig 4).

Family Tripterygiidae

The family Tripterygiidae comprising 32 genera and 164 species can be readily distinguished from the other blennioids with their divided dorsal fin with two spinous and one segmented sections (Fricke, 2009). Two subfamilies with 8 tribes are proposed based on morphological evidence (Fricke, 1994; Fricke, 2009) but their relationships are unclear. Reported phylogenetic relationships of triplefins are restricted to local scales and limited taxon sampling (Geertjes et al., 2001; Carreras-Carbonell et al., 2005; Hickey and Clements, 2005). In this study, we included representatives of 4 tribes, 8 genera and 19 species from the subfamily Tripterygiinae (Table 1 and 2). Although the monophyly of triplefins is strongly supported by our data, there is a general pattern of low node support values at the intra-family relationships (Fig 2). This might be due to incomplete data sampling (i.e. missing data for some markers) as well as numerous insertions and deletions observed in the sequences of RAG1 which can complicate the interpretation of evolutionary information (Loytynoja and Goldman, 2008). In addition, our sample size of 19 species is likely under-representative for investigating intra-family relationships among this extremely diverse family (Fricke, 2009). Consistent with our findings for labrisomids, relationships of some triplefin lineages have been regarded as difficult to resolve because of possible rapid evolution (Carreras-Carbonell et al., 2005).

In this study, the monophylies of the tribes with more than one genus available (Norfolkiini and Tripterygiini) are not supported (Fig 2). Also, none of the genera with

more than one representative were monophyletic in this analysis except for *Helcogramma*. Relationships among these and other genera are very different from what have been suggested based on morphological similarities (Rosenblatt, 1959; Fricke, 1994; Fricke, 2009). In the Tripterygiinae, two major clades are recovered although with limited support. One clade includes three Tripterygiin genera *Axoclinus*, *Crocodilichthys*, and *Enneanectes*, and one Norfolkiin genus *Lepidonectes*. The second clade includes a lineage of the Helcogrammini, *Helcogramma* and the Trianectini *Ruanoho*, the Tripterygiini *Enneapterygius*, and the Norfolkiini *Cremnochorites*. Clearly much broader taxon sampling is needed to resolve relationships within the triplefins.

Family Blenniidae

Among the blennioid families, the Blenniidae comprising 57 genera and 387 species, is the largest and its systematics is the most well studied due to their accessibility and wide distribution (Hastings and Springer, 2009a). The combtooth blennies share several unique features including incisoriform teeth in a single comblike row on the dentaries and premaxillaries (Springer, 1968; Williams, 1990; Springer, 1993). Although easily distinguished from the other blennioids, the classification within the Blenniidae has a long and complicated history that was recently reviewed by Hastings and Springer (2009a). Following their classification, we have samples of 20 genera and 49 species, representing both the subfamilies Blenninae and Salariinae, and 4 out of the 6 tribes, (Table 1 and 2).

Relationships of the six blenniid tribes have been proposed based on morphological evidence (reviewed by Hastings and Springer, 2009). The tribe Nemophini

together with a sister clade of the tribes Omobranchini and Phenablenniini form a monophyletic group based on unbranched central caudal-fin rays and a reduced number of epurals (Smith-Vaniz, 1976) (Fig 5a). Tribes Parablennini and Blenniini were first placed under a large, polyphyletic genus *Blennius* by Norman (1943) and others. Based on the possession of strongly sutured dentaries, a few taxa from this group were designated as the tribe Blenniini (Springer, 1968; Smith-Vaniz, 1976; Bath, 1977) while the remainders were placed in the tribe Parablenniini (Bock and Zander, 1986). The tribe Blenniini has a hypothesized close relationship with the Omobranchini, Phenablenniini, and Nemophini (Smith-Vaniz, 1976). These four tribes as a group is recognized as the subfamily Blenniinae (Fig 5a). The monophyly of the tribe Parablennini has not been confirmed although it is hypothesized to be the sister group of the Salariini (Williams, 1990; Bath, 2001). These two tribes as a group are recognized as the subfamily Salariinae (Fig 5a).

In this study, we have representative taxa from two tribes of the Salariinae (Salariini and Parablennini) and two tribes of the Blenniinae (Omobranchini and Nemophini). Although the monophyly of Blenniinae is supported, the Parablennini is sister to the Blenniinae instead of Salariini thus the monophyly of Salariinae (sensu Hastings and Springer, 2009a) is not supported (Fig 5b). However, because this study included few genera of the Parablenniini (2 of 14) and did not include a member of the tribe Blennini, additional taxon sampling is necessary to resolve the relationships of major lineages of blenniids.

Tribe Nemophini

The Nemophini genera *Meiacanthus, Plagiotremus, Petroscirtus*, and *Xiphasia* form a monophyletic clade that is sister to the Omobranchini genus *Omobranchus* (Fig 3). Within the Nemophini, this analysis provides an alternative phylogeny to the provisional hypothesis proposed by Smith-Vaniz (1976) in which *Meicanthus* and *Petroscirtes* together were sister to *Plagiotremus*, *Xiphasia* and *Aspidontis* (Smith-Vaniz, 1976). In our analysis, *Meiacanthus* and *Plagiotremus* form a strongly supported sister clade to *Petroscirtus* and *Xiphasia* (Fig 3). Relationships of the three species of *Plagiotremus* included in this study are consistent with those proposed by Smith-Vaniz (1976, Fig 81).

Tribe Omobranchini

Only two out of the 34 species are included and these two species form a strongly supported sister group (Fig 3).

Tribe Salariini

The Salariini is the largest blenniid tribe and we sampled thirteen out of its 28 genera. All included genera with more than one species are monophyletic except *Andamia* and *Salarias* (Fig 3). Within the Salariini, the *Salarias* group and the *Rhabdoblennius* group were recognized by Williams (1990) based on a highly modified dentary and numerous premaxillary teeth, features that are shared by the former group (Smith-Vaniz and Springer, 1971). In this study, the division of these two groups is not supported. The only genus of the *Rhabdoblennius* group available for this study is *Rhabdoblennius* and is nested within the *Salarias* group (Fig 3).

Generic relationships of the Salariini in this study (Fig 6b) are very similar and better resolved than the hypotheses based on morphological characters by Smith-Vaniz and Springer (1971) and later partially revised by Springer and Williams (1994) (Fig 6a). In both topologies (Fig 6a and b), the genus *Ecsenius* is sister to the remaining salariinins (node A), *Cirripectes* and *Ophioblennius* are sister (node B), *Atrosalarias* and *Salarias* are sister (node C), *Blenniella* and *Istiblennius* are sister (node D), and *Praealticus* is sister (node E) to a clade including *Andamia* and *Alticus* (node F). In the morphology-based tree (Fig 6a), the *Praealticus* clade shares a most recent common ancestor with *Blenniella* and *Istiblennius*, that is sister to the genus *Entomacrodus* (node G). However, the molecular analysis includes *Entomacrodus*, as well as *Rhabdoblennius* in this clade (node G'). Finally in the morphological hypothesis, *Cirripectes* and *Ophioblennius* (node B) are sister to node G, while in the molecular hypothesis, the *Salarias* clade (node C+*Nannosalarias*) is sister to node G'.

Ecsenius. Four *Ecsenius* species are included in this study and each of them belongs to different species group recognized by Springer (1988). The *opsifrontalis* group, represented by *E. opsifrontalis,* is sister to the *oculus* group, represented by *E. pardus*. Together these form an unresolved relationship with the *stigmatura* group, represented by *E. midas,* and the *yaeyammaensis* group, represented by *E. nalolo* (Fig 3).

Cirripectes. The topology within *Cirripectes* is consistent with the hypothesis of Williams (1988) in having *C. quagga* as sister to the other species, and *C. castanus* and *C. polyzona* as sister species (Fig 3). Relationships in this study differ in the placement of *C. stigmaticus* which is more closely related to *C. filamentosus* than to *C. castanus* and *C. polyzona* as hypothesized by Williams (1988).

Istiblennius. Relationships of the three *Istiblennius* species included in this study are different form the hypothesis of Springer and Williams (1994) in having *I. dussmieri* sister to *I. lineatus* instead of sister to *I. edentulus* (Fig 3).

Entomacrodus. This study includes two members of the *nigricans* group (*E. chiostictus* and *E. nigricans*) and two members of the *striatus* group (*E. striatus* and *niuafoouensis*) (Springer, 1967). These two groups were not recovered as monophyletic because *E. striatus* is nested within the *nigrican* group (Fig 3).

Praealticus. This study includes two of the three species groups defined by Bath (1992). The two members of the *striatus* group (*P. striatus* and *P. bilineatus*) are sister (Fig 3). Two members of the *bilineatus* group (*P. caesius* and *P. labrovittatus*) are sister, but the third (*P. margaritatus*) is sister to the members of the *striatus* group.

Tribe Parablennini

Two out of the fourteen Paraclinini genera *Hypsoblennius* and *Parablennius* are included in this study and these form a a monophyletic group (Fig 3). Six *Hypsoblennius* species included in this study show incongruent relationships from those suggested by Bath (2000) in having *H. brevipinnis* sister to *H. gilberti*, *H. jenkinsi* sister to *H. caulopus* and *H. gentilis*, and *H. hentzi* sister to the remaining species.

Family Clinidae

Two synapomorphic characters are hypothesized to unite the clinids, the presence of cycloid scales with radii in all fields and a cordlike ligament extending from the ceratohyal to the dentary (Hubbs, 1952; George and Springer, 1980; Springer, 1993).

Three tribes are recognized in this family (Hubbs, 1952; George and Springer, 1980) and a hypothesized relationship of the Myxodini as sister to the Clinini and the Ophiclinini is based on the reproductive pattern of internal fertilization with males possessing an intromittent organ shared by the later two tribes (George and Springer, 1980). This study covers the first two tribes and their sister relationship is well supported (Fig 4).

Tribe Myxodini

Two out of the five Myxodini genera are included in this study and their sister relationship is well supported (Fig 4). All of the three *Gibbonsia* species are included and *G. elegans* is sister to *G. metzi* and *G. montereyensis* which agrees with the previously hypothesized relationships based on 40 allozyme loci (Stepien and Rosenblatt, 1991). However, this is in conflict with a more recent study with expanded taxon sampling based on the same allozyme loci (Stepien et al., 1993) and 12SrDNA data (Stepien et al., 1997). That study reported an alternative relationship with *G. metzi* as the sister to the remaining two species.

Tribe Clinini

This study includes six out of the seventeen genera of the Clinini. The monophyly of each the four genera with more than one species available is not supported (Fig 4). However, the Australian genera *Heteroclinus* and *Cristiceps* form a well-supported sister group to the South African genera, *Clinus*, *Muraenoclinus*, *Blennophis*, and *Pavoclinus*.

Family Labrisomidae

Most of the 110 labrisomid species are generalized blennioids that do not fit in other well-defined families (Springer, 1993). The monophyly of the Labrisomidae has long been questioned because of the lack of any unique morphological characters (Springer, 1993; Springer and Orrell, 2004) and on the basing molecular evidence (Stepien et al., 1993; Stepien et al., 1997). In past studies, the members of this family usually have unresolved relationships with the clinids and chaenopsids. Also, relationships among the labrisomids are unknown and essentially unhypothesized except for several relatively well-defined tribes (Hastings and Springer, 2009b). This study includes representatives of all the five hypothesized tribes and 10 out of the 14 genera (Table 2). The only genera not included are the Eastern Pacific deepwater (>20m) genus *Cryptotrema* (Hubbs, 1952; Hubbs, 1954) and three rare and poorly known monotypic genera *Haptoclinus*, *Nemaclinus*, and *Cottoclinus* (Böhlke and Robins, 1974; Böhlke and Springer, 1975; McCosker et al., 2003). This study thus provides the most thorough investigation yet of the phylogenetics relationships of this family.

As suspected, the monophyly of the family Labrisomidae is not supported (Fig 1 and 4). Also, the monophyly of two of the tribes (Cryptotremini and Labrisomini) are not supported but that of the Starksiini and Paraclinini are confirmed. Except for the Mnierpini with only a single species available and whose relationship is unresolved within the clinioids *s.l.*, the phylogenetic relationships of each tribe are discussed further below.

Tribe Cryptotremini

The tribe Cryptotremini is the only antitropically distributed group in the Labrisomidae. Two northeastern Pacific genera *Alloclinus* and *Cryptotrema* were the only included members when this tribe was first described by Hubbs (1952). The tribe was later expanded with the addition of two southeastern Pacific genera *Auchenionchus* and *Calliclinus*. However their inclusion was based on the plesiomorphic condition of branched caudal-fin rays (Stephens and Springer, 1974) unbranched in all other labrisomids. None of the analytical methods used in this study support the monophyly of this tribe (Fig 4). With limited support, the northern *Alloclinus* is grouped with the southern *Auchenionchus* and as a group is sister to the clinioids *s.l.* excluding the Clinidae and another Cryptotremini genus *Calliclinus* which is sister to all the remaining clinioids *s.l.*. The evolutionary position of cryptotremins as early branching lineages of labrisomids and their close relationship with the Clinidae was also suggested by morphological (Stephens and Springer, 1974) and allozyme data (Stepien et al., 1993).

Tribe Labrisomini

The tribe Labrisomini including *Labrisomus* and *Malacoctenus* is not defined by synapomorphies (Springer, 1959). However, the monophyly of this tribe was supported by mitochondrial 12SrDNA of two *Labrisomus* and two *Malacoctenus* species (Stepien et al., 1997). With a much broader taxon sampling and multiple genetic markers in this study, the Labrisomini is evidently not monophyletic and the monophyly of the genus *Labrisomus* as currently construed is not supported (Fig 1 and 4).

Labrisomus. Based on our concatenated molecular data, the seven *Labrisomus* species are divided into two non-sister lineages (Fig 1 and 4). The first lineage is sister to

the other Labrisomini genus *Malacoctenus* and includes *L. nuchipinnis* and *L. xanti.* These were previously assigned to the subgenus *Labrisomus* (Hubbs, 1952; Hubbs, 1953; Springer, 1959). The second *Labrisomus* lineage can be further divided into two sub-lineages and as a group is sister to the tribe Starksiini in Bayesian Inference and Maximum Likelihood analyses. Members of the first sub-lineage include *L. nigricinctus* and *L. striatus* were previously assigned to the subgenus *Brockius* (Table 3; Hubbs, 1952; Hubbs, 1953; Springer, 1959). The second sub-lineage with *L. haitiensis* as a sister species to *L. bucciferus* and *L. guppyi* was previously assigned to the subgenus *Gobioclinus* (Table 3) (Springer, 1959). According to Springer's (1959) classification, the subgenus *Gobioclinus* can be distinguished from the other two by the presence of palatine teeth, several of which are considerably larger than those on vomer. Compared to the subgenus *Labrisomus*, the subgenus *Brockius* has fewer scales in the lateral line and all posterior lateral line scales have the anterior pore of canal exposed. Although this study only includes seven out of the twenty-one *Labrisomus* species, these three *Labrisomus* clades based on molecular data agree well with Hubbs' (1952) and Springer's (1959) three subgenera based on morphological similarity. As Springer (1959, p. 422) suspected, "I feel certain that some systematists would relegate each of the above subgenera (*Labrisomus*, *Brockius*, and *Gobioclinus*) to the rank of genus, as the differences separating them are trenchant." Here we confirm his assessment and recognize these three as full genera with the genus *Brockius* Hubbs, 1953 sister to the genus *Gobioclinus* Gill, 1860 and these are sister to the tribe Starksiini. In contrast, the restricted genus *Labrisomus* is sister to the genus *Malacoctenus* and together these form a

well-supported clade that is sister to the tribe Paraclinini. Species placed in each of these genera based on morphological criteria are listed in Table 3.

Malacoctenus. Ten out of the twenty-one *Malacoctenus* species are included in this study and these form a well-supported clade sister to the newly defined *Labrisomus* (see above) (Fig 4). No obvious generic division as suggested by Hubbs (1952) is observed in this analysis. One well-supported Eastern Pacific lineage is found comprising five species including *M. zaca*, *M. hubbsi*, *M. gigas*, *M. zonogaster* and *M. zonifer*.

Tribe Paraclinini

The two genera currently included in the tribe Paraclini, *Paraclinus* and *Exerpes*, share the unique characters of a spine on the posterior margin of the opercle and 0-2 segmented dorsal-fin rays (Hubbs, 1952; Rosenblatt and Parr, 1969; Brooks, 1992). Results from this study support the monophyly of the tribe (Fig 4). However, the monotypic *Exerpes asper* is nested within the four *Paraclinus* species, making the later genus paraphyletic. *Exerpes asper* can be distinguished from members of the genus *Paraclinus* for having greatly prolonged snout, no cirri on nape or eye, scales on anterior segment of lateral line consisting of a pore at each end of a tube, and the absence of suborbital lateral line canal (Springer, 1959). However, Brooks (1992) reported that this species shares several features with selected *Paraclinus* species, especially *P. infrons*. *Paraclinus infrons* also has an elongate snout, though less so than in *E. asper*. Additional study of relationships within this lineage are needed, but based on our findings and those of Brooks (1992), we synonomize the genus *Exerpes* Jordan and Evermann, 1896 with *Paraclinus* Mocquard, 1888.

Tribe Starksiini

The tribe Starksiini including two genera *Starksia* and *Xenomedea* uniquely shares a modification of the first anal-fin spine that functions as an intromittent organ in males and ovoviviparity as the reproduction mode (Hubbs, 1952; Rosenblatt and Taylor, 1971). Our molecular data supports the monophyly of this tribe (Fig 4), but the monotypic *Xenomedea* is nested within the nine *Starksia* species. Although possible divisions within *Starksia* are difficult to distinguish based on morphology (Rosenblatt and Taylor, 1971), one lineage including *S. ocellata*, *S. grammilaga* and *S. spinipenis* is well supported (Fig 4). Species of *Starksia* that would be included in the nominal genus *Brannerella* (1960) based on length of the gonopodium do not group together. Inclusion of additional species of this diverse lineage is needed to resolve the relationships.

Family Chaenopsidae

Within the Chaenopsidae, the subfamily Chaenopsinae is a strongly supported monophyletic group (Fig 1 and 4) and its phylogenetic relationships are analyzed in combination with morphological characters in chapter 2. As addressed in that study, the outgroup relationships of the Chaenopsinae are unclear. In this study, the three non-chaenopsin genera as a group are not the closest sister group to chaenopsins (Fig 1 and 4). Instead *Neoclinus* and *Mccoskerichthys* cluster together and as a group that is sister to the clinioids *s.l.* excluding the Clinidae and the Cryptotremini in Bayesian and Maximum Likelihood analyses (Fig 4). The other non-chaenopsin genus *Stathmonotus* shows an unexpected sister relationship with the family Dactyloscopidae. Therefore, the expansion

of the Chaenopsidae with the addition of *Neoclinus, Mccoskerichthys* and *Stathmonotus* (Hastings and Springer, 1994) is not supported by our molecular data. In order to maintain a monophyletic classification and recognize the distinctive lineages of dactyloscopids and chaenopsids, we recommend resurrection of the family Stathmonotidae Jordan and Evermann (1898) for this distinctive lineage of worm blennies.

Family Stathmonotidae

The phylogenetic relationships of the seven species of small (<55mmSL), cryptic, and eel-like fishes of the genus *Stathmonotus* have been controversial for many years (Hastings and Springer, 1994). *Stathmonotus* was considered closely related to chaenopsids based on sharing a scale-less body (Jordan, 1923). However, the later inclusion of one scaled species *Auchenistius stahli* excluded the *Stathmonotus* from chaenopsids (Springer, 1955). Later it was included in the non-monophyletic general blennioid family Labrisomidae (Nelson, 1984; Eschmeyer, 1990). The most recent revision of this lineage concluded a sister relationship with the Chaenopsinae (=Chaenopsidae Springer, 1963) based on six apomorphic morphological characters (Hastings and Springer, 1994). This study based on molecular data confirms its distinctiveness and offers a new hypothesis of the relationships of this enigmatic lineage as sister to the Dactyloscopidae (Fig 1 and 4). However its sister relationship with the Dactyloscopidae should be further examined with additional taxon sampling especially within the Dactyloscopida, as well as the evaluation of morphological evidence.

Family Dactyloscopidae

The dactyloscopids, also known as sand stargazers, are a distinctive group with several morphological adaptations for their sand or gravel dwelling behaviors (Hastings and Springer, 2009b). The monophyly of this family is supported by the presence of bony fimbriae extending from the ventral margin of the interopercle and posterodorsal margin of the opercle (Springer, 1993; Doyle, 1998). In this study, only 5 out of the 48 dactyloscopid species are included and they form a well-supported monophyletic group based on our molecular data (Fig 4).

Family Gobiesocidae (outgroup)

Although relatively few taxa of this diverse lineage are included (ten out of 140 species) (Nelson, 2006), this study provides support for the monophyly of this family and the genera *Gobiesox* and *Tomicodon* (Fig 2). Strongly supported sister relationship of these two genera is consistent with Briggs' (1955) inclusion of them in the largely New World tribe Gobiesocini.

Future work

Sequence data for several key taxa need to be included to further resolve relationships of the blennioid fishes (Fig 1-4). First, this study did not focus on the intra-family relationships of the Tripterygiidae, Clinidae and Dactyloscopidae because relatively few species were available. Of special interest would be members of the clinid tribe Ophicliniini that is hypothesized to be sister to the Clinini (George and Springer, 1980). Second, additional members of the Cryptotremin labrisomids are needed. This

study includes three of the four genera and three of the eight species. But given their position near the base of the clinioids *s.l.* (Fig 4), they may be key to resolving the relationships of clinioids *s.l.*. Third, relationships of the morphologically distinctive labrisomid *Dialommus* are unresolved and sequence data from the remaining two species in the tribe Mnierpini may help. Fourth, the nine species of the genus *Neoclinus* have unique distributions at the temperate waters of the northeastern and northwestern Pacific Ocean. Expanding taxon sampling is needed to confirm the monophyly of this genus and document a probable dispersal event across the north Pacific Ocean (Hastings, 2009). Finally, several key issues are remain regarding higher-level relationships within the Blenniidae. This will require inclusion of taxa from the tribe Blenniini (*Blennius* and *Spaniblennius*) and additional members of the diverse and likely non-monophyletic tribe Parablenniini.

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Table 1.1 Localities and sample IDs for terminal taxa used for phylogenetic analysis. SIO: Marine Vertebrate Collection of Scripps Institution of Oceanography, KU: Natural Hisotry Museum of University of Kansas, ASIZP: Biodiversity Research Museum at Academia Sinica, Taiwan, AM: Australian Museum, C: Gift from S. von der Heyden, University of Stellenbosch, South Africa. Abb: Abbreviation. Numbers in parentheses are the sample size.

Taxon	Sample number	Abb.	Locality
Family Tripterygiidae (19)			
Axoclinus storeye	SIO 01-49-1	Axca	Bahia Conejas, Mexico
Axoclinus lucillae	SIO 01-164-1	Axlu	Taboguilla, Panama
Axoclinus nigricaudus	SIO 02-16-1	Axni	La Paz, Mexico
Cremnochorites capensis	KU 6473	Crca	Port Alfred, South Africa
Crocodilichthys gracilis	SIO 06-54-1	Crgr	Bahia de los Angeles, Mexico
Enneanectes altivelis	KU 225	Enal	Carrie Bow Cay, Belize
Enneanectes boehlkei	KU 166	Enbo	Carrie Bow Cay, Belize
Enneanectes sp1	SIO 01-182-1	Ensp	Puerto Vallarta, Mexico
Enneanectes sp2	SIO 01-182-1	Enma	Puerto Vallarta, Mexico
Enneanectes pectoralis	KU 167	Enpe	Carrie Bow Cay, Belize
Enneanectes reticulatus	SIO 02-16-1	Enre	La Paz, Mexico
Enneanectes carminalis	SIO 01-164-1	Ense	Taboguilla, Panama
Enneapterygius abeli	KU 7026	Enab	Mahe, Seychelles
Enneapterygius gruschkai	KU 7150	Engr	Mahe, Seychelles
Enneapterygius tutuilae	KU 5483	Entu	Saipan, CNMI
Helcogramma ellioti	KU 801	Heel	Tongatapu, Tonga
Helcogramma fuscopinna	KU 7025	Hefu	Mahe, Seychelles
Lepidonectes corallicola	SIO 00-154-1	Leco	Pta Vincente, Galapagos Is.
Ruanoho whero	C74	Ruwh	
Family Blenniidae (48)			
Alticus saliens	KU 7531	Alsa	Pingtung, Taiwan
Andamia reyi	KU 7517	Anre	Pingtung, Taiwan
Andamia tetradactylus	KU 7519	Ante	Pingtung, Taiwan
Atrosalarias fuscus	KU 4125	Atfu	Viti Levu, Fiji
Blenniella chrysospilos	KU 4180	Blch	Viti Levu, Fiji
Blenniella cyanostigma	KU 5539	Blcy	Saipan, CNMI
Blenniella interrupta	KU 7566	Blin	Dededo, Guam
Cirripectes castaneus	KU 7056	Cica	Cap Ternay, Seychelles
Cirripectes filamentosus	KU 6917	Cifi	Mahe, Seychelles
Cirripectes polyzona	KU 4315	Cipo	Viti Levu, Fiji
Cirripectes quagga	KU 800	Ciqu	Tongatapu, Tonga
Cirripectes stigmaticus	KU 686	Cist	Tongatapu, Tonga
Ecsenius midas	KU 7206	Ecmi	Ils du Nord, Seychelles
Ecsenius nalolo	KU 7095	Ecya	Mahe, Seychelles
Ecsenius opsifrontalis	KU 5576	Ecop	Saipan, CNMI
Ecsenius pardus	KU 4090	Ecpa	Viti Levu, Fiji
Entomacrodus chiostictus	SIO 07-120-1	Enci	La Paz, Mexico
Entomacrodus nigricans	KU 139	Enni	Carrie Bow Cay, Belize
Entomacrodus niuafoouensis	KU 5534	Ennu	Saipan, CNMI
Entomacrodus striatus	KU 7061	Enst	Mahe, Seychelles
Hypsoblennius brevipinnis	SIO 01-41-1	Hybr	Huatulco, Mexico
Hypsoblennius caulopus	SIO 01-170-1	Hyca	Golfo de Fonseca, El Salvador

Table 1.1 Continued.

Taxon	Voucher number	Abb.	Locality
Hypsoblennius gentilis	SIO 06-51-1	Hyge	Bahia de los Angeles, Mexico
Hypsoblennius gilberti	SIO 05-81-1	Hygi	Point Loma, California
Hypsoblennius hentzi	KU 19	Hyhe	Charleston Harbor, South Carolina
Hypsoblennius jenkinsi	SIO 05-82-1	Hyje	Point Loma, California
Istiblennius dussumieri	KU 4801	Isdu	Vanua Levu, Fiji
Istiblennius edentulus	ASIZP 0800861	Ised	Kenting, Taiwan
Istiblennius lineatus	ASIZP 0800864	Isli	Kenting, Taiwan
Meiacanthus oualanensis	KU 4133	Meou	Viti Levu, Fiji
Nannosalarias nativitatus	KU 4041	Nana	Viti Levu, Fiji
Omobranchus anolius	AM 40863-002	Oman	Black Wattle Bay, Australia
Omobranchus obliquus	KU 4549	Omob	Viti Levu, Fiji
Ophioblennius macclurei	KU 136	Opat	Carrie Bow Cay, Belize
Ophioblennius steindachneri	SIO 01-43-1	Opst	Huatulco, Mexico
Parablennius marmoreus	SIO 00-181-1	Pama	Bocas del Toro, Panama
Petroscirtes mitratus	KU 7130	Pemi	Cap Ternay, Seychelles
Plagiotremus azaleus	SIO 01-50-1	Plaz	Reef la Entrega, Mexico
Plagiotremus rhinorhnychos	KU 5019	Plrh	Scottburgh, South Africa
Plagiotremus tapeinosoma	KU 4501	Plta	Viti Levu, Fiji
Praealticus caesius	KU 775	Prca	Tongatapu, Tonga
Praealticus labrovittatus	KU 7561	Prla	Mangilao, Guam
Praealticus margaritatus	ASIZP 0800867	Prma	Kenting, Taiwan
Praealticus striatus	ASIZP 0800872	Prst	Kenting, Taiwan
Praealticus tanegasimae	ASIZP 0800887	Prta	Kenting, Taiwan
Rhabdoblennius nitidens	KU 7544	Rhel	Yona, Guam
Salarias alboguttatus	KU 4418	Saal	Viti Levu, Fiji
Salarias fasciatus	KU 4005	Safa	Viti Levu, Fiji
Xiphasia setifer	ASIZP 0061269	Xise	Yilan, Taiwan
Family Labrisomidae (36)			
Alloclinus holderi	SIO 04-44-1	Alho	La Jolla Cove, California
Auchenionchus microcirrhis	SIO 03-84-2	Aumi	Valparaiso, Chile
Calliclinus geniguttatus	SIO 03-84-2	Cage	Valparaiso, Chile
Dialommus macrocephalus	SIO 03-1-1	Mnma	Guanacaste, Costa Rica
Exerpes asper	SIO 06-56-1	Exas	Bahia de los Angeles, Mexico
Labrisomus bucciferus	KU 162	Labu	Carrie Bow Cay, Belize
Labrisomus guppyi	KU 156	Lagu	Carrie Bow Cay, Belize
Labrisomus haitiensis	KU 228	Laha	Carrie Bow Cay, Belize
Labrisomus nigricinctus	KU 163	Lani	Carrie Bow Cay, Belize
Labrisomus nuchipinnis	SIO 07-80-1	Lanu	Los Farallones, Panama
Labrisomus striatus	SIO 07-39-2	Last	Gordo Point, Mexico
Labrisomus xanti	SIO 98-34-1	Laxa	Bahia de los Angeles, Mexico
Malacoctenus aurolineatus	KU 158	Maau	Carrie Bow Cay, Belize
Malacoctenus boehlkei	KU 182	Mabo	Carrie Bow Cay, Belize
Malacoctenus ebisui	SIO 01-48-1	Maeb	Huatulco, Mexico
Malacoctenus gigas	SIO 06-54-1	Magi	Bahia de los Angeles, Mexico
Malacoctenus hubbsi	SIO 06-54-1	Mahu	Bahia de los Angeles, Mexico
Malacoctenus tetranemus	SIO 01-40-1	Mate	Cacaluta, Mexico
Malacoctenus triangulatus	SIO 01-127-1	Matr	Dry Torrugas, Florida
Malacoctenus zacae	SIO 07-2-1	Maza	Cabo San Lucas, Mexico
Malacoctenus zonifer	SIO 01-170-1	Mazf	Gulf de Fonseca, El Salvador
Malacoctenus zonogaster	SIO 02-88-1	Mazo	Horpana Island, Galapagos Is.

Table 1.1 Continued.

Taxon	Voucher number	Abb.	Locality
Paraclinus integripinnis	SIO 04-46-1	Pain	Point Loma, California
Paraclinus marmoratus	KU 183	Pamr	Carrie Bow Cay, Belize
Paraclinus mexicanus	SIO 01-170-1	Pame	Gulf de Fonseca, El Salvador
Paraclinus sini	SIO 03-77-1	Pasi	Loreto, Mexico
Starksia atlantica	KU 165	Stat	Carrie Bow Cay, Belize
Starksia fasciata	KU 164	Stfa	Carrie Bow Cay, Belize
Starksia galapagensis	SIO 02-89-1	Stga	Floreana Island, Galapagos Is.
Starksia grammilaga	SIO 07-124-1	Stgr	Cabo San Lucas, Mexico
Starksia lepicoelia	KU 226	Stle	Carrie Bow Cay, Belize
Starksia nanodes	KU 184	Stna	Carrie Bow Cay, Belize
Starksia ocellata	KU 240	Stoc	Carrie Bow Cay, Belize
Starksia posthon	SIO 01-182-1	St po	Puerto Vallarta, Mexico
Starksia spinipenis	SIO 01-182-2	Stsp	Puerto Vallarta, Mexico
Xenomedea rhodopyga	SIO 04-121	Xerh	Las Cuevatas, Mexico
Family Chaenopsidae (38)			
Neoclinus blanchardi	SIO 00-73-1	Nebl	Redondo Beach, California
Mccoskerichthys sandae	SIO 01-167-1	Mcsa	Isla Montuosa, Panama
Stathmonotus culebrai	SIO 01-164-1	Stcu	Taboquilla, Panama
Stathmonotus lugubris	SIO 01-182-1	Stlu	Puerto Vallarta, Mexico
Stathmonotus stahli	KU 236	Stst	Carrie Bow Cay, Belize
Acanthemblemaria aspera	SIO 01-9-1	Acas	San Blas, Panama
Acanthemblemaria betinensis	SIO 03-141-1	Acbe	Bahia Azul, Panama
Acanthemblemaria castroi	SIO 02-89-1	Acca	Bartolome, Galapagos
Acanthemblemaria chaplini	SIO 03-141-1	Acch	Bahia Azul, Panama
Acanthemblemaria crockeri	SIO 03-82-3	Accr	Loreto, Mexico
Acanthemblemaria exilispinus	SIO 03-142-1	Acex	Isla Taboga, Panama
Acanthemblemaria greenfieldi	SIO 03-147-1	Acgr	Carrie Bow Cay, Belize
Acanthemblemaria hancocki	SIO 03-3-1	Acha	Playa Cocos, Costa Rica
Acanthemblemaria macrospilus	SIO 01-48-1	Acma	Huatulco, Mexico
Acanthemblemaria rivasi	SIO 01-9-1	Acri	San Blas, Panama
Acanthemblemaria spinosa	SIO 03-147-1	Acsp	Carrie Bow Cay, Belize
Ekemblemaria myersi	SIO 01-170-1	Ekmy	Fonseca, El Savador
Ekemblemaria nigra	SIO 03-141-1	Ekni	Bahia Azul, Panama
Chaenopsis alepidota	SIO 00-9-1	Chal	Santa Catalina, California
Chaenopsis limbaughi	SIO 03-149-1	Chli	Carrie Bow Cay, Belize
Chaenopsis schmitti	SIO 02-83-1	Chsc	Isla Rabida, Galapagos
Emblemaria diphyodontis	SIO 06-276-1	Emdi	Laguna Grande del Obispo, Venezuela
Emblemaria hypacanthus	SIO 06-52-1	Emhy	Bahia de los Angeles, Mexico
Emblemaria nivipes	SIO 01-165-1	Emni	Cocos Island, Costa Rica
Emblemaria pandionis	SIO 01-9-1	Empa	San Blas, Panama
Emblemaria piratica	SIO 01-182-1	Empi	Puerto Vallarta, Mexico
Hemiemblemaria simulus	SIO 05-2-1	Hesi	Florida keys, Florida
Lucayablennius zingaro	KU 110	Luzi	Carrie Bow Cay, Belize
Cirriemblemaria lucasana	SIO 05-141-1	Cilu	Bahia Bandaras, Mexico
Coralliozetus angelicus	SIO 05-124-1	Coan	Isla Danzante, Mexico
Coralliozetus boehlkei	SIO 01-52-1	Cobo	Huatulco, Mexico
Coralliozetus cardone	SIO 01-5-2	Coca	Soufrierre, St. Lucia
Coralliozetus micropes	SIO 03-82-2	Comi	Loreto, Mexico
Coralliozetus rosenblatti	SIO 07-120-1	Coro	La Paz, Mexico

Table 1.1 Continued.

Taxon	Voucher number	Abb.	Locality
Coralliozetus springeri	SIO 01-164-1	Cosp	Taboquilla, Panama
Emblemariopsis randalli	SIO 06-276-1	Emra	Laguna Grande del Obispo, Venezuela
Emblemariopsis signifera	SIO 01-171-1	Emsi	Rio de Janeiro, Brazil
Protemblemaria bicirrus	SIO 02-16-1	Prbi	La Paz, Mexico
Family Clinidae (14)			
Blenniophis stella	C24	Blse	
Blenniophis striatus	KU 6477	Blst	Port Alfred, South Africa
Clinus superciliosus	KU 6485	Clsu	Port Alfred, South Africa
Clinus cottoides	KU 6487	Clco	Port Alfred, South Africa
Cristiceps australis	C85	Crau	
Gibbonsia elegans	SIO 02-24-1	Giel	La Jolla, California
Gibbonsia metzi	SIO 06-267-1	Gime	Laguna Grande del Obispo, Venezuela
Gibbonsia montereyensis	SIO 06-41-1	Gimo	Vandenburg AFB, California
Heteroclinus adelaidae	C80	Head	
Heteroclinus nasutus	AM 41084-022	Hena	Watsons Bay, Australia
Heterostichus rostratus	SIO 01-179-1	Hero	Mission Bay, California
Muraenoclinus dorsalis	KU 6489	Mudo	Port Alfred, South Africa
Pavoclinus graminis	C23	Pagr	
Pavoclinus profundus	KU 6476	Papr	Port Alfred, South Africa
Dactyloscopidae (5)			
Dactyloscopus lacteus	SIO 02-88-1	Dala	Horpana Island, Galapagos Is.
Dactyloscopus pectoralis	SIO 01-182-1	Dape	Puerto Vallarta, Mexico
Gillelus sp.	SIO 07-78-1	Gisp	San Blas Islands, Panama
Gillelus uranidea	SIO 07-78-1	Giur	San Blas Islands, Panama
Platygillellus rubrocinctus	KU 206	Plru	Carrie Bow Cay, Belize
Family Gobiesocidae (10)			
Apletodon dentatus	GenBank	Apde	
Aspasma minima	Genbank	Asmi	
Gobiesox juradoensis	SIO 03-42-1	Goju	Golfo de San Miguel, Panama
Gobiesox pinniger	SIO 06-51-1	Gopi	Bahia de los Angeles, Mexico
Gobiesox rhessodon	SIO 09-170	Gorh	La Jolla, California
Lepadogaster lepadogaster	Genbank	Lele	
Tomicodon humeralis	SIO 02-1-1	Tohu	Bahia de los Angeles, Mexico
Tomicodon myersi	SIO 07-2-1	Tomy	Cabo San Lucas, Mexico
Tomicodon sp.	SIO 01-167-1	Tosp	Isla Montuosa, Panama
Tomicodon zebra	SIO 07-2-1	Toze	Cabo San Lucas, Mexico

Table 1.2 Currently recognized subfamilies and tribes of five families of the Blennioidei (excluding the Chaenopsidae which is discussed in Chapter 2). Only the genera with representative taxa are listed.

Family (total genera/ total species)	Subfamily	Tribe (total genera/ sampled genera)	Genera (total species/ sampled species)
Tripterygiidae (32/164)			
	Notoclininae		
		Notoclinini (2/0)	
	Tripterygiinae		
		Trianectini (5/1)	
			Ruanoho (2/1)
		Norfolkiini (4/2)	
			Cremnochorites
			Lepidonectes
		Tripterygiini (8/4)	
			Axoclinus (6/3)
			Crocodilichthys (1/1)
			Enneanectes (8/7)
			Enneapterygius (53/3)
		Forsterygiini (5/0)	
		Karalepini (2/0)	
		Helcogrammini (3/1)	
			Helcogramma (39/2)
		Blennodontini (3/0)	
Blenniidae (57/387)			
	Blenniinae		
		Blenniini (2/0)	
		Nemophini (5/4)	
			Meiacanthus (25/1)
			Petroscrites (11/1)
			Plagiotremus (11/3)
			Xiphasia (2/1)
		Omobranchini (7/1)	
			Omobranchus (21/2)
		Phenablenniini (1/0)	
	Salariinae		
		Parablenniini (14/2)	
			Hypsoblennius (14/6)
			Parablennius (27/1)
		Salariini-Salarias group (20)	
			Alticus (10/1)
			Andamia (7/2)
			Atrosalarias (3/1)
			Blenniella (9/2)
			Cirripectes (22/5)
			Ecsenius (53/4)

Table 1.2 Continued.

Family (total genera/ total species)	Subfamily	Tribe (total genera/ sampled genera)	Genera (total species/ sampled species)
			Entomacrodus (25/4)
			Istiblennius (14/3)
			Nannosalarias (1/1)
			Ophioblennius (5/2)
			Praealticus (13/5)
			Salarias (13/2)
		Salariini-Rhabdoblennius group (8/1)	
			Rhabdoblennius (5/1)
Labrisomidae (14/109)			
		Cryptotremini (4/3)	
			Alloclinus (1/1)
			Auchenionchus (3/1)
			Calliclinus (2/1)
		Labrisomini (2/2)	
			Labrisomus (20/7)
			Malacoctenus (21/10)
		Paraclinini (2/2)	
			Exerpes (1/1)
			Paraclinus (23/4)
		Starksiini (2/2)	
			Starksia (30/9)
			Xenomedea (1/1)
		Mnierpini (2/2)	
			Dialommus (2/1)
		Uncertain (2/0)	
Family Clinidae (26/85)			
		Clinini (17/6)	
			Blenniophis (2/2)
			Clinus (17/2)
			Cristiceps (3/1)
			Heteroclinus (15/2)
			Muraenoclinus (1/1)
			Pavoclinus (9/2)
		Myxodini (5/2)	
			Gibbonsia (3/3)
			Heterostichus (1/1)
		Ophiclinini (4/0)	
Family Dactyloscopidae (9/48)			
		Dactyloids (3/1)	
			Dactyloscopus (20/2)
		Gillelloids (3/1)	
			Gillelus (10/2)
		Uncertain (3/1)	
			Platygillellus (6/1)

Table 1.3 Species allocated to three genera previously placed in the genus *Labrisomus* based on Springer, 1959 and details presented in the original species description ([a]). * = type species; [#] = placement tentative; [+] = included in this study.

Labrisomus Swainson, 1837
Labrisomus conditus Sazima, Carvalho-Filho, Gasparini and Sazima, 2009 [a]
Labrisomus cricota Sazima, Gasparini and Moura, 2002 [a]
Labrisomus fernandezianus (Guichenot, 1848)[#]
Labrisomus jenkinsi (Heller and Snodgrass, 1903)
Labrisomus multiporosus Hubbs, 1953
Labrisomus nuchipinnis (Quoy and Gaimard 1824)*[+]
Labrisomus philippii (Steindachner, 1866)
Labrisomus pomaspilus Springer and Rosenblatt, 1965 [a]
Labrisomus socorroensis Hubbs, 1953
Labrisomus wigginsi Hubbs, 1953
Labrisomus xanti Gill, 1860[+]
Brockius Hubbs, 1953
Brockius albigenys (Beebe and Tee-Van, 1928)
Brockius nigricinctus (Howell Rivero, 1936) [+]
Brockius striatus (Hubbs, 1953)* [+]
Gobioclinus Gill, 1860
Gobioclinus bucciferus (Poey, 1868) [+]
Gobioclinus dendriticus (Reid, 1935)
Gobioclinus filamentosus (Springer, 1960)
Gobioclinus gobio (Valenciennes 1836)*
Gobioclinus guppyi (Norman, 1922) [+]
Gobioclinus haitiensis (Beebe and Tee-Van, 1928) [+]
Gobioclinus kalisherae (Jordan, 1904)

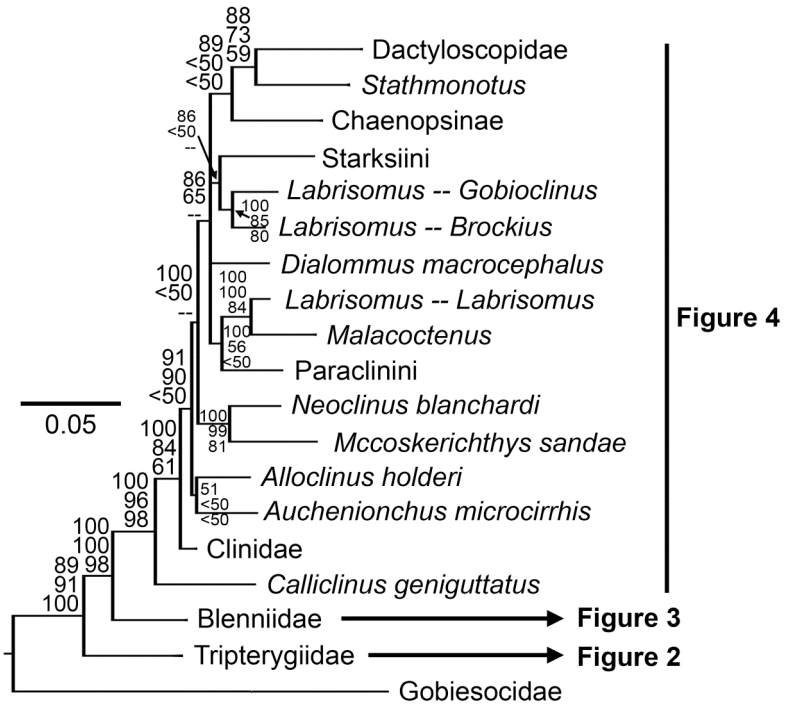

Fig. 1.1 Phylogenetic relationships among major blennioid clades based on Bayesian analysis. Numbers at nodes from top to bottom are supporting values of Bayesian posterior probability, Maximum Likelihood bootstrap, and Maximum Parsimony jackknifing. -- = not supported.

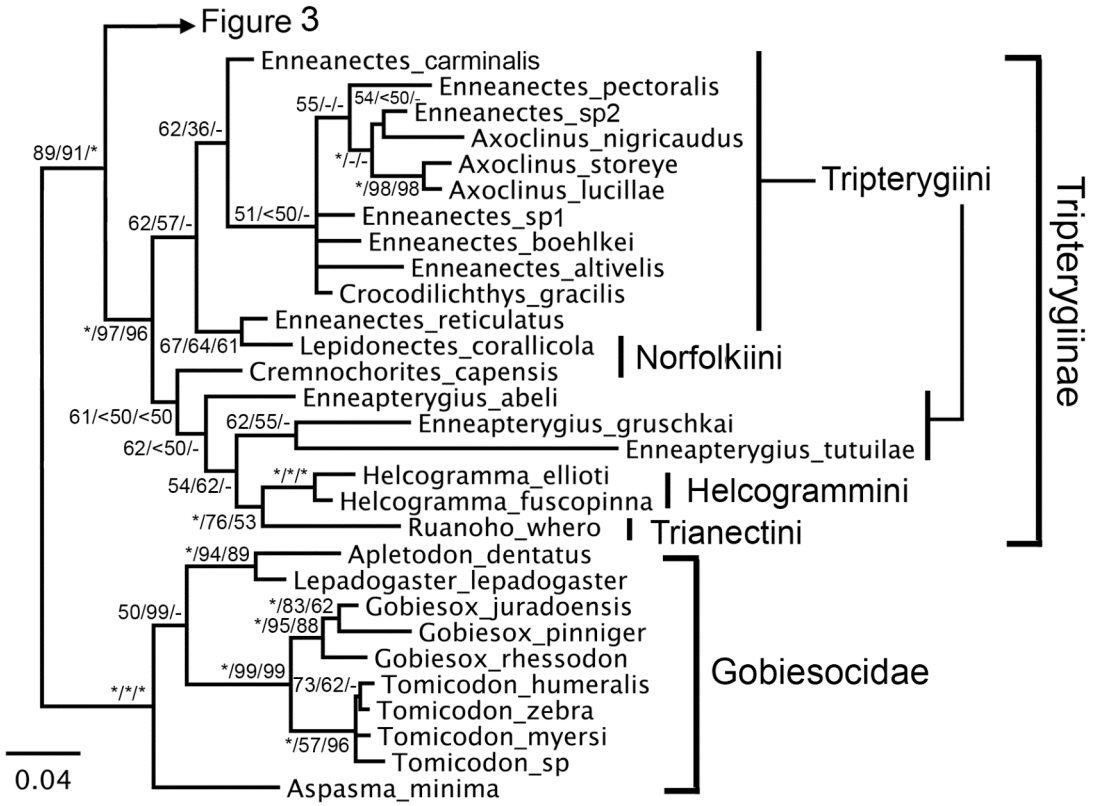

Fig. 1.2 Phylogenetic relationships of the Tripterygiidae with gobiesocids as the outgroup based on Bayesian analysis. Node supports are BI posterior probability/ML bootstrap/MP jackknifing. *=100. - = not supported.

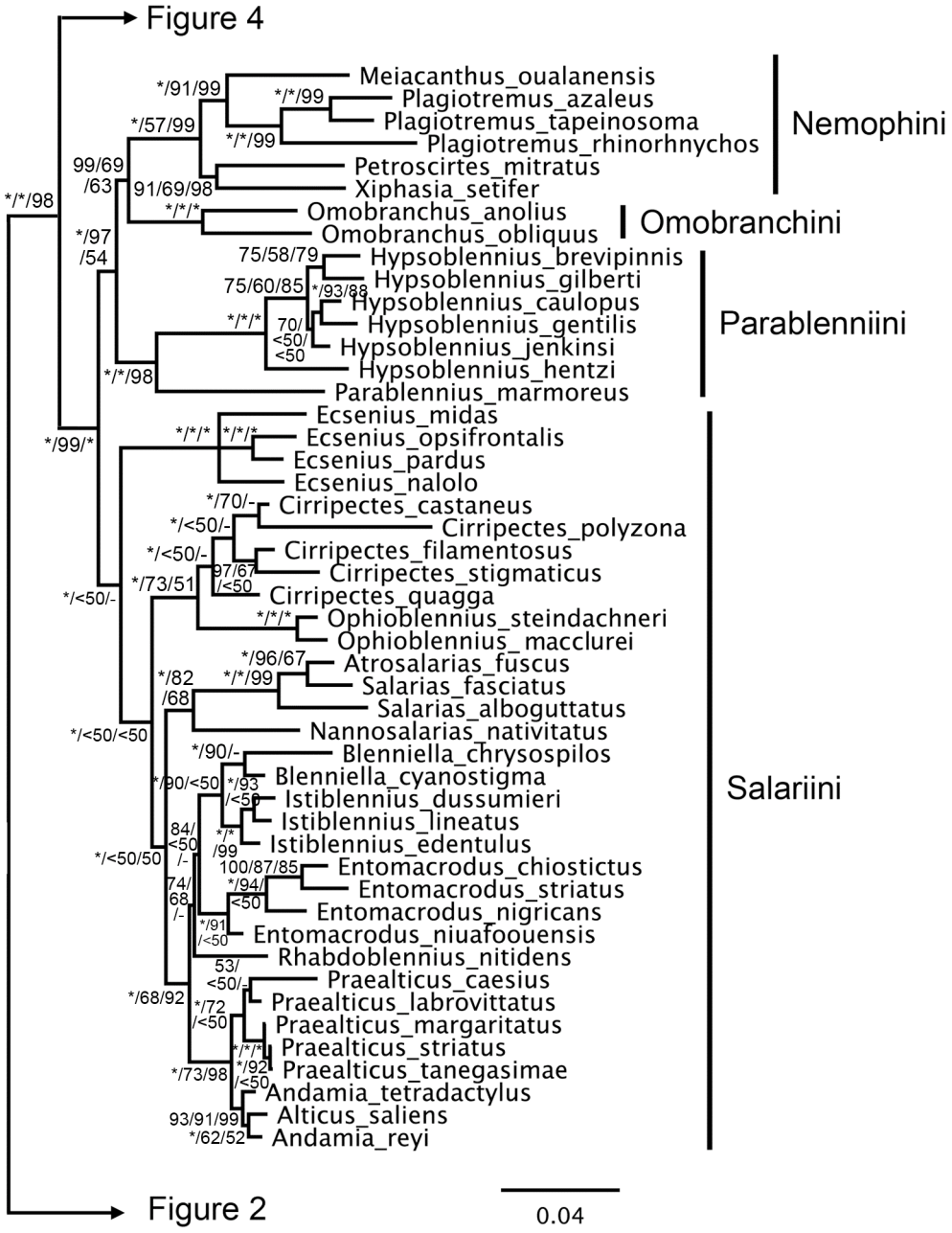

Fig. 1.3 Phylogenetic relationships of the Blenniidae with triplefins as the outgroup based on Bayesian analysis. Node supports are BI posterior probability/ML bootstrap/MP jackknifing. *=100. - = not supported.

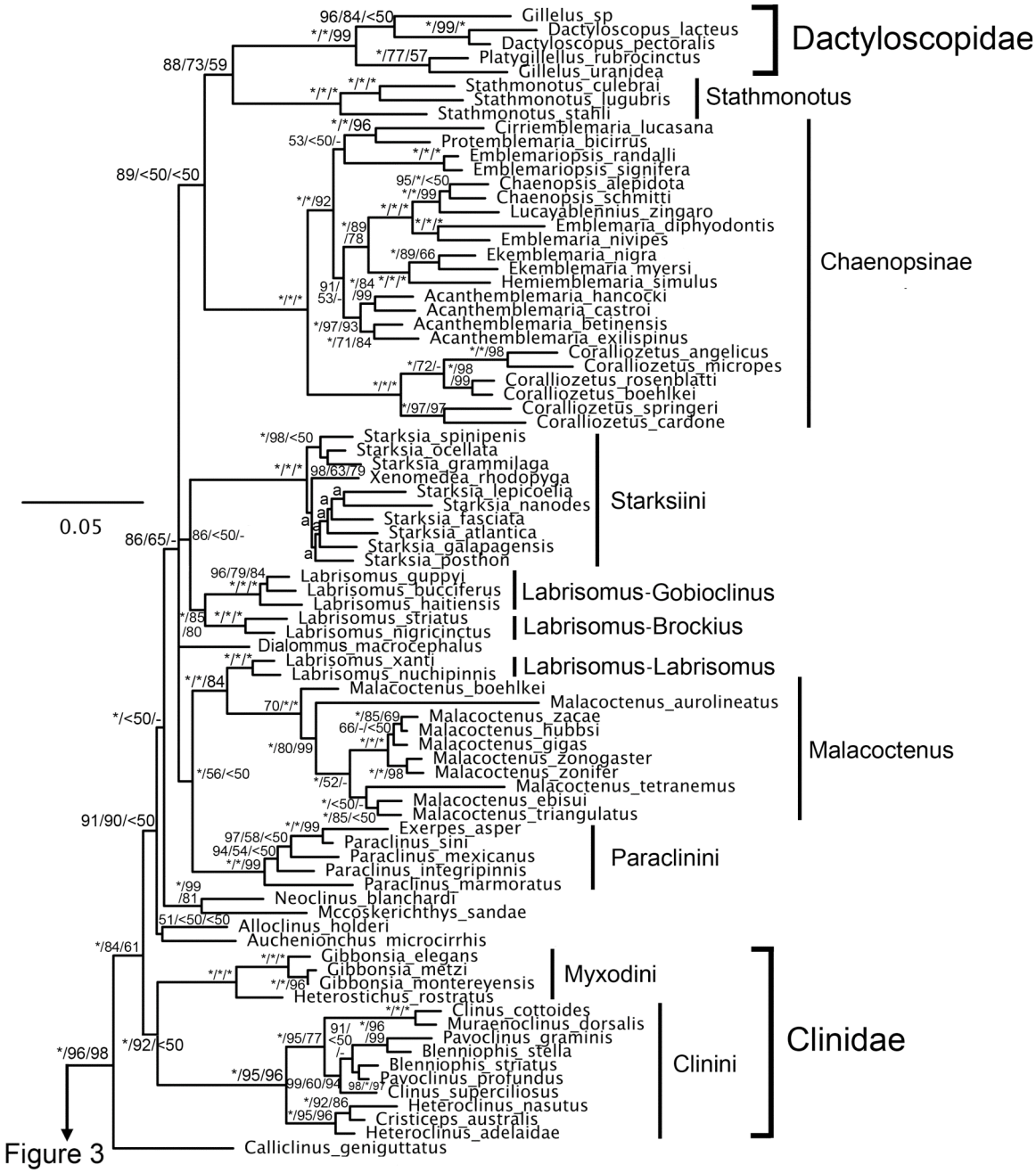

Fig. 1.4 Phylogenetic relationships of the Clinidae, Labrisomidae, Chaenopsidae and Dactyloscopidae based on Bayesian analysis. Node supports are BI posterior probability/ML bootstrap/MP jackknifing. *=100. - = not supported. a=node supports from three methods are all lower than 50.

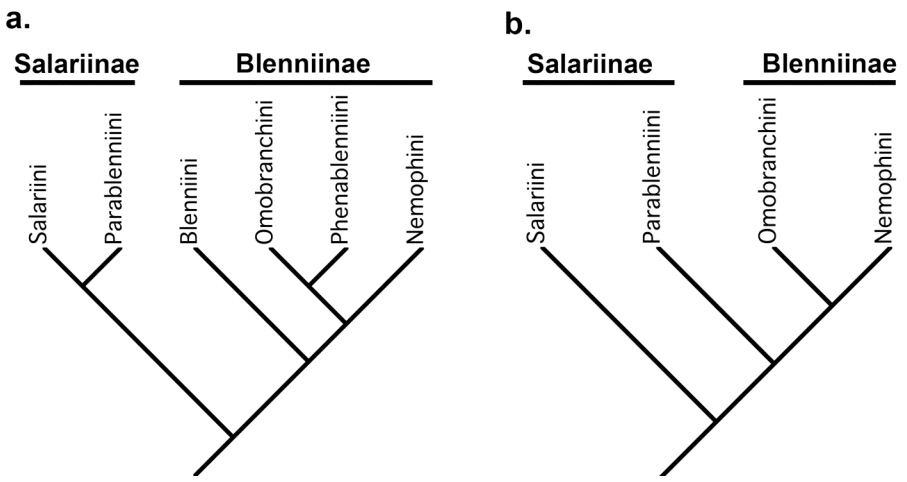

Fig 1.5 Phylogenetic relationships of the tribes of Blenniidae based on (a) morphological evidence by Smith-Vaniz and Springer (1976) and Williams (1990), and (b) molecular data in this study.

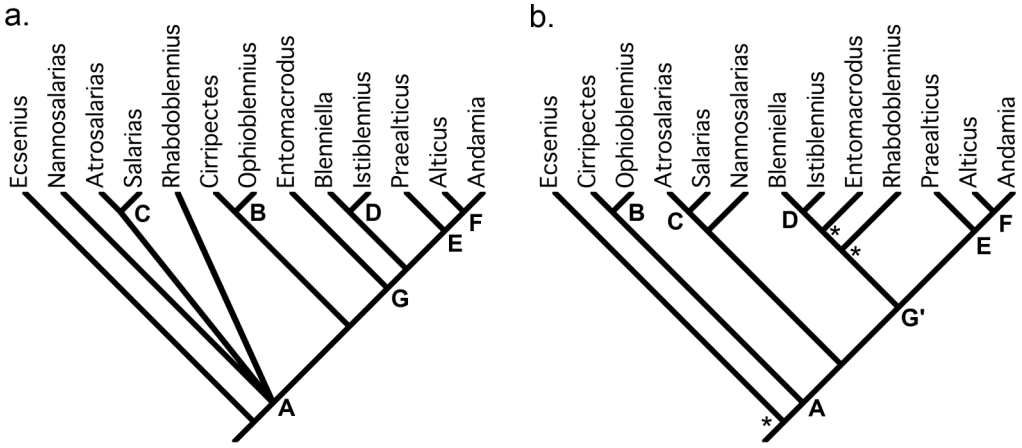

Fig 1.6 Generic relationships of the blenniid tribe Salariini. (a) Hypothesized relationship based on Smith-Vaniz and Springer, 1971 and updated in Springer and Williams, 1994. (b) Consensus relationship based on Bayesian Inference, Maximum Likelihood, and Maximum Parsimony in this study. *= node not supported by Maximum Parsimony method. Nodes labeled with the same letters (A-G) in both (a) and (b) are identical. Node G' in (b) is different from node G in (a) by including *Rhabdoblennius*.

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CHAPTER 2

Phylogenetic relationships of a New World marine fish lineage (Family Chaenopsidae, Suborder Blennioidei) based on combined molecular and morphological data.

Abstract

The family Chaenopsidae is composed of 14 genera and 91 species with mostly Neotropical distributions. These reef-associated small fishes are known as tube blennies for occupying empty tests of invertebrates. The phylogenetic relationships of the Chaenopsidae have been hypothesized based entirely on morphological characters. This incorporates multiple molecular markers (mitochondrial DNA: COI; nuclear DNA: TMO-4C4, RAG1, Rhodopsin, and Histone H3) and morphological characters in reconstructing relationships of a blennioid family with dense taxon sampling (13 genera and 40 species). The total of 3354 base pairs and 145 morphological characters are combined to reconstruct the total-evidence based topology which is well resolved and mostly reflects the molecular signal. The monophyly of the subfamily Chaenopsinae and all chaenopsid genera are confirmed by both molecular and morphological data. However, the recent expansion of Chaenopsidae with the inclusion of three chaenopsid genera, *Stathmonotus*, *Mccoskerichthys* and *Neoclinus*, is not supported and needs further study within the context of a broader blennioid taxon sampling. Within the Chaenopsinae, four main clades are hypothesized with *Coralliozetus* (Clade 1) as sister to an unresolved polytomy of Clades 2, 3 and 4. Because both molecular and morphological data do not resolve this polytomy, it may represent a rapid radiation event. Additional genetic markers are needed to provide further insight into this and into the relationships among the six species of *Coralliozetus*, for which different genes and morphology support alternative topologies. Osteological characters related to an elongate head shape shared by *Hemiemblemaria* and the lineage of *Chaenopsis* and *Lucayablennius* may likely be a result of convergent evolution for free swimming.

Introduction

The family Chaenopsidae comprises a primarily Neotropical marine fish lineage including 14 genera and 91 species (Hastings, 2009; Hastings and Springer, 2009). The only exceptions are one Peruvian species *Emblemaria hudsoni* and one *Chaenopsis* species and nine northern Pacific *Neoclinus* species from California to Japan and Taiwan (Stephens, 1963; Hastings, 2000). Chaenopsids are commonly known as tube blennies, associated with their behavior of occupying empty tests of invertebrates, such as worms, barnacles, and mollusks (Stephens, 1963). These small (usually less than 50 mm) reef-associated fishes occur mostly in shallow waters. The closure of Panama Isthmus was proposed as a likely evolutionary mechanism in shaping the current amphi-American distribution with 38 species (42%) in the Pacific and 51 species (56%) in the Atlantic (Hastings, 2000; Hastings, 2009).

Chaenopsids are significant models for the study of ecology and evolution. They are site attached, often abundant (Thomson and Gilligan, 2002) and consequently readily observed. As a consequence, they have been the focus of numerous studies on species interactions, habitat selection, mate choice, and feeding behavior (Lindquist, 1980; Lindquist, 1985; Hastings, 1986; Hastings, 1988b; Hastings, 1988a; Hastings, 1992b; Clarke, 1999; Hastings, 2002). In addition, their life-history traits, such as possessing demersal eggs (Hastings, 1988a), short pelagic larval duration (Almany and Baldwin, 1996), near-shore development (Brogan, 1994), and limited adult dispersal ability increase the possibilities of genetic differentiation. Thus chaenopsids are ideal systems for studying historical evolutionary events (Bernardi et al., 2003; Riginos, 2005; Lin et al., 2009). Within the Chaenopsidae, the broad spectrum of sexual dimorphism (Stephens,

1963; Hastings, 1991), body size (maximum length: 25 mm in *Coralliozetus springeri*, 300 mm in *Neoclinus blanchardi*), aggressive displays (Hastings, 2001), courtship displays (Thomson et al., 2000), microhabitat use (Hastings, 2002), and feeding habits that include aggressive mimicry for prey approach in *Lucayablennius zingaro* and *Hemiemblemaria simulus* (Greenfield, 1972; Colin and Gomon, 1973; Böhlke and Chaplin, 1993) provide numerous opportunities for studying character evolution. Studies of these and other features of chaenopsids will be greatly facilitated by detailed knowledge of their phylogenetic relationships.

The taxonomic status of the chaenopsids has undergone several changes (reviewed by Stephens, 1963; Hastings and Springer, 1994) since the family was first described in 1865 (Gill, 1865). Recently, the Chaenopsidae was expanded to include the Chaenopsidae of Stephens (1963, 1970) and *Stathmonotus*, *Mccoskerichthys* and *Neoclinus* as its serial outgroups (Hastings and Springer, 1994). The systematics of the redefined Chaenopsidae were recently summarized by Hastings and Springer (2009). The Chaenopsidae are one of the six currently recognized families of the perciform suborder Blennioidei (Springer, 1993), along with the Blenniidae, Tripterygiidae, Dactyloscopidae, Labrisomidae, and Clinidae. The inter-family relationships of these blennioid lineages are incompletely resolved based on morphological characters (reviewed by Hastings and Springer, 2009) but are currently under study based on molecular data (Lin and Hastings, in prep.).

This study focuses on phylogenetic relationships within the Chaenopsinae. The Chaenopsinae includes 11 genera and 74 species and can be distinguished from non-chaenopsin blennioids by three unique synapomorphic characters: the mesopterygoid

(when present) is posterior rather than parallel to the ectopterygoid, one rather than two lobes of testicular tissue, a single-lobed testicular accessory gland (Hastings and Springer, 1994; Patzner and Lahnsteiner, 2009). They also are characterized by four homoplastic characters: two infraorbitals, a truncate ossification of the median fin spines, a broad posteriormost branchiostegal, and a single lateral ridge on the dorsal-fin pterygiophores (Hastings and Springer, 1994). Phylogenetic relationships among the chaenopsin genera and species were hypothesized based on morphological similarities (Stephens, 1963; Stephens, 1970; Acero, 1984) and later revised through the application of phylogenetic systematics (Hastings, 1990; Hastings, 1992c; Hastings, 1992a; Hastings and Springer, 1994; Hastings, 1997). Three monophyletic lineages within Chaenopsinae were proposed including the *Acanthemblemaria* clade (Hastings, 1990; Hastings, 1992a) with two genera and 23 species, the *Chaenopsis* clade (Hastings, 1992c) with five genera and 29 species, and the *Coralliozetus* clade (Hastings, 1997) with four genera and 22 species. The *Acanthemblemaria* clade was hypothesized to be sister to the remaining two clades albeit with little morphological support (Hastings and Springer, 1994).

This study is the first attempt in reconstructing the phylogenetic relationships of a blennioid lineage based on dense taxon sampling and a combined dataset of molecular and morphological characters. One mitochondrial and four nuclear markers were sequenced from forty chaenopsid taxa and combined with 145 morphological characters assembled from former studies. This total evidence-based phylogeny aims to test former hypotheses based on morphology and investigate parsimony-informative morphological characters.

Materials and methods

Taxon sampling

Molecular data for forty terminal taxa were collected to reconstruct the phylogenetic relationships of chaenopsids (see Table 1 for details of included species, their collection localities and disposition of voucher specimens). We covered the three main chaenopsin clades and included representatives of 13 out of 14 total genera. The only genus not included was the monotypic *Tanyemblemaria* described from a single specimen from Isla Perlas, Panama (Hastings, 1992c). Most samples were collected by snorkeling or SCUBA diving, preserved in the field with 95-100% ethanol and subsequently deposited in the Marine Vertebrate Collection, Scripps Institution of Oceanography. Two tissue samples are courtesy gifts from the University of Kansas Natural History Museum.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from muscle tissue with a Qiagen (Chatsworth, CA) QIAquick Tissue Kit by following the manufacturer's instructions. DNA sequences of one mitochondrial DNA marker, Cytochrome C Oxidase I (COI), and four nuclear markers, TMO-4C4, RAG1, Rhodopsin and Histone H3, were used to reconstruct the phylogenetic relationships. Primers used in amplifying sequences in polymerase chain reaction (PCR) and sequencing were given in Table 2. The PCR was performed on a Mastercycler EP Gradient S (Eppendorf, Hamburg, Germany) with the following conditions: 94°C for one minute for initial denaturing, 35 cycles of 94°C for 30 sec, 52-56°C for 45 seconds, and 72°C for 45 sec, follow by 72°C for 5 minutes as the

final extension. Resulting amplicons were purified with Exonuclease I (20U/μl, New England Biolabs) and Shrimp Alkaline Phosphatase (1U/μl, Roche) which can remove single-stranded DNA and unincorporated dNTPs. Sequencing was done in both directions with the amplification primers and DYEnamicTM ET dye terminator sequencing kit on an automated MegaBACE^{TM} 500 DNA sequencer (Amersham Biosciences Corp., Piscataway, NJ).

DNA sequence alignment and analysis

Sequences were assembled and edited with Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI), then aligned with CLUSTAL X (Thompson et al., 1997) and adjusted by eye in MacClade 4.07 (Maddison and Maddison, 1997). Nucleotide sequences were checked on NCBI database (http://www.ncbi.nlm.nih.gov/) for possible gaps and translated for possible stop codons as an indication of pseudogenes. Prior to phylogeny reconstruction, substitution saturation tests were performed in DAMBE 5.1.1 (Xia et al., 2003; Xia and Lemey, 2009) on first and second codons and third codon of each gene. Substitution number, including transition and transversion versus pairwise TN93 (Tamura and Nei, 1993) sequence distance plot were used to explore the degree of saturation present in the dataset. A plateau is expected with increasing distance if saturation has been reached. In addition, by comparing the Iss (Index of Substitution Saturation) with Iss.c (critical Iss value), dataset with significant larger Iss values than Iss.c, indicative of severe substitution saturation (Xia et al., 2003) were discarded for subsequent phylogenetic analysis. The Akaike Information Criterion (AIC) (Akaike, 1974) implemented in MrModeltest v2.2 (Nylander, 2004) was used to select the best-fit

evolutionary model for each marker. The General Time Reversible model (GTR) (Tavaré, 1986) was selected as the best-fit nucleotide substitution model by AIC for COI1+2, TMO-4C4, RAG1 and Histone H3. The Hasegawa-Kishino-Yano 1985 model (HKY85) (Hasegawa et al., 1985) was selected for Rhodopsin. The evolutionary divergence of each gene between four hypothesized geminate species pairs across the Isthmus of Panama, *Acanthemblemaria exilispinus* and *A. betinensis*, *A. castroi* and *A. rivasi*, *Ekemblemaria myersi* and *E. nigra*, *Coralliozetus springeri* and *C. cardone* (Hastings, 2000), was estimated using PAUP 4.0b10 (Swofford, 2002) with uncorrected p-distance and individual best-fit model.

Morphological characters

One hundred forty five morphological characters were assembled from previous studies on various lineages of chaenopsids (Hastings, 1990; Hastings, 1992c; Hastings, 1992a; Hastings and Springer, 1994; Hastings, 1997). Only species with molecular data were included in the analysis. Character and character states are listed in Appendix Table 1 and the data matrix is given in Appendix Table 2. More detailed descriptions of characters are available in the original publications. Where new relationships were suggested by molecular data, we examined selected specimens in the SIO Marine Veretebrate Collection in search of morphological synapomorphies.

Phylogenetic analyses

For all analyses, five non-chaenopsin chaenopid taxa, *Stathmonotus culebrai*, *S. lugubris*, *S. stahli*, *Mccoskerichthys sandae*, and *Neoclinus blanchardi*, were specified as the outgroups. Because model-based tree methods are more accurate compared to parsimony methods when the phylogeny contains long branches or complex evolutionary

histories (Swofford et al., 1996), we used Bayesian Inference as the main method and compared the results with those from both Maximum Likelihood and Maximum Parsimony.

Bayesian Metropolis coupled Markov chain Monte Carlo (MCMCMC) estimation of phylogeny was carried out using MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). Bayesian Inference (BI) of phylogeny is based on a quantity called the posterior probability distribution of trees, which is the probability of a tree conditioned on the observations. For BI analyses, best-fit evolutionary models selected by MrModeltest and standard discrete model were applied to each genetic marker and morphological data, respectively. Two simulated independent runs were performed starting from different random trees. Each run comprised four chains (one cold and three heated) and was sampled every 100 generations. The total generation number was determined by the average standard deviation of split frequencies between the two independent runs while approached to zero and Effective Sample Size (ESS) larger than 200 in Tracer v1.4 (Rambaut and Drummond, 2007). The total generation numbers were 6×10^6 for COI 1st and 2nd codon, 2×10^6 for TMO-4C4 and Rhodopsin, 8×10^6 for RAG1, 4×10^6 for Histone H3, and 2×10^6 for morphological data. In all analyses, the sampled parameter values from Bayesian MCMC were evaluated in Tracer and the first 20% of generations from each run were discarded as burnin. The convergence of topologies after burnin was tested within and between runs by the AWTY (Are We There Yet) system (Wilgenbusch et al., 2004; Nylander et al., 2008). Samples from the stationary phase of the two runs were then pooled to produce one 50% majority rule consensus tree for each analysis. The MT+NUC dataset comprising the five genes was run for 10×10^6 generations with a partitioned

mixed-model Bayesian phylogenetic analysis, where the optimal molecular evolution model was applied to each of the five data partitions and model parameter values were "unlinked" among partition (Ronquist and Huelsenbeck, 2003). The combined molecular and morphological dataset (MT+NUC+morph) was run under the same settings and a standard discrete model was used for the morphological data.

Maximum likelihood (ML) tree searching for all datasets except morphology and MT+NUC+morph was executing through RAxML with 1000 non-parametric bootstrapping on Cipres Portal v1.14 (Stamatakis, 2006; Stamatakis et al., 2008). The MT+NUC dataset was partitioned by genes and substitution model parameters selected by MrModeltest were used. Parsimony analyses (MP) of individual gene, MT+NUC, morphological data, and MT+NUC+morph were conducted using PAUP 4.0b10 (Swofford, 2002). Heuristic searches to find the most parsimonious tree(s) were performed using tree bisection reconnection (TBR) branch-swapping from 1000 random-addition-sequence replicates to avoid entrapment in local optima. All sites were equally weighted and gaps treated as missing characters. Bremer or decay support on node represents steps required to be inconsistent compared to the shortest tree (Bremer, 1988). Bremer index of MT+NUC tree was calculated by executing the constrained analysis in PAUP with TreeRot v3 (Sorenson and Franzosa, 2007) generated command file. Additional replicate heuristic searches were added to better explore tree space and confirm that the same island(s) of trees were consistently found.

Feasibility of combining phylogenetic information of the five molecular markers and the morphological matrix was evaluated by reviewing each gene tree and looking for strongly supported but conflicting clades (Wiens, 1998). Clades were considered strongly

supported if both Bayesian posterior probability was ≥95% in BI method and bootstrap support was ≥70% in ML method (Leaché and Reeder, 2002).

Results

Sequence analysis

After trimming sequence ends, total sequences of 3,544 base pairs (bp) including 570bp in COI, 418bp in TMO-4C4, 1,491bp in RAG1, 737bp in Rhodopsin and 328bp in Histone H3 were aligned unambiguously. A single putative amino acid deletion (three nucleotides) was observed in TMO-4C4 of *Coralliozetus springeri* (FJ381597, three nucleotides after 285bp). No other indels or stop codons were observed in all markers for the remaining species. All sequences were deposited in the GenBank (Table 1). Among these five molecular markers, only the nucleotide substitutions of the third codon position of COI showed a plateau, with the increase of TN93 distance (Fig 1) and the observed Iss value (0.732) significantly higher than the Iss.c value (0.321, $p<0.005$). Therefore the third codon of COI was discarded from the matrix and the remaining 3,354bp were used for all subsequent phylogenetic analyses. The sequences of the five molecular markers provided different evolutionary information (Table 3). COI has the highest proportion of constant sites after removing the 3rd codon position (COI1+2, 83.68%) and TMO-4C4 has the lowest (55.98%). The five markers presented a range of parsimony informative sites from 11.32% to 36.36%. Among the five genetic markers, COI1+2 is the least compelling because it produced a mixture of outgroup and ingroup taxa and displayed no monophyletic grouping of any genera (Fig 3a). The less variable and parsimony-informative nuclear markers (Table 3), Rhodopsin and Histone H3, contributed to the

resolution of deeper nodes, such as the monophyly of the five outgroup taxa and the subfamily Chaenopsinae (Fig 3d and e). Instead, the more variable and parsimony-informative markers, TMO-4C4 and RAG1 provided most of the resolution power at shallower nodes (Fig 3b and c). RAG1 was especially informative in resolving the general topology of chaenopsids.

Phylogenetic relationships

For phylogenies reconstructed based on MT+NUC, overall similar topologies were found using BI, MP and ML approaches. Also, the majority of nodes were well resolved except within the genus *Acanthemblemaria* (Fig 2). Parsimony analysis yielded three equally parsimonious trees with 2,396 steps (CI: 0.47 and RI: 0.64). The score of the best ML trees found was 17202.2788. The majority of the nodes had Bayesian posterior probabilities higher than 95% and ML bootstrap values higher than 85%.

Strong evidence was found for the monophyly of the subfamily Chaenopsinae and all the currently recognized genera with the exception that the monotypic *Lucayablennius* was nested within *Chaenopsis* (Fig 2). Generic relationships were mostly resolved in the 85% majority con*sensu*s BI (Fig 5c). *Coralliozetus* was the sister clade to the remaining chaenopsin genera which formed an unresolved trichotomy. The first branch contained a single genus, *Acanthemblemaria*, the second branch contained two low diversity genera, *Cirriemblemaria* (1 species) and *Protemblemaria* (3 species), and the third branch contained the remaining five genera *Emblemariopsis*, *Emblemaria*, *Chaenopsis*, *Lucayablennius*, *Ekemblemaria* and *Hemiemblemaria* (Fig 5c).

The 50 consensus BI tree constructed based on morphological data was less resolved at the deeper nodes (Fig 3f) compared to MT+NUC (Fig 2). However, the majority of nodes within genera had posterior probabilities higher than 95%. The genus-level relationships based on morphology were very different from those based on molecular data (Fig 5c and d). *Chaenopsis* was grouped with *Lucayablennius*, *Hemiemblemaria* and *Emblemaria* serially and formed an unresolved polytomy with *Protemblemaria*, a lineage of *Acanthemblemaria* and *Ekemblemaria*, and a polytomous lineage of *Coralliozetus*, *Emblemariopsis* and *Cirriemblemaria* (Fig 5d).

The 50 consensus BI tree and MP tree based on the combined dataset MT+NUC+morph showed very similar, but better resolved, topology (Fig 4) compared to MT+NUC (Fig 2). Parsimony analysis yielded three equally parsimonious trees with 3,037 steps (CI: 0.51 and RI: 0.66). The phylogenetic relationships within *Acanthemblemaria* were more fully resolved, but the trichotomy at the deeper node within the Chaenopsinae remained unresolved. All the BI node supports of the MT+NUC+morph-based tree were higher than 90% except the clade including *Chaenopsis, Lucayablennius*, *Emblemaria*, *Ekemblemaria*, *Hemiemblemaria* and *Emblemariopsis* (70%) (Fig 4).

Conflicts among markers

There was no evidence of significant topological conflicts among the BI (Fig. 3), ML, and MP (topologies not shown for ML and MP analyses) methods for each dataset. However, two conflicting clades among the gene trees had strong support in both BI and ML analyses (Fig 3). The first conflict was the relationship between *Ekemblemaria* and *Hemiemblemaria*. *H. simulus* was nested within the genus *Ekemblemaria* based on TMO-

4C4 (Fig 3b). In contrast, Histone H3, morphology, MT+NUC and MT+NUC+morph all strongly supported the monophyly of *Ekemblemaria* (Figs. 2, 3 and 4). The second conflict was the relationship among the six *Coralliozetus* species. TMO-4C4 placed *C. boehlkei*, *C. rosenblatti*, *C. springeri*, and *C. cardone* into one clade and *C. angelicus* and *C. micropes* into another (Fig 6c). However, both RAG1 and Rhodopsin placed *C. springeri* and *C. cardone* into a sister clade to the remaining species (Fig 6d). In addition, morphological data placed *C. boehlkei*, *C. micropes,* and *C. rosenblatti* as the sister clade to the remaining three species (Fig 6b).

Divergence between geminate species

The sister relationship of four putative geminate species pairs across the Isthmus of Panama (Hastings, 2000) was strongly supported by MT+NUC (Fig 2), morphology (Fig 3f), and MT+NUC+morph (Fig 4). The uncorrected p-distance between *Acanthemblemaria exilispinus* and *A. betinensis*, *A. castroi* and *A. rivasi*, *Ekemblemaria myersi* and *E. nigra*, and *Coralliozetus springeri* and *C. cardone* ranged from 17.0 to 25.6 in COI, 0.4 to 6.1 in TMO-4C4, 0.8 to 3.1 in RAG1, 1.1 to 1.9 in Rhodopsin, and 0.0 to 1.6 in Histone H3 (Table 4). With individual best-fit model, the distance ranged from 61.0 to 1450.0 in COI, 1.3 to 12.8 in TMO-4C4, 0.6 to 4.0 in RAG1, 1.2 to 2.3 in Rhodopsin, and 0 to 1.9 in Histone H3 (Table 4).

Discussion

Total Evidence Tree

A prolonged debate has focused on how best to incorporate morphological and molecular data to provide "total evidence" in reconstructing phylogenies (e.g. Gura,

2000; Scotland et al., 2003; Wiens, 2004). With the rapid advance of molecular techniques, there is a significantly increasing ratio of available molecular data to morphological data for many groups (e.g. 28:1 in Su et al., 2008). Under the circumstances of discordant signals, the minor morphological data tend to be swamped by molecular data, thus morphology's contribution to the phylogenetic signal is diminished in total evidence analyses. This appears to be the case in this study on chaenopsid blennies. The highly mirrored topologies based on molecular data (MT+NUC) (Fig 2) and total evidence (MT+NUC+morph) (Fig 4) are quite different from the morphology-based topology (Fig 3f), suggesting that the molecular signal swamped the morphological signal. In fact, the ratio of available molecular characters to morphological characters in this analysis is 21.5:1 (4.4:1 for parsimony-informative characters only). We believe both molecular and morphology have strength in unraveling evolutionary history, therefore adopt a "total evidence" approach (Kluge, 1998) but discuss instances where the data sets are in conflict.

Monophyly of the Chaenopsinae and outgroup relationships

The monophyly of Chaenopsinae (= Chaenopsidae of Stephens 1963, 1970) is well supported (100% posterior probabilities) by the total evidence (MT+NUC+morph), molecular (MT+NUC), and morphological datasets. This clade shares six unique morphological apomorphies including two infraorbitals in males (character 41, Appendix Tables 1 and 2), incomplete ossification of both dorsal- and anal-fin spines (52), one ridge along the lateral margin of the proximal dorsal-fin pterygiophores (54), expanded posteriormost branchiostegal (74), single-lobed testis (138), and single-lobed testicular accessory organ (139).

The outgroup relationships of the Chaenopsinae are, however, unresolved. This study included three non-chaenopsin chaenopsid genera, *Stathmonotus*, *Mccoskerichthys,* and *Neoclinus* as outgroup taxa based on the morphological analysis of Hastings and Springer (1994). These three genera share eight hypothesized apomorphies with all chaenopsins, most of which are not unique to the Chaenopsidae (Hastings and Springer, 1994). Although all the outgroup taxa were grouped together in this study, a molecular phylogeny with a much broader taxa sampling including other blennioid families suggests that as a group they are not the closest relatives to Chaenopsinae (Lin and Hastings in preparation). Therefore the expansion of Chaenopsidae by Hastings and Springer (1994) needs to be carefully reviewed. This issue is related to the persistent unresolved relationships among the Chaenopsidae, Labrisomidae, Clinidae and Dactyloscopidae, as well as the questionable monophyly of Labrisomidae (reviewed by Hastings and Springer, 2009).

Monophyly and species relationships of chaenopsid genera

Four included genera (*Mccoskerichthys*, *Cirriemblemaria*, *Lucayablennius* and *Hemiemblemaria*) are monotypic, while only single species of *Neoclinus* and *Protemblemaria* were available for this study. Based on our total evidence analysis, the monophyly of all other currently recognized chaenopsid genera were fully supported (posterior probability = 100%) (Fig 4).

Stathmonotus. Three out of the six *Stathmonotus* species were included in this study. These three species were grouped together as a strongly-supported monophyletic group. They share twelve unique morphological character states including the presence of

a bony shelf on the metapterygoid (4), posterior margin of preopercle covered with skin (8), reduced numbers of procurrent rays (51), dorsal fin composed only of spines (53), fewer anterior anal-fin pterygiophores than associated hemal spines (59), presence of a ventrally projecting flange on the anterior margin of the first anal-fin pterygiophore (60), proximal epineural ribs contacting the vertebra (70), lateral line represented by a single tubular bone (91), no teeth on the palatine (111), no third pelvic-fin ray (124), a deep notch on the dorsal margin of upper lip (127), and a worm-like body shape (140). The sister relationship of *S. culebrae* and *S. lugubris*, two of the three members of the subgenus *Parastathmonotus* (Hastings and Springer, 1994), was well supported by molecular and morphological data. The latter includes expansion of the proximal end of the epipleural ribs (71), reduced numbers of cephalic sensory pores in the anterior infraorbital (96), posterior infraorbital (97), preopercular (98) and mandibular series (103), a lateral spur on lateral side of the articular (118), and the presence of a dark male morph (141).

Coralliozetus. The genus *Coralliozetus* includes six species and is quite distinctive both genetically (Fig 2) and morphologically (Fig 3f). There are eleven unique synapomorphies: the scapula is higher than it is wide (45), the proximal radials of all dorsal-fin pterygiophores pass through a hole in the base of the associated spine and are fused with the anterior margin of the pterygiophore (57), the posterior anal-fin pterygiophores lie anterior of a hemal spine (62), the branchiostegal anterior of the posteriormost is expanded and bladelike (75), the upper pharyngeal cartilage rod is reduced or absent (84), the outer row of palatine is short, bearing usually less than six teeth (113), the pelvic-fin spine is reduced (120), the pelvis is broad, its width

approximately equal to its length (123), dorsal margin of upper lip with a shallow notch (127), the lips of females rounded and protruding (128), and a fleshy pad near the mandibular symphysis (129).

All extant *Coralliozetus* species are included in our phylogenetic analyses and the species relationships are fully resolved by the total evidence dataset. Three pairs of sister species were found: *C. angelicus* and *C. micropes*, *C. boehlkei* and *C. rosenblatti*, *C. springeri* and *C. cardone*. The first pair is sister to the other two (Fig 4 and 6c). Morphologically, only the sister relationship of the *springeri* - *cardone* pair is supported by unique synapomorphic characters included in our morphological data matrix. These species have a slightly elevated anterior dorsal fin in females (64), a bladelike first pleural rib (72), vomerine teeth distributed in a straight row perpendicular to the long axis of body (106), and two supraorbital cirri (133).

The relationships proposed here based on total evidence are the same as the concatenated molecular data, but quite different from what have been hypothesized based on morphological characters except for the well-supported *springeri – cardone* pair (Fig 6). In 1963, Stephens first hypothesized the relationships within *Coralliozetus* and recognized the *boehlkei* - *rosenblatti* pair with *C. micropes*, *C. angelicus*, and *C. cardone* as its serial sister species (Stephens, 1963) (Fig 6a). The *springeri – cardone* pair was later hypothesized as geminate in the original description of *C. springeri* which is known from the eastern Pacific coast from Panama to Ecuador (Stephens et al., 1966) (Fig 6a). Hastings (1997) hypothesized the phylogenetic relationships of the *Coralliozetus* clade based on a parsimony analysis of 53 morphological characters. He hypothesized one clade with the *springeri – cardone* pair sister to *C. angelicus*, and second clade with *C.*

rosenblatti as a sister species to the species pair of *C. micropes* and *C. boehlkei* (Fig 6b). In general, these relationships were well supported by morphological synapomorphies. This was especially true for the sister-group relationship of *micropes* and *boehlkei*, a relationship supported by several features of the pharyngeal jaws (Hastings, 1997).

The discordant phylogenetic relationships among the *Coralliozetus* species found in this study were not only observed between molecular and morphological data but also among subsets of the molecular dataset. TMO-4C4 supports the topology based on total evidence (Fig 6c), but RAG1 and Rhodopsin show alternative relationships among the three pairs with the *springeri – cardone* pair sister to the other two (Fig 6d). COI and Histone H3 incompletely resolved these species level relationships. Although the alternative topology (Fig 6d) is supported by more genetic markers, choosing a species tree through "demographic vote" can be misleading because the real relationship is not guaranteed to be the most common one (Degnan and Rosenberg, 2006). Discordance of tree branching pattern among molecular markers has been well recognized when additional markers are used in estimating phylogenetic relationships (recently reviewed by Degnan and Rosenberg, 2009). Incomplete lineage sorting, horizontal gene transfer, gene duplication and loss, hybridization and recombination have been proposed as possible explanations for this discordance among markers (Degnan and Rosenberg, 2009). However, possible factors underlying the discordant gene trees of *Coralliozetus* and the real species relationship can only be identified with additional study. For example, incomplete lineage sorting is likely to be common where rapid radiation may have resulted in polymorphic genotypes in common ancestors that are retained in current species (Takahashi et al., 2001). If so, this phenomenon may be observed through

genotyping multiple individuals of each *Coralliozetus* species (Heckman et al., 2007). Although our proposed relationships of *Coralliozetus* species is based on total evidence, the discordance among genetic markers and the lack of supporting morphological characters indicate a complicated evolutionary history and the need for further examination.

Acanthemblemaria. The number of described species of *Acanthemblemaria* has more than doubled since Stephens' revision in 1963 and descriptions of new species are still ongoing (e.g. Lin & Galland in preparation). Currently, twenty species are recognized (Patzner et al., 2009), thirteen of which were included in this study. The monophyly of this genus is supported by combined molecular and morphological data including at least four unique synapomorphies: spines along the margin of the frontal ridge (16), serrations or spines present along the supraorbital margin of the frontal (22), spines or ridges at the lateral aspect of the first infraorbital (30), and a "keyhole" shaped dentary (107). Partial relationships within this most speciose genus of the Chaenopsidae (Patzner et al., 2009) were hypothesized based on morphological similarities (Stephens, 1963; Smith-Vaniz and Palacio, 1974; Rosenblatt and McCosker, 1988) and later further resolved with phylogenetic systematics by Hastings (1990). In this study, we retrieved a topology similar to that of Hastings (1990) except that *A. crockeri* is included within the "*hancocki* species group" as the sister to *A. macrospilus* and *A. hancocki* (Fig 4). The two hypothesized tranisthmian geminate species pairs, *A. castroi* and *A. rivasi*, and *A. betinensis* and *A. exilispinus* (Hastings, 1990), were also recognized in this analysis. Although well resolved by total evidence and morphology, molecular data showed limited power in supporting the monophyly of *Acanthemblemaria* (Bremer

support/Bayesian posterior probability/ML bootstrap: 3/59/99) and resolving species relationships (Fig 2). The unresolved nodes and limited support values based on molecular data suggest the need of additional sequence information. Relationships within this genus are currently under study by R. Eaton with broader taxon sampling and different genetic markers (pers. comm.).

Emblemariopsis. Only two of the twelve species of *Emblemariopsis* were available for this study, but their clustering provides tentative support for the monophyly of this entirely Western Atlantic genus. Two character states for *Emblemariopsis* have been regarded as synapomorphies: the distal portion of the neural spine of the penultimate vertebra is truncate (50) and the single epural is expanded proximally occupying the position of the neural spine (Hastings, 1997; Tyler and Tyler, 1997).

Ekemblemaria. Two of the three species of *Ekemblemaria* are included in this analysis and their sistergroup relationship is well supported by genetic data and a number of morphological characters. These include the presence of a simple frontal ridge (16), numerous pits along the midline of the frontal bones (18), a dark median fin with the distal margin unpigmented (143), clear unpigmented spots on the isthmus (144), and clear spots on the dorsal fin (145).

Emblemaria. Five of the sixteen species of *Emblemaria* were available for this study and these formed a well-supported monophyletic clade. Morphological characters supporting the monophyly of *Emblemaria* include lateral wing-like projections on the first three proximal dorsal-fin pterygiophores (58), slightly separated tooth patches on the vomer and palatines but bones adjacent (104), and males with a large pelvic-fin membrane (125). Relationships within *Emblemaria* are only partially consistent with one

Pacific clade and one Atlantic clade hypothesized by Stephens (1963, 1970). Instead, the eastern Pacific species *E. nivipes* is sister to a lineage with both Pacific and Atlantic clades (Fig 4). The later two clades have a well-developed fleshy flap along the first dorsal-fin spine of males (65). The two Pacific sister species *E. hypacanthus* and *E. piratica* share relatively thin infraorbitals in females (43) compared to other included *Emblemaria* species. However, no morphological characters were observed to support the Atlantic clade.

Chaenopsis. The monophyly of the three out of ten *Chaenopsis* species included in this study is well supported based on molecular and several unique morphological characters including short neural spines on caudal vertebrae (49), first basibranchial weakly to strongly fuses to the urohyal (78), the presences of a short ascending process of premaxilla ends before the first anterofrontal pore (115), and very elongate body shape (140). In this analysis, the Atlantic species *C. limbaughi* is sister to the two Pacific species *C. alepidota* and *C. schmitti*.

Four clades of Chaenopsinae

This study identified four major lineages within the Chaenopsinae. The first lineage (Clade 1) includes a single genus, *Coralliozetus,*and is sister to the remaining genera. While the monophyly of *Coralliozetus* is strongly supported morphologically (see above), currently there are no known morphological synapomorphies supporting the monophyly of it sister lineage. These remaining chaenopsin genera can be further divided into three lineages (Fig 4 and 5c). Two out of the three lineages are composed of few genera. Clade 2 includes only the genus *Acanthemblemaria* and is morphologically well-supported (see above). Clade 3 includes *Cirriemblemaria* and *Protemblemaria.* Its

monophyly is supported by at least two morphological features: two dorsal-fin pterygiophores inserted anterior to the first vertebra (55), and two pairs of supraorbital cirri on each eye (133). This relationship is identical to that proposed by Stephens (1963) when he placed his new species (*C. lucasana*) together with *Emblemaria bicirris* into his newly described genus *Protemblemaria*. The remaining genera, *Emblemariopsis*, *Emblemaria*, *Chaenopsis*, *Lucayablennius*, *Ekemblemaria* and *Hemiemblemaria*, are grouped as Clade 4, but with limited node support (70% posterior probability) and no known morphological synapomorphies.

The relationships between Clades 2, 3 and 4 are unresolved in this analysis (Fig 4 and 5c). The potential causes of lack of resolution may include insufficient data collecting and/or badly chosen analytical methods (soft polytomy), or it may reflect a true nearly simultaneous speciation event (hard polytomy) (Walsh et al., 1999). A true (hard) polytomy can be indicated by extremely short branches in independent gene trees of the same relationships and the lack of nonrandom topological congruence among independent gene trees (Poe et al., 2004). For this polytomy (Clades 2, 3 and 4) within the Chaenopsinae, the comparison among gene trees is compromised since the resolving power of individual genes is limited (Fig 3). However, the branch lengths of Clades 2, 3 and 4 to their most recent common ancestor are shorter compared to Clade 1 in the molecular-based tree (Fig 2). Chaenopsids may have increased potential for simultaneous radiation because of their higher-than-average mutational rates. For instance, the Kimura-two-parameter (K2P) percent differences between other fish geminate species that likely diverged at the final closure of Panama Isthmus range from 3.2 to 5.5 in COI, 0.3 in RAG1, and 2.6 in Histone H3 (reviewed by Lessios, 2008). The TN93 percent

differences between chaenopsin transisthmian geminates are much higher, especially in mitochondrial DNA, i.e. 23.6 to 41.8 in COI, 0.6 to 3.3 in RAG1, and 0.0 to 3.1 in Histone H3 (Table 4). Alternatively the ten-fold higher molecular divergence in COI of chaenopsids may indicate a much earlier divergence (i.e., the hypothesized geminates diverged well before the final closing of the isthmus (Knowlton and Weigt, 1998)). However, a more plausible explanation is less gene exchange thus higher molecular variance in chaenopsids as a consequence of their restricted life-history characters including demersal eggs (Hastings, 1988a), short pelagic larval duration (Almany and Baldwin, 1996), nearshore early larval development (Brogan, 1994), and tube-dwelling adults with limited mobility (Stephens, 1963). As similar rapid radiation revealed by unresolved polytomy was hypothesized for another blennioid lineage *Tripterygion* (Family Tripterygiidae) with similar life history features (Carreras-Carbonell et al., 2005).

The topology of the Chaenopsinae proposed by our total evidence analysis is very different from earlier phylogenetic hypotheses of chaenopsid relationships based on morphology. Stephens (1963, 1970) proposed two almost symmetrical clades within the Chaenopsinae by comparing a few focal characters, such as nasal bones and cranial sensory pores, with clinids and labrisomids representing presumed ancestral states. He proposed one clade with *Ekemblemaria*, *Coralliozetus*, and *Protemblemaria* as serial sister genera to *Acanthemblemaria*; and a second clade with *Lucayablennius*, *Hemiemblemaria*, *Emblemaria*, and *Emblemariopsis* as serial sister genera to *Chaenopsis* (Fig 5a). Using phylogenetic systematics, Hastings hypothesized relationships of various lineages of chaenopsids (1990, 1992a, 1992c, 1994, 1997) and later combined and

expanded the topologies to the whole family (2000, Fig 5b). Hypothesized relationships are similar between Stephens' and Hastings' except the three most basal genera (*Coralliozetus*, *Protemblemaria*, and *Emblemariopsis*) of Stephens' two clades were drawn out as another clade in Hastings' topology, the *Coralliozetus* clade (Hastings, 1997). The remaining species of Stephens' two clades were identified as the *Acanthemblemaria* clade (Hastings, 1990; Hastings, 1992a) and the *Chaenopsis* clade (Hastings, 1992c), respectively (Fig 5b). In addition, two newly described genera, *Cirriemblemaria* and *Tanyemblemaria*, were assigned to the *Coralliozetus* and *Chaenopsis* clade, respectively (Hastings, 1992c; Hastings, 1997). Hastings' three-clade topology is recovered by our morphological dataset except *Protemblemaria* is not included in the *Coralliozetus* clade (Fig 5d). Although the monophylies of *Coralliozetus*- and *Acanthemblemaria*- clades are not recovered with our total evidence or concatenated molecular dataset, four out of the five genera in the *Chaenopsis* clade (*Tanyemblemaria* is not included in this study) are grouped together with the addition of *Ekemblemaria* (Fig 5c).

Generic relationships in Clade 4

Except for the sister relationship of *Emblemariopsis* to the remaining genera of Clade 4, all the generic relationships are well supported in the total evidence tree (Fig 4 and 5C). The remaining genera include four out of the five *Chaenopsis* clade genera (Stephens, 1970; Hastings, 1992c; Hastings, 2000) and *Ekemblemaria* as the sister genus to the monotypic *Hemiemblemaria*. One morphological synapomorphy, loss of the neural

spur, a lateral projection on the anterior portion of the neural arch (46) unites these genera compared to other chaenopsins.

Chaenopsis and *Lucayablennius.* The sister relationship of *Chaenopsis* and the monotypic *Lucayablennnius* is well supported by molecular and morphological characters including presence of foramen in the upper scapular arm (44), dorsal-fin pterygiophores with a thin sheet of bone anterior to the primary lateral ridge (56), three anal-fin pterygiophores anterior to the first hemal spine (61), an elongate urohyal (77), an unossified third hypobranchial (80), a broad and rectangular vomer (104), the pectoral fin inserted above the midline (119), the third pelvic-fin ray splintlike and shorter than the pelvic-fin spine (124), upper lip interrupted medially (127), and no cirrus on the anterior nostril (132). *Lucayablennius zingaro* (Böhlke, 1957) is distinctive and distinguishable from the *Chaenopsis* species with nine autapomorphic characters (Hastings, 1992c) reflecting its unique coloration and behavior as an aggressive mimic of its prey, *Coryphopterus* gobies (Greenfield, 1972; Colin and Gomon, 1973). However, we did not find comparable distinctiveness in the molecular data for *Lucayablennius* (Fig 2). This implies that the distinctive morphology and behavior of this mimetic species evolved without radical genetic shifts. Some of the unique characters of *Lucayablennius* are paedomorphic (Hastings, 1992c). Similar heterochronic changes have been responsible for rapid morphological evolution in a variety of species (Gould, 1977; West-Eberhard, 2003) including females of the chaenopsid genus *Coralliozetus* (Hastings, 2002).

Emblemaria, Chaenopsis and *Lucayablennius.* The sister relationship of *Emblemaria* to the lineage of *Chaenopsis* and *Lucayablennius* is well supported by total evidence (Fig 4) and molecular data (Fig 2). Unique shapes of pelvic fin rays are shared

by these three genera: threadlike or large membrane in males (125), and threadlike in females (126).

Ekemblemaria and *Hemiemblemaria.* The newly proposed sister relationship of *Ekemblemaria* and *Hemiemblemaria* based on total evidence makes the formerly morphology-based monophyletic clade including *Chaenopsis*, *Lucayablennnius*, and *Hemiemblemaria* paraphyletic (Stephens, 1963; Stephens, 1970; Hastings, 1992c). *H. simulus* has distinct dentition, general body shape and fin counts (Stephens, 1963) likely related to mimicking the parasite-picking bluehead wrasse, *Thalassoma bifasciatum*, for better prey access (Böhlke and Chaplin, 1993). Compared to other chaenopsins, *Chaenopsis* spp, *L. zingaro*, and *H. simulus* are relatively free swimmers and often hover above the substrate (Böhlke and Chaplin, 1993). In addition, they share several morphological similarities, most of which reflect their uniquely elongated head shape. These include a long glossohyal (76), presence of a secondary canal on the ventral side of preopercular sensory canal (100), lower jaw projecting anteriorly beyond the upper jaw (117), and palatine separated from the vomer by a space (104). If the sister relationship of *Ekemblemaria* and *Hemiemblemaria* proposed by total evidence is true, the above character states in *H. simulus* would be a result of convergent evolution with the lineage of *Chaenopsis* and *Lucayablennius* probably associated with their independently evolved active swimming behavior.

Two synapomorphic characters between *Ekemblemaria* and *Hemiemblemaria* have been hypothesized: the presence of simple frontal ridge (16) and pits all over the surface of frontal bones (18). In addition, two features uniquely common to these two genera are identified (PAH pers. obser.). First, both have the surfaces of most bones

densely pitted; this pitting is especially prominent on the infraorbitals, but is also present on most cranial bones and those of the pectoral girdle (Appendix Fig 1a). Second, in both the dorsalmost pectoral-fin radial is broad proximally rather than narrow as in other chaenopsids (Appendix Fig 1b).

Conclusions

One mitochondrial, four nuclear markers and 145 morphological characters were combined together in a total evidence analysis of the phylogenetic relationships within the fish family Chaenopsidae. This well resolved total-evidence tree mostly reflects the molecular signal rather than the morphological data. The monophyly of the subfamily Chaenopsinae and all chaenopsid genera are confirmed with both molecular and morphological data. However, the recent expansion of Chaenopsidae with the addition of the genera, *Stathmonotus*, *Mccoskerichthys* and *Neoclinus*, needs to be revisited with an analysis of a broader array of blennioid taxa, a project currently underway. Within the Chaenopsinae, instead of recovering the *Acanthemblemaria*-, *Chaenopsis*-, and *Coralliozetus*- clades as hypothesized in former morphological studies, an incompatible four-clade topology is proposed here, with the genus *Coralliozetus* (Clade 1) sister to the unresolved trichotomy of Clade 2, 3 and 4. Additional genetic markers will have to be sampled to resolve the relationships among *Coralliozetus* spp. as conflicts among the molecular markers and with morphology remain. Osteological characters related to elongate head shape shared by *Hemiemblemaria* and the lineage of *Chaenopsis* and *Lucayablennius* might be a result of convergent evolution for a relatively free swimming lifestyle.

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Table 2.1 Localities, voucher IDs and Genbank accession numbers for chaenopsid terminal taxa used for phylogenetic analysis. SIO: Marine Vertebrate Collection of Scripps Institution of Oceanography, KU: Natural History Museum of University of Kansas. Abb: Abbreviation.

Taxon	Abb.	Locality
Outgroup		
Neoclinus blanchardi Girard, 1858	Nebl	Redondo Beach, California
Mccoskerichthys sandae Rosenblatt & Stephens, 1978	Mcsa	Isla Montuosa, Panama
Stathmonotus culebrai Seale, 1940	Stcu	Taboquilla, Panama
Stathmonotus lugubris Böhlke, 1953	Stlu	Puerto Vallarta, Mexico
Stathmonotus stahli (Evermann & Marsh, 1899)	Stst	Carrie Bow Cay, Belize
Ingroup		
Acanthemblemaria clade		
Acanthemblemaria aspera (Longley, 1927)	Acas	San Blas, Panama
Acanthemblemaria betinensis Smith-Vaniz & Palacio, 1974	Acbe	Bahia Azul, Panama
Acanthemblemaria castroi Stephens & Hobson, 1966	Acca	Isla Bartolome, Galapagos
Acanthemblemaria chaplini Böhlke, 1957	Acch	Bahia Azul, Panama
Acanthemblemaria crockeri Beebe & Tee-Van, 1938	AccrS	La Paz, Mexico
Acanthemblemaria crockeri Beebe & Tee-Van, 1938	AccrN	Loreto, Mexico
Acanthemblemaria exilispinus Stepehens, 1963	Acex	Isla Taboga, Panama
Acanthemblemaria greenfieldi Smith-Vaniz & Palacio, 1974	Acgr	Carrie Bow Cay, Belize
Acanthemblemaria hancocki Myers & Reid, 1936	Acha	Playa Cocos, Costa Rica
Acanthemblemaria macrospilus Brock, 1940	AcmaM	Huatulco, Mexico
Acanthemblemaria macrospilus Brock, 1940	AcmaG	Espiritu Santo, Mexico
Acanthemblemaria rivasi Stephens, 1970	Acri	San Blas, Panama
Acanthemblemaria spinosa Metzelaar, 1919	Acsp	Carrie Bow Cay, Belize
Ekemblemaria myersi Stephens, 1963	Ekmy	Fonseca, El Savador
Ekemblemaria nigra (Meek & Hildebrand, 1928)	Ekni	Bahia Azul, Panama
Chaenopsis clade		
Chaenopsis alepidota (Gilbert, 1890)	Chal	Santa Catalina, California
Chaenopsis limbaughi Robins & Randall, 1965	Chli	Carrie Bow Cay, Belize
Chaenopsis schmitti Böhlke, 1957	Chsc	Isla Rabida, Galapagos
Emblemaria diphyodontis Stephens & Cervigón, 1970	Emdi	Laguna Grande del Obispo, Venezuela
Emblemaria hypacanthus (Jenkins & Evermann, 1889)	Emhy	Bahia de los Angeles, Mexico
Emblemaria nivipes Jordan & Gilbert, 1883	Emni	Cocos Island, Costa Rica
Emblemaria pandionis Evermann & Marsh, 1900	Empa	San Blas, Panama
Emblemaria piratica Ginsburg, 1942	Empi	Puerto Vallarta, Mexico
Hemiemblemaria simulus Longley & Hildebrand, 1940	Hesi	Florida keys, Florida
Lucayablennius zingaro (Böhlke, 1957)	Luzi	Carrie Bow Cay, Belize
Coralliozetus clade		
Cirriemblemaria lucasana (Stephens, 1963)	Cilu	Bahia Banderas, Mexico
Coralliozetus angelicus (Böhlke & Mead, 1957)	Coan	Isla Danzante, Mexico
Coralliozetus boehlkei Stephens, 1963	Cobo	Huatulco, Mexico
Coralliozetus cardone Evermann & Marsh, 1899	Coca	Soufrierre, St. Lucia
Coralliozetus micropes (Beebe & Tee-Van, 1938)	Comi	Loreto, Mexico
Coralliozetus rosenblatti Stephens, 1963	Coro	La Paz, Mexico
Coralliozetus springeri Stephens & Johnson, 1966	Cosp	Taboquilla, Panama
Emblemariopsis randalli Cervigón, 1965	Emra	Laguna Grande del Obispo, Venezuela
Emblemariopsis signifera (Ginsburg, 1942)	Emsi	Rio de Janeiro, Brazil
Protemblemaria bicirrus (Hildebrand, 1946)	Prbi	La Paz, Mexico

Table 2.1 Continued.

Voucher ID	COI	TMO-4C4	RAG1	Rhodopsin	Hstione H3
SIO 00-73-1	FJ381462	FJ381610	FJ381538	FJ381574	FJ381500
SIO 01-167-1	FJ381461	FJ381609	FJ381537	FJ381573	FJ381499
SIO 01-164-1	FJ381463	FJ381612	FJ381540	FJ381576	FJ381502
SIO 01-182-1	FJ381465	FJ381613	FJ381541	FJ381577	FJ381503
KU 236	FJ381464	N/A	FJ381542	FJ381578	FJ381504
SIO 01-9-1	FJ381429	FJ381579 [a]	FJ381505	FJ381543	FJ381466
SIO 03-141-1	FJ381430	FJ381580	FJ381506	FJ381544	FJ381467
SIO 02-89-1	FJ381431	FJ381581	FJ381507	FJ381545	FJ381468
SIO 03-141-1	FJ381432	N/A	FJ381508	FJ381546	FJ381469
SIO 07-120-3	FJ381434	N/A	FJ381510	N/A	FJ381471
SIO 03-82-3	FJ381433	FJ381582	FJ381509	FJ381547	FJ381470
SIO 03-142-1	FJ381435	FJ381583	FJ381511	FJ381548	FJ381472
SIO 03-147-1	FJ381436	FJ381584 [a]	FJ381512	FJ381549	FJ381473
SIO 03-3-1	FJ381437	FJ381585	FJ381513	FJ381550	FJ381474
SIO 01-48-1	FJ381439	FJ381586	FJ381515	FJ381551	FJ381475
SIO 03-79-1	FJ381438	FJ381587	FJ381514 [b]	N/A	FJ381476
SIO 01-9-1	FJ381440	FJ381588	FJ381516	FJ381552	FJ381477
SIO 03-147-1	FJ381441	N/A	FJ381517	N/A	FJ381478
SIO 01-170-1	FJ381450	FJ381598	FJ381527	FJ381562	FJ381489
SIO 03-141-1	FJ381451	FJ381599	N/A	FJ381563	FJ381490
SIO 00-9-1	FJ381442	FJ381589	FJ381517	FJ381553	FJ381479
SIO 03-149-1	FJ381443	N/A	FJ381518	FJ381554	FJ381480
SIO 02-83-1	FJ381444	FJ381590	FJ381519	FJ381555	FJ381481
SIO 06-276-1	FJ381452	FJ381600	FJ381528	FJ381564	FJ381491
SIO 06-52-1	FJ381453	FJ381601	FJ381529	FJ381565	FJ381492
SIO 01-165-1	FJ381454	FJ381602	FJ381530	FJ381566	FJ381493
SIO 01-9-1	FJ381455	FJ381603	FJ381531	FJ381567	FJ381494
SIO 01-182-1	FJ381456	FJ381604	FJ381532	FJ381568	FJ381495
SIO 05-2-1	FJ381459	FJ381607	FJ381535	FJ381571	FJ381497
KU 110	FJ381460	FJ381608	FJ381536	FJ381572	FJ381498
SIO 05-141-1	FJ381445	FJ381591	FJ381520	FJ381556	FJ381482
SIO 05-124-1	FJ381446	FJ381592	FJ381521	FJ381557	FJ381483
SIO 01-52-1	FJ381447	FJ381593 [a]	FJ381522	FJ381558	FJ381484
SIO 01-5-2	FJ381448	FJ381594	FJ381523	FJ381559	FJ381485
SIO 03-82-2	FJ381449	FJ381595	FJ381524	FJ381560	FJ381486
SIO 07-120-1	FJ381429	FJ381596	FJ381525	N/A	FJ381487
SIO 01-164-1	FJ381429	FJ381597 [a]	FJ381526	FJ381561	FJ381488
SIO 06-276-1	FJ381458	FJ381606	FJ381534	FJ381570	N/A
SIO 01-171-1	FJ381457	FJ381605	FJ381533 [b]	FJ381569	FJ381496
SIO 02-16-1	FJ381429	FJ381611	FJ381539	FJ381575	FJ381501

[a] Partial TMO-4C4 sequence. [b] Partial RAG1 sequence.

Table 2.2 Primers used for PCR amplification and sequencing.

Gene	Primer name	Sequence (5'--> 3')	Reference
Mitochondrial			
COI	Fish-F1	TCAACCAACCACAAAGACATTGGCAC	(Ward et al., 2005)
	Fish-R2	ACTTCAGGGTGACCGAAGAATCAGAA	(Ward et al., 2005)
Nuclear			
TMO-4C4	TMO-F2	GAKTGTTTGAAAATGACTCGCTA	(Near et al., 2004)
	TMO-R2	AAACATCYAAMGATATGATCATGC	(Near et al., 2004)
	TMO-F3	GTGAAGTGGTTCTGCAA	This study
	TMO-R3	GTGTACTCNGGRATRGT	This study
RAG1	Of2	CTGAGCTGCAGTCAGTACCATAAGATGT	(Holcroft, 2004)
	Or2	CTGAGTCCTTGTGAGCTTCCATRAAYTT	(Holcroft, 2004)
	Rag-F1	AGCAGGCTCATCCTGTCCAT	This study
	Rag-R2	GGGTGATGGAGTGCAGCACCATGTT	This study
Rhodopsin	RhodF	CCGTCATGGGCGCCTAYATGTTYYT	(Taylor and Hellberg, 2005)
	RhodR	CAGCACAGGGTGGTGATCATRCARTG	(Taylor and Hellberg, 2005)
Histone H3	H3a-L	ATGGCTCGTACCAAGCAGACVGC	(Colgan et al., 1999)
	H3b-H	ATATCCTTRGGCATRATRGTGAC	(Colgan et al., 1999)

Table 2.3 Summary of datasets, model and model parameters of DNA substitution obtained by Akaike Information Criterion (AIC) implemented in MrModeltest, and scores of MP analyses.

	Data set				
	COI (1^{st} and 2^{nd} codon)	TMO-4C4	RAG1	Rhodopsin	Histone H3
Sequence length (bp)	380	418	1491	737	328
Constant sites	318 (83.68%)	234 (55.98%)	1073 (71.97%)	599 (81.28%)	261 (79.57%)
MrModeltest					
DNA substitution model	GTR[b]	GTR	GTR	HKY85[c]	GTR
No. substitution types	6	6	6	2	6
Invariant site?	Yes	Yes	Yes	Yes	No
Substitution rates[a]	Γ distributed	Γ distributed	Γ distributed	Γ distributed	Γ distributed
Maximum parsimony					
Parsimony-informative	43 (11.32%)	152 (36.36%)	295 (19.78%)	101 (13.70%)	51 (15.55%)
CI/RI[d]	0.31/0.47	0.54/0.68	0.53/0.69	0.45/0.63	0.57/0.82

[a] Among-site rate variation.
[b] General time reversible model.
[c] Hasegawa-Kishino-Yano 1985 model.
[d] CI: Consistency Index; RI: Retention Index.

Table 2.4 Estimates of evolutionary divergence between geminate species at either side of the Central America. The number of base substitution per site (%) was analyzed with uncorrected p-distance and its best-fit model selected by AIC.

			p-distance				best-fit model				
Genus	E. Pacific	W. Atlantic	COI	TMO-4C4	RAG1	Rhodopsin	Histone H3	COI	TMO-4C4	RAG1	Rhodopsin	Histone H3
Acanthemblemaria	*exilispinus*	*betinensis*	17.2	4.6	0.8	1.9	0.3	63.2	10.3	0.6	2.3	0.3
Acanthemblemaria	*castroi*	*rivasi*	17.0	0.4	0.8	1.1	1.0	61.0	1.3	0.9	1.2	1.3
Ekemblemaria	*myersi*	*nigra*	17.5	1.4	N/A	1.9	0.0	60.4	3.4	N/A	2.3	0.0
Coralliozetus	*springeri*	*cardone*	25.6	6.1	3.1	1.4	1.6	1450	12.8	4.0	1.5	1.9

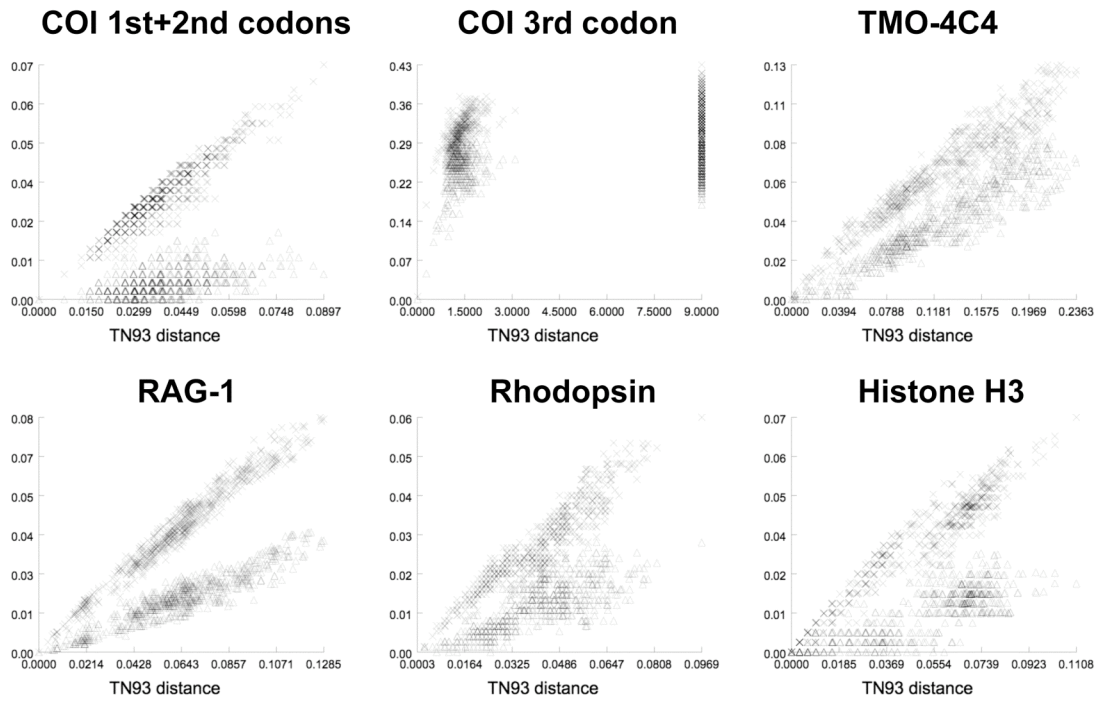

Fig. 2.1 Substitutions versus divergence plots for the mitochondrial COI gene (1st and 2nd codon, 3rd codon) and nuclear TMO-4C4, RAG1, Rhodopsin, and Histone H3 genes. The estimated number of transitions and tansversions for each pairwise comparison is plotted against the genetic distance calculated with the TN93 model. (×:transition, Δ: transversion)

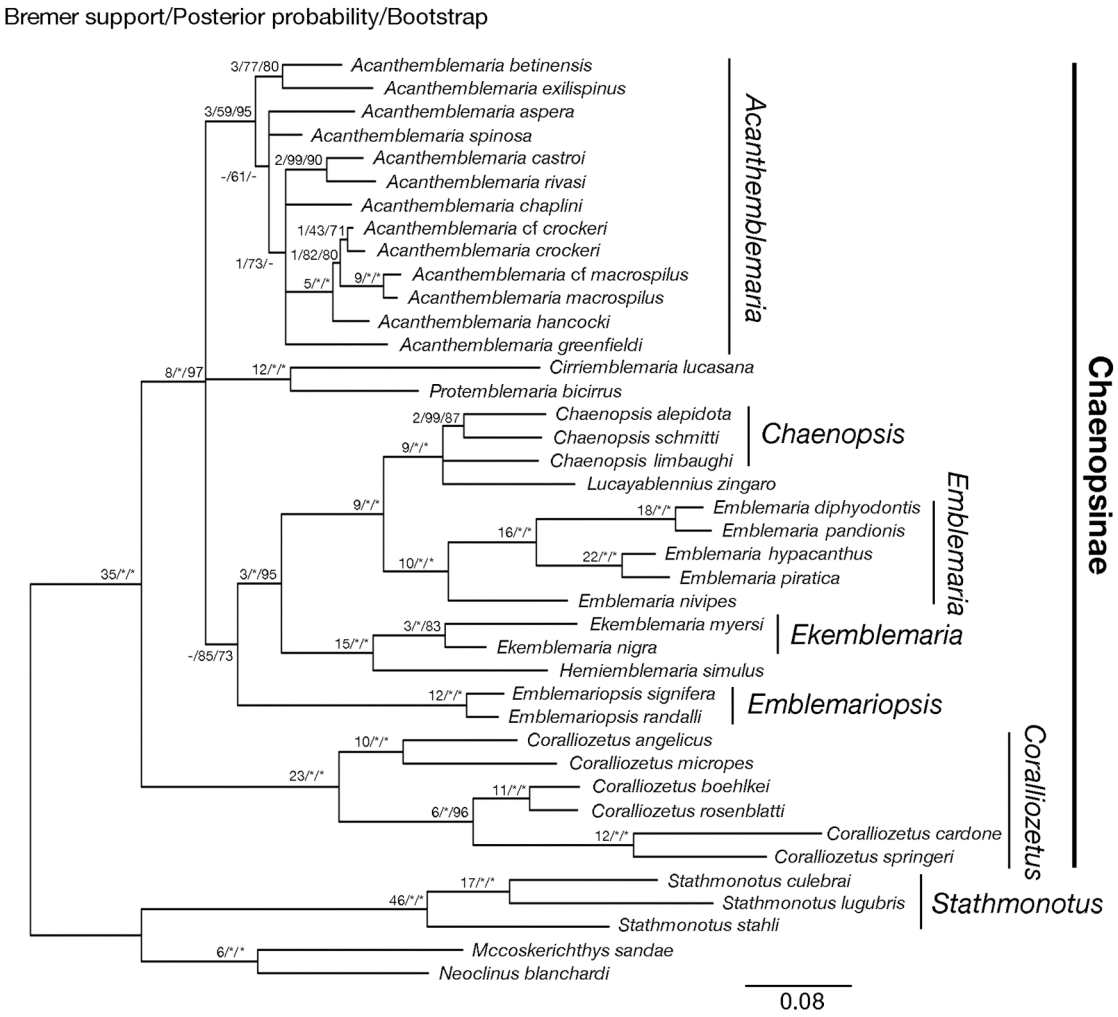

Fig. 2.2 50% majority consensus tree of the concatenated molecular dataset (MT+NUC) with Bayesian inference approach. Five non-chaenopsin chaenopsid taxa were used as outgroups. Numerals by nodes are Bremer support/posterior probability/bootstrap values. (*: 100; -: not supported)

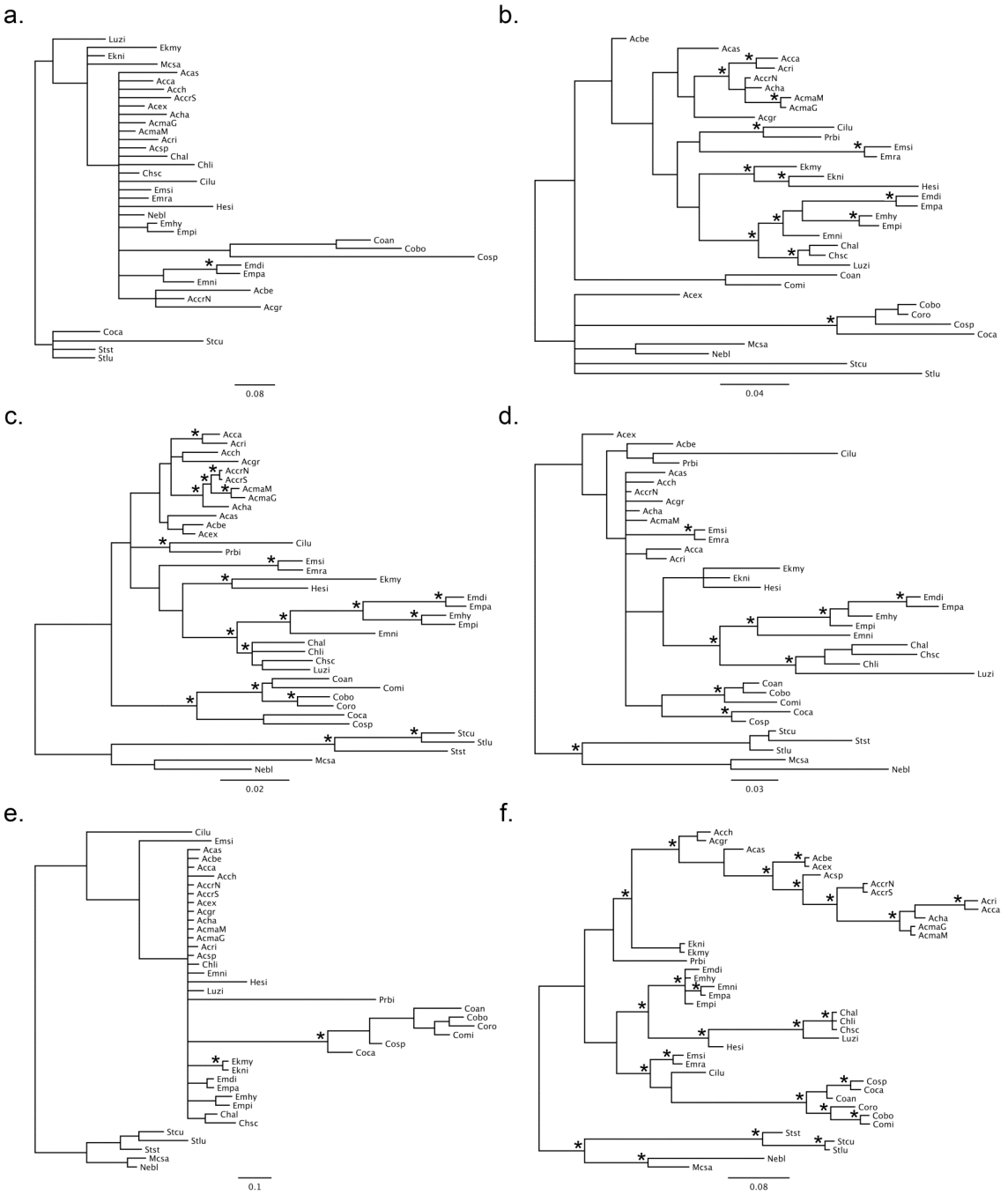

Fig 2.3 50% majority consensus trees of (a) COI1+2 (b) TMO-4C4 (c) RAG1 (d) Rhodopsin (e) Histone H3 and (f) morphological data with Bayesian inference approach. The abbreviations of terminal taxa are as listed in Table 1. Asterisks indicate strongly supported nodes with both Bayesian posterior probability ≥95% and ML bootstrap values ≥70% (only Bayesian posterior probability in f).

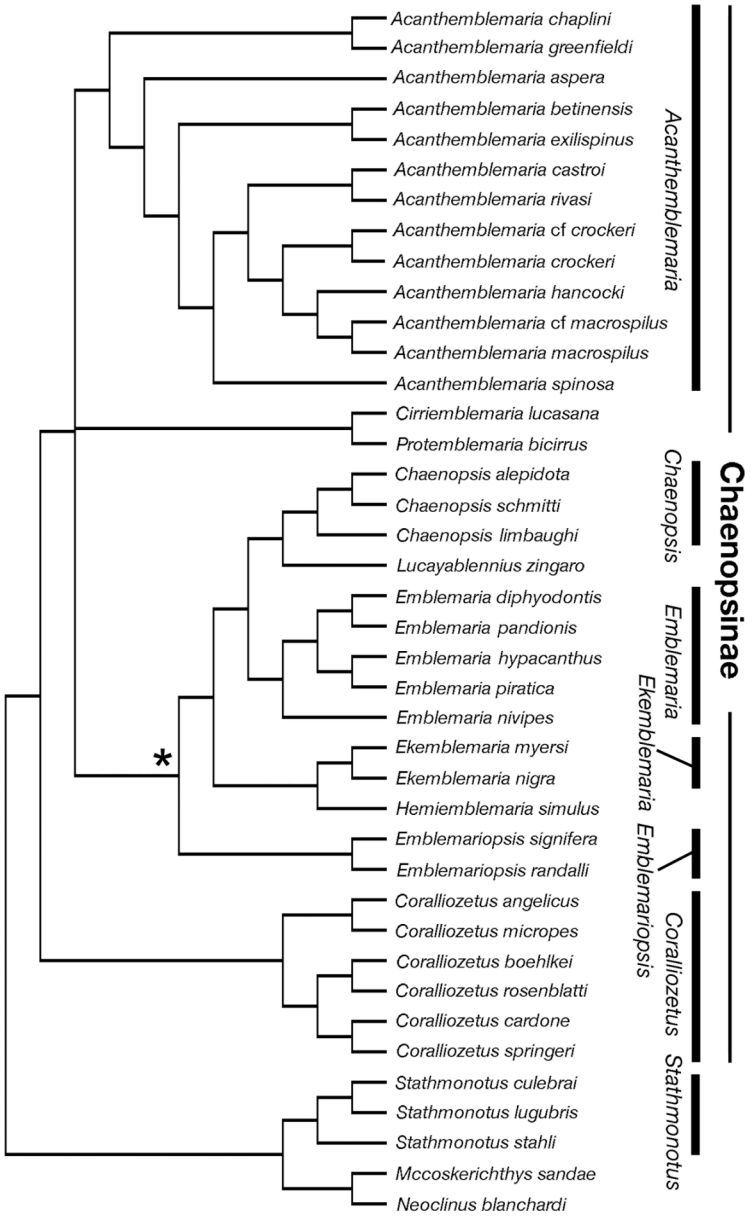

Fig. 2.4 Reconstructed phylogeny of Chaenopsidae using Bayesian inference approach based on total evidence (MT+NUC+morph). All the nodes are well supported with higher than 90% posterior probability except the one marked with * is 70%.

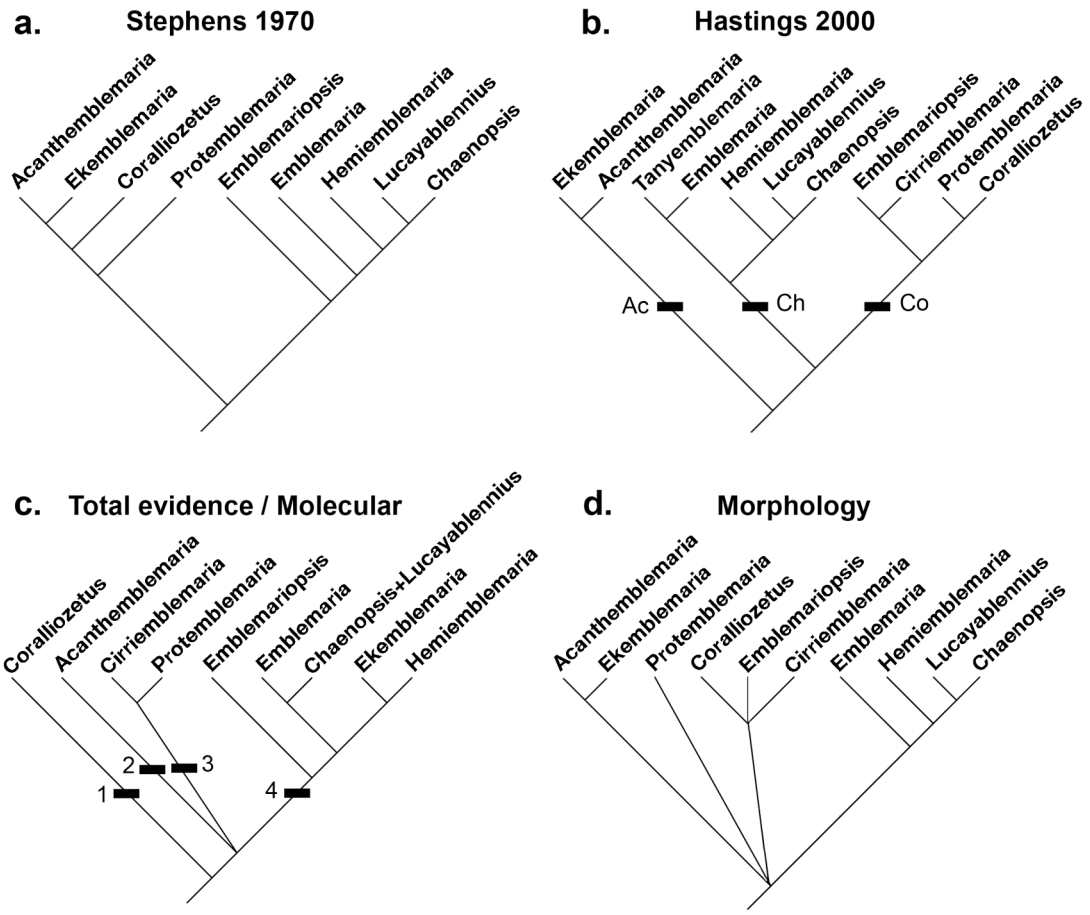

Fig. 2.5 Hypothesized genus-level relationships of Chaenopsinae by (a) Stephens 1970 and (b) Hastings 2000, and 85 % majority consensus Bayesian inference trees based on (c) total evidence (MT+NUC+morph) and concatenated molecular data (MT+NUC), and (d) morphology. Filled bars indicate major clades in the topology. Ac: *Acanthemblemaria* clade; Ch: *Chaenopsis* clade; Co: *Coralliozetus* clade; 1-4: Clade 1 to 4. Clade 4 in (c) has 70% node support in total evidence tree.

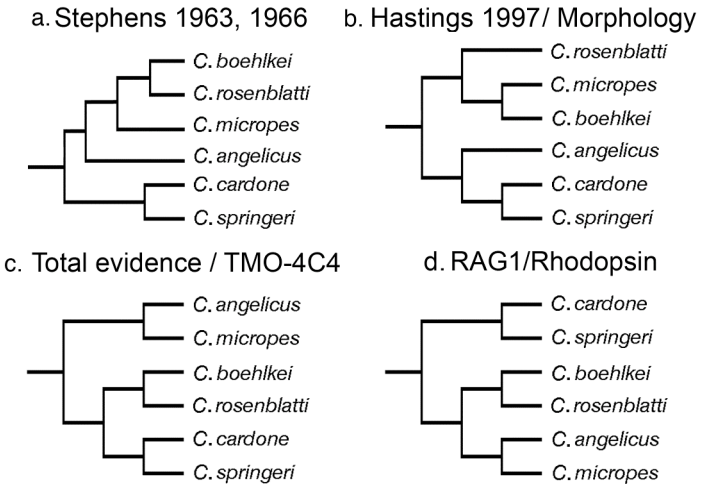

Fig. 2.6 Alternative species-level relationships within genus *Coralliozetus* hypothesized by (a) Stephens 1963 and 1966, (b) Hastings 1997 and morphological characters from this study, (c) total evidence and TMO-4C4, and (d) RAG1 and Rhodopsin.

Table 2.A1 List of morphological characters and character states for the Chaenopsidae. Primary citations describing characters are given in brackets [ ] followed by the character number in the original publication where: 90 = Hastings, 1990; 92 = Hastings, 1992; 92B = Hastings, 1992B; 94 = Hastings and Springer, 1994; 97 = Hastings, 1997. 98 = Smith et al., 1998.

1.	Mesopterygoid: (0) present; (1) absent. [97-35]
2.	Mesopterygoid position: (0) paralleling ectopterygoid; (1) posterior to ectopterygoid. [94-23]
3.	Metapterygoid flap: (0) absent to small; (1) large. [94-6]
4.	Metapterygoid shelf: (0) absent; (1) present. [94-33]
5.	Hyomandibular hook: (0) absent; (1) present. [94-16]
6.	Spenotic spur: (0) absent; (1) present. [94-7]
7.	Belophram of the basisphenoid: (0) present; (1) absent. [94-42]
8.	Posterior margin of preopercle: (0) free; (1) covered with skin. [94-37]
9.	Nasal bones (males): (0) separate; (1) fused medially. [94-10]
10.	Nasal bones (females): (0) separate; (1) fused medially. [94-10]
11.	Position of nasal bones: (0) over ascending processes; (1) lateral of ascending processes. [92-1]
12.	Nasal ridges: (0) absent; (1) present. [90-26]
13.	Anterior margin of nasals: (0) smooth; (1) spines or serrations present. [90-1]
14.	Number of nasal spines: (0) none; (1) two to three; (2) four or more. [90-2]
15.	Anterofrontal (AFO) spine: (0) absent; (1) papillose; (2) ossified. [90-3]
16.	Frontal ridge: (0) absent; (1) present, simple; (2) present, spines along margin. [90-36]
17.	Lateral extent of frontal ridge: (0) intersects middle of supraorbital region;(1) intersects at or posterior to upper insertion of infraorbital. [90-7]
18.	Surface of frontal bones: (0) smooth; (1) spines; (2) ridges; (3) pits. [92-37]
19.	Midline of frontals: (0) no spines; (1) spines present to midline. [90-8]
20.	Size of frontal spines: (0) short; (1) long. [90-11]
21.	Shape of frontal spines: (0) pointed; (1) rounded; (2) fused as ridges. [90-10]
22.	Margin of supraorbital: (0) smooth; (1) serrate; (2) spines present. [90-12]
23.	Angle of interorbital margin: (0) lateral; (1) inclined upward. [90-13]
24.	Central row of interorbital spines: (0) absent; (1) present, low; (2) present, on raised ridge. [90-14]
25.	Lateral row of interorbital spines: (0) absent; (1) present. [90-15]
26.	Orbital margin of lateral ethmoid: (0) smooth; (1) serrate; (2) spines present. [90-17]
27.	Spacing of lateral ethmoid spines: (0) even; (1) clustered. [90-18]
28.	Lateral margin of lateral ethmoid: (0) thin; (1) thick. [92-13]
29.	Position of first infraorbital and nasal bone: (0) separate; (1) touching. [92B]
30.	Lateral aspect of first infraorbital: (0) smooth; (1) spines or ridges present. [90-19]
31.	Orbital margin of first infraorbital: (0) smooth; (1) serrate; (2) spines present. [90-20]
32.	Ventral margin of lateral infraorbital: (0) smooth; (1) serrate. [90-21]
33.	Ventro-posterior extent of first infraorbital: (0) raised above jaw; (1) expanded downward over lateral portion of jaw. [90-22]
34.	Shape of anterior margin of first infraorbital: (0) thin; (1) expanded. [90-7]
35.	First infraorbital near juncture with lateral ethmoid: (0) no spine present; (1) spine present. [90-24]
36.	First infraorbital margin with nostril: (0) even; (1) ridges or spines present. [90-25]
37.	Lateral surface of second infraorbital: (0) smooth; (1) spines or ridges present. [90-26]
38.	Orbital margin of second infraorbital: (0) flat; (1) raised flange present. [90-27]
39.	Posterior margin of second infraorbital: (0) straight; (1) expanded near second posterior infraorbital sensory pore. [90-29]
40.	Posterior extent of second infraorbital: (0) not prolonged; (1) prolonged. [90-32]
41.	Number of infraorbitals: (0) four; (1) two. [94-26, 97-30]
42.	Size of infraorbitals (males): (0) thick; (1) thin. [97-32]

Table 2.A1 Continued.

43. Size of infraorbitals (females): (0) thick; (1) thin. [97-32]
44. Foramen in scapular arm: (0) absent; (1) present. [92-22]
45. Shape of scapula: (0) broad basally with a stout arm; (1) narrow. [97-20]
46. Neural spur: (0) absent; (1) present. [94-9]
47. Fifth hypural: (0) present; (1) absent. [90-32]
48. Epural: (0) sheathed in bone; (1) free, not sheathed. [94-30]
49. Neural spines on caudal vertebrae: (0) long; (1) short. [92]
50. Neural spine of penultimate vertebra: (0) normal; (1) truncate. [92]
51. Number of procurrent rays: (0) many; (1) few or none. [94-34]
52. Median fin spines: (0) ossified to tip; (1) ossification truncate. [94-27]
53. Dorsal fin elements: (0) spines and rays present; (1) only spines present. [94-38]
54. Lateral margin of dorsal-fin pterygiophores: (0) two ridges present; (1) one ridge present. [94-29]
55. Dorsal-fin pterygiophore pattern: (0) three inserted anterior of first vertebra; (1) two anterior of first vertebra; (2) one anterior of first vertebra; (3) one before second vertebra; (4) one before third vertebra. [94-39]
56. Shape of dorsal-fin pterygiophores: (0) no sheet of bone anterior to primary lateral ridge; (1) thin sheet of bone anterior to primary lateral ridge. [90-28]
57. Dorsal-fin pterygiophore relation to fin spines: (0) open, fingerlike projection extends through hole in spine base; (1) closed, projection fused anteriorly to pterygiophore in double ring joint. [97-18]
58. Anterior three dorsal-fin pterygiophores: (0) no projections; (1) lateral projection present. [92]
59. Number of anterior anal-fin pterygiophores: (0) greater than associated hemal spines; (1) equal to the associated hemal spines; (2) less than number of associated hemal spines. [94-31]
60. Ventrally projecting flange on anterior margin of first anal-fin pterygiophore: (0) absent; (1) present. [94-32]
61. Number of anal-fin pterygiophores anterior of first hemal spine: (0) one or two; (1) three. [92-31]
62. Anal-fin pterygiophore position: (0) all immediately posterior to a hemal spine; (1) those in posterior fin anterior to a hemal spine. [97-19]
63. Dorsal fin shape (males): (0) low, even; (1) elevated, sail-like; (2) slightly elevated; (3) spikelike. [90-49, 92-24, 97-40]
64. Dorsal fin shape (females): (0) low, even; (1) elevated; (2) slightly elevated; (3) spikelike. [90-49, 92-24, 97-40]
65. Dorsal-fin flap on first spine (males): (0) absent; (1) present. [92-15]
66. Dorsal-fin flap on first spine (females): (0) absent; (1) present. [97-27]
67. Dorsal-fin notch: (0) absent; (1) present. [92-27]
68. Membrane attachment posterior to anal fin (males): (0) to caudal peduncle; (1) near procurrent rays or contiguous with caudal fin. [97-36]
69. Membrane attachment posterior to anal fin (females): (0) to caudal peduncle; (1) near procurrent rays or contiguous with caudal fin. [97-26]
70. Proximal epineural ribs: (0) not contacting vertebra; (1) contacting vertebra. [94-35]
71. Proximal end of epipleural ribs: (0) narrow; (1) broad. [94-47]
72. Shape of first pleural rib: (0) narrow; (1) broad. [97-38]
73. Suture between anterior and posterior ceratohyals: (0) complex; (1) simple. [94-22]
74. Shape of posteriormost branchiostegal: (0) narrow; (1) broad. [94-28
75. Shape of next to posteriormost branchiostegal: (0) narrow; (1) broad. [97-16]
76. Basihyal length: (0) short; (1) long. [92-11]
77. Urohyal shape: (0) triangular; (1) elongate; (2) crescentic. [97-5]
78. First basibranchial and urohyal: (0) separate; (1) weakly to strongly fused. [92]
79. Relative thickness of branchial arches: (0) thick; (1) thin. [94-18]
80. Third hypobranchial: (0) ossified; (1) not ossified. [94-43]
81. Second basibranchial: (0) ossified; (1) not ossified. [94-44]

Table 2.A1 Continued.

82. Third basibranchial: (0) ossified; (1) not ossified. [94-19]
83. Shape of upper pharyngeal: (0) oval; (1) circular. [97-43]
84. Size of upper pharyngeal cartilage rod: (0) elongate; (1) reduced; (2) absent. [97-24]
85. Shape of fifth ceratobranchial (lateral aspect): (0) narrow wedge; (1) deep wedge. [97-41]
86. Size of fifth ceratobranchial: (0) elongate, rounded; (1) short, truncate. [97-42]
87. Ventral margin of fifth ceratobranchial: (0) even; (1) notch present. [97-45]
88. Posterior margin of fifth ceratobranchial: (0) thin; (1) thick. [97-46]
89. Relative size of third and fourth epibranchials: (0) third larger; (1) fourth larger. [97-47]
90. Insertion of second epibranchial: (0) lateral of pharygobranchial; (1) on pharyngobranchial. [97-48]
91. Lateral line on body: (0) present, several pores anteriorly; (1) single tube present; (2) absent. [94-3]
92. Otic pore: (0) present; (1) absent. [94-21]
93. Second frontal pore: (0) present; (1) absent. [92-4]
94. Second anterofrontal pore: (0) pair of pores present; (1) single, median pore present; (2) absent. [97-34]
95. Second nasal pore: (0) present; (1) absent. [94-55]
96. Commissural pore: (0) present; (1) absent. [94-53]
97. Number of anterior infraorbital pores: (0) three; (1) two. [94-51]
98. Number of posterior infraorbital pores: (0) four or more; (1) three; (2) two. [94-52]
99. Number of preopercular pores: (0) three or more; (1) two. [94-50]
100. Secondary canal on ventral side of preopercular canal: (0) absent; (1) present. [92-10]
101. Number of common pores: (0) one; (1) two or more. [90-56]
102. Mandibular pore 1B: (0) present; (1) absent. [94-20]
103. Mandibular pore 3: (0) present; (1) absent. [94-49]
104. Tooth patches on vomer and palatines: (0) contiguous; (1) slightly separate, but bones adjacent; (2) well separate, a gap between bones. [92-2]
105. Shape of vomer: (0) narrow, diamond-shaped; (1) broad, rectangular. [92-19]
106. Shape of vomerine tooth patch: (0) crescentic; (1) circuler; (2) two separate patches; (3) scattered, irregularly spaced teeth; (4) single straight row; (5) absent. [94-56]
107. Shape of dentary: (0) rami uniformly diverging; (1) “keyhole”, rami constricted posterior of outer row of enlarged teeth. [92B]
108. Symphysial teeth on dentary: (0) similar in size to surrounding teeth; (1) enlarged. [90-37]
109. Dentary symphysis: (0) thin, left and right sides not sutured; (1) thick, left and right sides sutured. [97-44]
110. Teeth on dentary rami: (0) one row; (1) two rows. [90-38]
111. Teeth on palatine: (0) one row; (1) two rows; (2) absent. [90-40]
112. Inner row of palatine teeth: (0) smaller than outer row; (1) equal to outer. [90-41]
113. Outer row of palatine teeth: (0) short, six or fewer teeth; (1) long, more than six teeth. [97-23]
114. Maxillary: (0) posterior tip visible when mouth closed; (1) posterior tip slides under infraorbital region, thus hidden when mouth closed. [94-60]
115. Ascending process of premaxilla: (0) long, to mid orbit; (1) short, ending before first anterofrontal pore. [92-33]
116. Jaw length (females): (0) short, not extending to level of posterior orbital margin; (1) long, extending to or beyond level of posterior orbit. [94-5]
117. Lower jaw length: (0) equal to upper jaw; (1) longer than upper jaw, tip protruding beyond snout. [92-8]
118. Lateral side of articular: (0) smooth, spur absent; (1) lateral spur present. [94-48]
119. Position of pectoral fin: (0) inserted near midline; (1) inserted above midline. [92-18]
120. Shape of pectoral fin (females): (0) rounded; (1) pointed. [97-33, 92-6]
121. Pelvic spur: (0) present; (1) absent. [90-47]
122. Size of pelvic spine: (0) long, thin; (1) short, shaft reduced or absent. [97-21]
123. Pelvis: (0) slender, width less than length; (1) broad, width equal to length. [97-22]

Table 2.A1 Continued.

124.	Third pelvic-fin ray: (0) well-developed; (1) reduced, splintlike, length less than pelvic spine length; (2) absent. [94-41]
125.	Pelvic-fin shape (males): (0) normal; (1) threadlike; (2) large membrane present. [98]
126.	Pelvic-fin shape (females): (0) normal; (1) threadlike. [92-6]
127.	Dorsal margin of upper lip: (0) even; (1) shallow notch present; (2) deep notch present; (3) divided. [94-36, 97-14]
128.	Lip shape (females): (0) flat; (1) rounded, protruding. [97-25]
129.	Fleshy pad ventrally near mandibular symphysis: (0) absent; (1) present. [97-15]
130.	Adductor mandibularis muscles on nape (males): (0) absent; (1) present. [94-17]
131.	Adductor mandibularis muscles on nape (females): (0) absent; (1) present. [97-31]
132.	Cirrus on anterior nostril: (0) present; (1) absent. [92-29]
133.	Number of supraorbital cirri: (0) one; (1) none; (2) two; (3) more than two. [97-50]
134.	Shape of supraorbital cirri: (0) pinnate; (1) palmate; (2) unbranched; (3) basal branches; (4) flaplike. [90-52]
135.	Length of primary supraorbital cirrus (males): (0) less than half orbital diameter; (1) half to one orbital diameter; (3) greater than one orbital diameter. [90-53]
136.	Length of primary supraorbital cirrus (females): (0) less than half orbital diameter; (1) half to one orbital diameter; (2) greater than one orbital diameter. [97-29]
137.	Papilla on dorsal margin of eye: (0) absent; (1) present. [90-55]
138.	Testis: (0) two lobes; (1) one lobe. [94-24]
139.	Testicular accessory organ: (0) paired located along each testicular lobe; (1) single lobe; (2) absent. [94-25]
140.	Body shape: (0) moderately elongate; (1) very elongate; (2) worm-like; (3) short. [92-34, 97-37]
141.	Dark morph (males): (0) absent; (1) present. [94-54]
142.	Band on chin (females): (0) no band; (1) single dark band; (2) light bands on dark background; (3) dark bands on light background; (4) dots on light background. [90-59]
143.	Median fin coloration: (0) variously pigmented to distal margin; (1) dark with distal margin unpigmented. [92B]
144.	Spots on isthmus: (0) absent; (1) present. [92B]
145.	Clear unpigmented spots on dorsal fin: (0) absent; (1) present. [92B]

Table 2.A2 Data matrix of morphological characters of the chaenopsid taxa included in the phylogenetic analysis.

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                                  10        20        30        40        50        60        70        80        90        100       110       120       130       140
                                  .         .         .         .         .         .         .         .         .         .         .         .         .         .
Acanthemblemaria aspera           0100110011000122010102100200112100010000100001100001010000000000110110001100001001000000002101100100010001100010110100001000000001100111011000000
Acanthemblemaria betinensis       0100110011001122110101101200111110011010100001000001010000000000110110000100001001000000002100100000110000110011110100001000000001100011011000000
Acanthemblemaria castroi          0100110011001122021021010110110110111011000010000010100000000000010000001000010010000000021001000001100021101111101000000000000011002110110000000
Acanthemblemaria chaplini         0100111011000012000??1000?701100000000001000011000010100000000001101100011000001001000000021001001001100001000101101000010000000011000111110000000
Acanthemblemaria crockeri         0100110011001222110002011210112100111100100001000001010000000000111000000100001001000000002100100100010002110111110100001000000001100111011000000
Acanthemblemaria cf crockeri      0100110011001222110002011210112110111100100001000001010000000000111000000100001001000000002100100100010002110111110100001000000001100111011000000
Acanthemblemaria exilispinus      0100110011001122110101101200111110011010100001000001010000000000110110000100001001000000002100100000110000110011110100001000000001100011011000000
Acanthemblemaria greenfieldi      0100111011000012000??2000?701100000000001000011000010100000000001101100011000001001000000002100100100010001100010110100001000000001100011111000000
Acanthemblemaria hancocki         0100110011001222111100101121011111101111100100001000001010000000000010000001000010010000000021001000001100021000111101000000000000011002000110010000
Acanthemblemaria cf macrospilus   0100110011001222111001021210111110111100100001000001010000000000010000001000010010000000021001000000100021000111101000000000000011002110110010000
Acanthemblemaria macrospilus      0100110011001222111001021210111110111100100001000001010000000000010000001000010010000000021001000000100021000111101000000000000011002110110010000
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Acanthemblemaria spinosa          0100110011001222111020112001121101111101000010000010100000000001101100001000010010000000021011001000100001000111101000010000000011000110110000000
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Chaenopsis limbaughi              0100110000100000?00??00000?100000100000011110011100101010000101000011000110111110100000000211211010101021200000?1111101010011130011111???0111000000
Chaenopsis schmitti               0100110000100000?00??00000?100000100000011110011100101010000101000011000110111?0100000000211211010101021200000?1111101010011130011111???0111000000
Cirriemblemaria lucasana          1?001000000100000?00??00000?000000000000001010010100010111000000000101110001100001010000000021001101000100000000?1100000110000000010023110110000000
Coralliozetus angelicus           1?100000100000000?00??00000?000000000000001010111000101001000010010100000111020101101000000210211010001000000000100100000101100011110002200113030000
Coralliozetus boehlkei            1?100000100000000?00??00000?000000000000001010111010001010010000100101000011102010111211111121021101000100000000100?0100000101100011110002100110030000
Coralliozetus cardonae            1?100000100000000?00??00000?000000000000001010111100010100100001021010000111102010110100000021021101000100040000100100000101100011110022000113000000
Coralliozetus micropes            1?100000100000000?00??00000?000000000000001010111100010100100001101011000011102010112111111210211010001000000100?0100000101100011110002200110040000
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Coralliozetus springeri           1?100000100000000?00??00000?000000000000001010110100010100100001021010000111102010110100000021021101000100040000?0100000101100011110022000113000000
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Lucayablennius zingaro            0100110000100000?00??00000?100000100000011110011000101010000100000000001101101111000000002112100101010212000007110010101001130000112???0110000000
Mccoskerichtys sandae             0000110011000000?00??00000?000000000000000000011000000021000100000011000000000000000000000200000000000000001000?1101000000000000000033000021000000
Neoclinus blanchardi              0010017000000000?00??00000?000000000000000000000000000000010000000000000000000000000000000000000000000000000000000?1001000000000000000037110000000000
Protemblemaria bicirris           0110100011000000?00??00000?000000000000000010000101000101100000000011111000010020101100000000021121101010001000000000?1101000000000000001102011011002000
Stathmonotus culebrae             0001111111000000?00??00000?000000000000000010011000101040001100000071111010002011110000000011121112100117050000227000010010020020011017??000210000
Stathmonotus lugubris             0001111111000000?00??00000?000000000000000010011000101040001100000071111010002011110000000011121112100117050000227000010010020020011004000000200000
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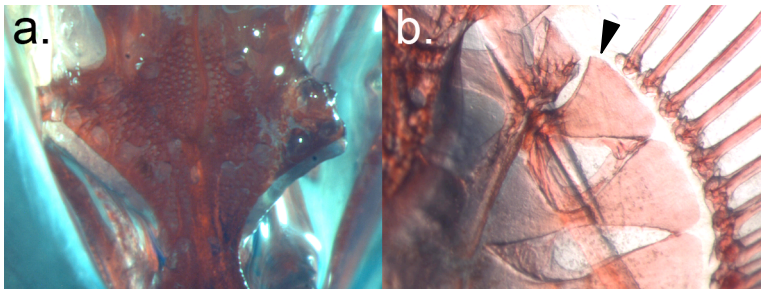

Fig. 2.A1 Two synapomorphic characters shared by *Ekemblemaria* and *Hemiemblemaria*. (a) dorsal view of densely pitted neurocranium of *Ekemblemaria myersi* (PAH8222); (b) lateral view of left pectoral girdle of *Hemiemblemaria simulus* (UMML15055); arrow indicates the dorsalmost radial.

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CHAPTER 3

Colour variation is incongruent with mitochondrial lineages: cryptic speciation and subsequent diversification in a Gulf of California reef fish (Teleostei: Blennioidei).

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Colour variation is incongruent with mitochondrial lineages: cryptic speciation and subsequent diversification in a Gulf of California reef fish (Teleostei: Blennioidei)

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Abstract

The Gulf of California endemic reef fish, *Acanthemblemaria crockeri* (Blennioidei, Chaenopsidae), reportedly has two colour morphs, one with melanic lateral spots ('Gulf' morph) and one with orange spots ('Cape' morph). In this study, we recorded colour morph in both males and females and collected mitochondrial DNA sequence data for cytochrome *c* oxidase I (COI) and tRNA-Pro/D-loop of specimens from throughout the Gulf to explore the genetic basis of the colour morphs. Two highly divergent (HKY + I distance = 11.9% for COI), reciprocally monophyletic lineages were identified, consistent with the presence of two parapatric species. A 30-km gap between the distributions of mitochondrial lineages roughly corresponds to a hypothesized former seaway across the Baja California peninsula north of La Paz, although the estimated divergence time (1.84 million years ago) is more recent than the hypothetical seaway (3–4 million years ago). Surprisingly, the distribution of mitochondrial species is not congruent with the distribution of either male or female colour morphs. Our analysis also revealed significant population differentiation within both species and no shared haplotypes among populations. The northern Gulf species includes four populations (NB, CB, NM and CM) corresponding to northern and central Baja and northern and central mainland sites, while the Cape species includes two populations (SB and SM) corresponding to the Baja and mainland sides of the southern Gulf. The NB/CB division corresponds to a hypothesized Plio–Pleistocene mid-peninsular seaway. The level of genetic divergence documented in this lineage is extraordinary for a marine fish with a pelagic larval stage within a semi-enclosed basin.

Keywords: browncheek blenny, Chaenopsidae, colour morphs, population structure

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Introduction

Cryptic or sibling species are common across taxa in marine environments (Knowlton 1993; Miya & Nishida 1997). Even for those without apparent morphological differences, genetics can often provide a useful tool to distinguish them (Palumbi 1996; Belfiore *et al*. 2003; Rocha *et al*. 2007). Mitochondrial DNA (mtDNA) markers are especially useful because of their matrilineal inheritance, absence of recombination, and higher evolution rate compared to nuclear DNA (reviewed by Avise 2004). For example, several cryptic species have been identified using mtDNA cytochrome *b* in the globally distributed fish genus *Albula* (bonefish) which has highly conserved morphology and ecology (Colborn *et al*. 2001; Pfeiler *et al*. 2008). Cryptic species may also provide unique insights into the underlying mechanisms of speciation that have drawn much attention from evolutionary biologists. The two main problems of studying speciation are identifying the isolation barriers that restrict gene flow and the evolutionary forces that produce the barriers (Coyne & Orr 2004). Absolute geographical barriers to dispersal of marine organisms such as land masses have frequently been implicated in speciation, especially when they separate multiple species pairs with concordant differentiation patterns. Examples include the

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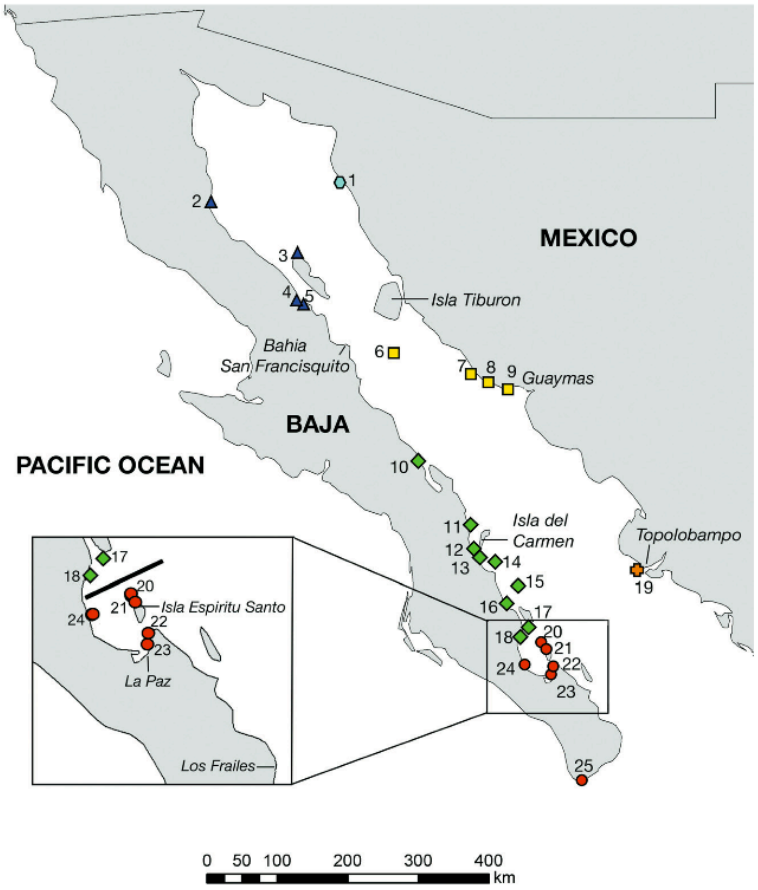

Fig. 1 Collecting sites in the Gulf of California. The site labels, latitude and longitude information are as listed in Table 1. The blow-up window enlarges the region around the boundary of the Gulf form and Cape form indicated by a line. Sites from the six biogeographic regions are labelled with hexagons (NM), triangles (NB), squares (CM), diamonds (CB), crosses (SM) and circles (SB).

Isthmus of Panama (Collins 1996; Bermingham *et al*. 1997; Knowlton & Weigt 1998; Lessios 2008) and the Baja California peninsula (Present 1987; Huang & Bernardi 2001; Stepien *et al*. 2001; Bernardi *et al*. 2003; Bernardi & Lape 2005). Less obvious marine biogeographic barriers, such as the Mona Passage in the Caribbean, can be inferred after the observation of large genetic gaps across taxa (Taylor & Hellberg 2006). On the other hand, there is increasing evidence showing that geographical barriers are unnecessary to build a species-level phylogenetic break (Irwin 2002; Rocha *et al*. 2007). Ecological traits (e.g. dispersal period, nearshore development of larva, habitat specialization, colour morphs), and intra- and interspecies interactions (e.g. competition, predation, mate choice) are potent evolutionary forces that may initiate speciation. These evolutionary forces not only act on species-level differentiation but also act at the intraspecific or population level (Irwin 2002; Maan *et al*. 2004; Bernardi 2005; Rocha *et al*. 2005; Langerhans *et al*. 2007; Mank 2007).

The Gulf of California, also known as the Sea of Cortés, is a semi-enclosed marginal sea of the eastern Pacific Ocean. The Gulf separates the Baja California peninsula from the mainland of Mexico along a 1070 km northwest–southeast axis and ranges in width from 100 to 200 km. The ichthyofauna of the Gulf includes over 900 species, most of which are of tropical origin (Hastings *et al*. 2009). Rocky reefs are ubiquitous and mostly continuous along the Baja coast but less extensive along the mainland coast except for the central Gulf (Thomson *et al*. 2000). Based on changes in fish community composition along the Gulf, three biogeographic subregions (northern, central and southern) were designated by Walker (1960). These were separated by a line from Bahia San Francisquito to the southern tip of Isla Tiburon between the northern and central Gulf and a line from La Paz to Guaymas between the central and southern Gulf (Fig. 1). In addition to the north–south biogeographic subdivisions, East (mainland coast)–West (Baja coast) subdivisions have also been proposed based on

significant population structure in one small Gulf reef fish (Riginos & Nachman 2001). These biogeographic differences possibly result from oceanographic heterogeneity such as water temperature and circulation patterns (Pegau *et al*. 2002; Marinone 2003), and also palaeogeography of the Baja peninsula (reviewed by Murphy & Aguirre-Léon 2002). For instance, genetic studies on marine fishes have implicated the Pleistocene mid-peninsula seaway [about 1 million years ago (Ma)] proposed from herpetofauna palaeobiogeography (Grismer 2000; Riddle *et al*. 2000; Murphy & Aguirre-Léon 2002) as a factor in the population-level differentiation between the upper Gulf (northern Gulf) and lower Gulf (central and southern Gulf) (Riginos 2005).

The patterns and underlying processes of genetic differentiation of marine organisms within the Gulf are not fully understood. Earlier genetic sampling of Gulf species is typically inadequate to reveal biogeographic regions within the Gulf or does not fully cover the distributional range of most species. Exceptions include intertidal snails that show no population structure within the Gulf (Hurtado *et al*. 2007) and an endemic triplefin fish (Riginos & Nachman 2001) that exhibits significant differentiation congruent with Walker's (1960) subregions. Although less thoroughly sampled, mtDNA data for some other Gulf fishes reveal population structure partially corresponding to Walker's subregions (Riginos & Nachman 2001; Riginos 2005), while others show little or no structure within the Gulf (Terry *et al*. 2000; Riginos & Victor 2001; Bernardi *et al*. 2003; Craig *et al*. 2006). Early life-history traits, such as pelagic larval duration, egg type (demersal/pelagic), and locations of larval development (nearshore/offshore), have been proposed as the underlying factors resulting in different patterns of genetic divergence in some of these species (Riginos & Victor 2001).

The blennioid family Chaenopsidae is well-represented in the Gulf of California. Chaenopsids are found primarily on rocky and coral reefs in tropical and subtropical areas of America (Stephens 1963; Hastings 2000). The browncheek blenny, *Acanthemblemaria crockeri* Beebe and TeeVan, is endemic to the Gulf of California and is found throughout the Gulf, from Puertocitos to Cabo San Lucas along the Baja California coast, and from Puerto Lobos to Isla San Ignacio de Farallon along the Sonoran and Sinaloan coasts of mainland Mexico (Thomson *et al*. 2000; Thomson & Gilligan 2002). This small reef fish (usually less than 3 cm) inhabits vacant invertebrate tests as shelters (Lindquist 1985; Thomson *et al*. 2000) and feeds primarily on mobile planktonic crustaceans (Kotrschal & Thomson 1986). Its breeding season ranges from May to July in the central Gulf but can be much extended in the southern Gulf (Mesnick 1996). During breeding seasons, males court from their shelters where females deposit eggs. Eggs are guarded by males until they hatch in 4–5 days (Hastings 1988). Larvae are likely planktonic for 22 to 25 days (Almany & Baldwin 1996) but may be retained near shore instead of drifting offshore (Brogan 1994). These life-history traits have the potential to increase self-recruitment, decrease gene flow and thus increase the possibility of population differentiation (Riginos & Victor 2001; Carreras-Carbonell *et al*. 2007).

Past studies have indicated that this may be the case for the browncheek blenny. *A. crockeri* exhibits prominent geographical variation along the Gulf in its ecology, behaviour, size, and morphology (Stephens 1963; Lindquist 1980; Mesnick 1996). Stephens (1963) reported two colour patterns, the northern 'Gulf' morph and southern 'Cape' morph which occur exclusively at the two ends of the Gulf but have a limited sympatric area without apparent hybridization from Isla Espiritu Santo (24°32′N) to Bahia Los Frailes (23°24′N; Stephens 1963). Stephens considered recognizing these as two distinct species based on the significant colour variation but declined to do so because other morphological differences were not evident. Lindquist (1980) examined the relationship of colour variation with geography and sex in greater detail, reporting a sympatric zone of the two colour morphs from Isla del Carmen (26°04′N) to Los Frailes. Nevertheless, the zone of sympatry comprised mostly Gulf males and Cape females, further obscuring the relationship between the two colour morphs.

Molecular techniques have been greatly advantageous in re-examining closely related species even when traditional methods are ambiguous (reviewed by Avise 2004). To solve the enigma of variation within *A. crockeri*, we analysed two mtDNA markers, COI and tRNA-Pro/D-loop, of individuals collected throughout the Gulf of California to explore the differentiation pattern and underlying evolutionary mechanisms. The goals of this study are to answer the following. What is the intraspecific differentiation pattern of *A. crockeri* within the Gulf of California based on molecular data? Is there a cryptic species as proposed by Stephens (1963)? If so, is it concordant with the two colour morphs and what are the possible mechanisms underlying the speciation? Are there more biogeographic subdivisions within the species? If so, do they correspond to formerly reported boundaries?

Materials and methods

Sampling of Acanthemblemaria crockeri

We obtained samples of *Acanthemblemaria crockeri* from throughout its geographical distribution within the Gulf of California (Fig. 1 and Table 1). *Acanthemblemaria macrospilus* from La Paz was used as the outgroup. Samples were collected by snorkelling or SCUBA diving using the anaesthetic quinaldine. Specimens were preserved in the field with 95–100% ethanol and subsequently deposited in the Marine Vertebrate Collection, Scripps Institution of Oceanography. To avoid sex-biased genetic data, we collected samples from both sexes.

Table 1 Study sites, biogeographic regions and sample sizes of *Acanthemblemaria crockeri* for this study. Voucher specimens of all are catalogued in the Marine Vertebrate Collection, Scripps Institution of Oceanography

Site	Locality	Latitude	Longitude	Biogeographic region*	COI	D-loop
1	Puerto Lobos	30°15′	112°51′	NM		4
2	Puertocitos	30°21′	114°38′	NB	1	1
3	Puerto Refugio	29°32′	113°34′	NB	2	6
4	La Gringa	29°02′	113°32′	NB	4	7
5	Isla la Ventana	28°59′	113°30′	NB	16	15
6	Isla San Pedro Mártir	28°22′	112°20′	NB	2	3
7	Isla San Pedro Nolasco	27°58′	111°23′	CM	2	1
8	Caleta Venecia	28°07′	111°18′	CM	1	4
9	Bahia San Carlos	27°57′	111°04′	CM	5	9
10	Mulege	26°47′	111°51′	CB	3	3
11	El Bajo	26°05′	111°19′	CB	5	3
12	Puerto Escondido	25°48′	111°18′	CB		4
13	Isla Danzante	25°45′	111°14′	CB	2	2
14	Islotes las Galeras	25°44′	111°02′	CB	2	2
15	Isla Santa Cruz	25°17′	110°43′	CB	2	2
16	Isla la Habana	25°07′	110°51′	CB	2	6
17	Isla San Francisco	24°49′	110°34′	CB	4	4
18	Punta Coyote	24°42′	110°41′	CB	2	2
19	Isla San Ignacio de Farallon	25°24′	108°54′	SM		6
20	Los Islotes	24°35′	110°24′	SB	4	7
21	Isla Espiritu Santo	24°33′	110°23′	SB	3	3
22	Isla Gaviota	24°17′	110°20′	SB	1	7
23	Tecolote	24°20′	110°18′	SB	1	1
24	La Paz Bay	24°23′	110°40′	SB	3	5
25	Cabo San Lucas	22°52′	109°53′	SB	1	8
	Total				68	115

*assigned by prior knowledge and supported by genotypes and pairwise distance values. NM, Northern Gulf mainland; NB, Northern Gulf Baja; CM, Central Gulf mainland; CB, Central Gulf Baja; SM, Southern Gulf mainland; SB, Southern Gulf Baja.

DNA sequencing

Total genomic DNA was extracted from muscle tissue with a QIAGEN QIAquick Tissue Kit by following the manufacturer's instructions. The polymerase chain reaction (PCR) of two mitochondrial DNA markers was used to analyse the genetic variation. The PCR was performed on a Mastercycler EP Gradient S (Eppendorf) with the following conditions: 94 °C for 1 min for initial denaturing, followed by 35 cycles of 94 °C for 30 s, 52–60 °C for 45 s, and 72 °C for 45 s. Universal primers FishF2 and FishR2 (Ward *et al*. 2005) and A and E (Lee *et al*. 1995) were used in amplifying 558 bp cytochrome *c* oxidase I (COI) and 422 bp tRNA-Pro/D-loop, respectively. Resulting amplicons were purified with Exonuclease I (20 U/μL, New England Biolabs) and Shrimp Alkaline Phosphatase (1 U/μL, Roche) to remove unwanted single-stranded DNA and dNTPs. Sequencing was done in both directions with the amplification primers and an additional internal sequencing primer for tRNA-Pro/D-loop (DloopF1: 5′-GGGGGTCAAGGACATATATG-3′) and DYEnamic ET dye terminator sequencing kit (Amersham Bioscience) on an automated MegaBACE 500 DNA sequencer (Amersham Biosciences Corp.). Sequences were assembled and edited with Sequencher 4.5 (Gene Codes Cop.), then aligned with Clustal_X (Thompson *et al*. 1997) using default settings and adjusted by eye in MacClade 4.07 (Maddison & Maddison 1997). All sequences are available from GenBank (Tables S1 and S2, Supporting information). We also surveyed four commonly used nuclear intron markers to provide independent evidence of intraspecific variation. Alpha-Tropomyosin, Gonadotropin-releasing hormone 3–2 (GnRH3–2), Lactate dehydrogenase B (LDH-B), and S7 first intron (S7–1) of 12 representatives from throughout the Gulf were sequenced using published primers (Chow & Hazama 1998; Friesen *et al*. 1999; Ruzzante *et al*. 2006).

Genetic divergence within A. crockeri

Partial sequence of a barcode gene, COI, was amplified from 68 specimens to test for possible cryptic species within *A. crockeri*. To increase the resolution of subsequent

diversification, more individuals were sampled and an additional sequence including partial tRNA-Pro/D-loop was amplified (Table 1).

Genealogical relationships within *A. crockeri* were constructed based on COI data by maximum- parsimony (MP) method implemented in PAUP 1.0b 10 (Swofford 2002) with *A. macrospilus* as the outgroup. Heuristic tree searches were conducted using 1000 replicates of random sequence addition. Statistical consistency of the nodes was estimated with bootstrap values. The differentiation level within *A. crockeri* was estimated by comparing with COI divergence between congeneric species pairs reported in recent barcode surveys (Hebert *et al*. 2003b; Ward *et al*. 2005) and phylogenetic study of the genus *Acanthemblemaria* (R. Eytan, unpublished). Uncorrected p-distance (the numbers of different nucleotides between sequences from the two lineages divided by the total number of nucleotides examined) and the best-fit model HKY + I (Hasegawa *et al*. 1985) selected by MrModeltest version 2.2 (Nylander 2004) were used as the distance model. The divergence time was estimated using the evolutionary rate as 4.94% p-distance per million years (R. Eytan, unpublished) based on the Panama-isthmus geminate species pair *A. betinensis* and *A. exilispinus* that hypothetically diverged at the final closure of the trans-isthmian seaway 3.5 Ma (Hastings 2000).

Several approaches were used to explore subsequent diversification within *A. crockeri* based on a faster evolving region, tRNA-Pro/D-loop. Hierarchical *F*-statistics in analyses of molecular variance (AMOVA) (Excoffier *et al*. 1992) based on Tamura–Nei distance method (TN93) (Tamura & Nei 1993) were performed in Arlequin version 3.11 (Excoffier & Schneider 2005) to provide evidence of population subdivisions by evaluating the amount of sequence variation hierarchically. Statistical significances of these analyses were determined on the basis of the distribution of values obtained from 10 000 permutations. The evolutionary divergence over sequence pairs between study sites was estimated with TN93 method in MEGA4 (Tamura *et al*. 2007). A tRNA-Pro/D-loop haplotype network was constructed based on parsimony criteria (Templeton *et al*. 1992) with 95% probability limit implemented in the TCS version 1.2 software (Clement *et al*. 2000). This network analysis is useful for intraspecific data in revealing multiple connections between haplotypes and indicating possible missing mutational connections. In addition, one specialized program, Migrate version 3.0.3 (Beerli & Felsenstein 1999; Beerli 2006) was used to estimate population size theta ($\theta = 2N_f\mu$, N_f: number of heritable copies in female population, μ: mutation rate per site per generation) and migration rate M ($M = m/\mu$; m: migration rate per generation). The number of migrants per generation (N_em, N_e: effective population size) can be inferred by multiplying M times θ of the recipient population. The Bayesian mode of Migrate using Markov chain Monte Carlo algorithm was conducted first with the default settings to obtain priors of parameters. Then we set these priors and ran three replicates of one single long chain with 1 million recorded steps and 1 million burn-in steps. Under the assumption of similar mutation rates, M and θ are comparable among populations.

Colour morphs of A. crockeri

We recorded sex and colour characters of most specimens used for molecular analysis. Twenty out of the 115 specimens have no vouchers available. For the remaining 95 voucher samples, we determined the sex and observed the left side of the bodies under a dissecting scope and from digital photographs. The sexes were determined by either the presence of a simple genital papilla in males or plicate villi surrounding the vent in females (Böhlke 1957). According to Stephens's (1963) definition, we categorized both males and females into the northern Gulf morph and the southern Cape morph. For males preserved in alcohol, the Gulf morph has eight to 11 black, dash-like markings along the midline, while the Cape morph is generally lighter with less distinct mottling on the body, and has a series of 12 ring-like spots on the midline and a second, more ventral series of spots anteriorly. For females, the Cape morph is similar to Cape males, while the Gulf morph has three to six dark brown bars on the anterior half of the body that become progressively smaller posteriorly. In addition to the female Gulf and Cape morphs recognized by Stephens (1963) and Lindquist (1980), we recognized two intermediate morphs due to ambiguities in assigning some females to the two described morphs. Intermediate 1 (I1) differs from the Gulf morph in having melanic spots instead of bars on the midline. Intermediate 2 (I2) differs from the Cape morph in having ring-like spots along the midline without a second (ventral) series.

Results

Genealogical relationships within *Acanthemblemaria crockeri* based on the two mitochondrial markers, COI and tRNA-Pro/D-loop, indicate the presence of two distinct lineages and subsequent diversification within both of them. Three nuclear intron markers we surveyed had few polymorphic sites. Among the 12 specimens from NB, CB and SB, there was no polymorphic site in the 145 bp GnRH3–2, four in the 367 bp α-Tropomyosin, and two in the 202 bp LDH-B. The fourth marker, 630 bp S7–1, had seventeen polymorphic sites but all the substitutions were unique to single individuals.

Maximum-parsimony analysis of 558 bp COI sequences (Table S1) with *A. macrospilus* (Accession no. EU626025) as the outgroup showed two well-supported monophyletic lineages corresponding to individuals collected from north and south of the line between southwest Bahia de la Paz

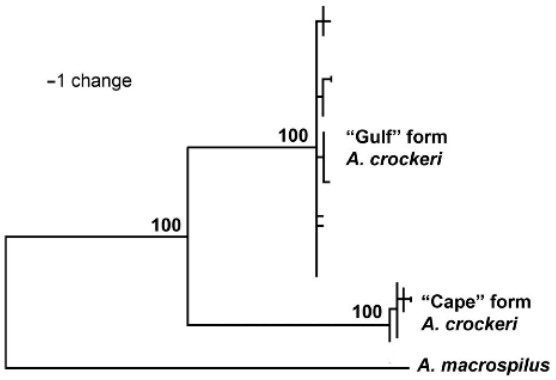

Fig. 2 Maximum parsimonious (MP) phylogeny based on 558 bp mtDNA COI data of 68 *Acanthemblemaria crockeri* with *A. macrospilus* as the outgroup. Numbers above the nodes are bootstrap values above 50. Gulf form and Cape form individuals are from north and south of the line between southwest Bahia de la Paz and south of Guaymas, respectively.

and south of Guaymas (Fig. 2). These two exclusive lineages will be referred to as the Gulf form (north of the line) and the Cape form (south of the line) in the following content. This lineage separation was also indicated by the haplotypes of 422 bp tRNA-Pro/D-loop (Table S2) which were too different to be placed in one network by TCS with 95% probability limit (Fig. 3). The COI divergence between the two lineages was estimated as 9.1% (uncorrected p-distance) and 11.9% (HKY + I distance). A divergence time of 1.84 Ma between the two lineages was further estimated using the evolutionary rate as 4.94% (p-distance) per million years.

tRNA-Pro/D-loop haplotypes of 115 *A. crockeri* and pairwise distance values among sites showed partitions according to both Walker's (1960) and Riginos & Nachman's (2001) biogeographic subdivisions. To simplify discussion in the following text, these biogeographic regions were assigned into NM, NB, CM, CB, SM, and SB corresponding to the Baja (B) and mainland (M) sides of the Gulf and the northern (N), central (C) and southern (S) regions of the Gulf. In the tRNA-Pro/D-loop haplotype networks, none of the haplotypes were shared by more than one population under the 95% probability limit (Fig. 3). Thirty-two haplotypes were found within the Gulf form with a general pattern of NB and CB haplotypes connected through CM. The four NM individuals only presented one haplotype that was connected to both CB and NB haplotypes. Twelve haplotypes were identified within the Cape form. There were two most frequent haplotypes in SB and these were connected to SM haplotypes with at least three mutational changes. In both the Gulf and Cape forms, pairwise Φ_{ST} values of TN93 distance among 18 study sites (25 minus 7 with less-than-three sample size) are mostly significant ($P < 0.05$) between but not within the six biogeographic groups (Table 2). In addition, pairwise TN93 distance values among sites within populations are at least twofold smaller (not available for single-site NM and SM) than between populations (Table 2). Although sites 2, 7, 13, 14, 15, 18 and 23 were not included in these pairwise divergence analyses due to low sample size, they can be assigned to the six groups easily both geographically and genotypically in the haplotype network (Fig. 3). Two ambiguities were the division between NM (site 1) and NB (sites 3–5), and the population assignment of site 6. The TN93 values within NB (0.6–0.7%) were not much smaller than the values between NM and NB (0.8–0.9%; Table 2). However, the single haplotype of NB was closer to CB (two mutational changes away from the main haplotype of CB) than NB (Fig. 3) and the Φ_{ST} values were all significantly different ($P < 0.05$) between sites from NM and NB (Table 2). Therefore, NM was considered as a separate population from NB. The population assignment of site 6 was ambiguous because the Φ_{ST} values between site 6 and sites from each of the four geographical neighbouring groups (NM, NB, CM and CB) can be either significant or insignificant. Based on the single-substitution difference in the haplotype network (Fig. 3) and the much smaller pairwise TN93 distance values (0.3–0.4) with CM, we assigned site 6 as CM. After subdividing the Gulf form into the four populations (NM, NB, CM, and CB) and the Cape form into the two populations (SM and SB) (Table 1), 87.76% ($\Phi_{CT} = 0.88$, $P < 0.001$) of the total TN93-distance variation can be explained between forms and 8.08% ($\Phi_{SC} = 0.66$, $P < 0.001$) among populations within forms.

The estimated demographic parameters of the three replicate runs of Migrate were very similar and no multiple peaks were found in the posterior distribution plots (data not shown), implying that Migrate had run long enough and our data coverage was sufficient. The acceptance ratios for all parameters were 1.000 and the Harmonic mean likelihood value was –1288.67. The mode of θ values for NM, NB, CM, CB, SM, and SB were 0.00025, 0.01525, 0.00275, 0.00675, 0.00025, and 0.00725, respectively. Because Migrate lacks power in estimating migration rates for very small populations ($\theta < 0.001$) or when migration rates are low (Beerli 2006), we only estimated the migration rates between NB, CM and CB in the Gulf form. The migration rates (M) were NB to CB: 22.5; NB to CM: 2.5; CB to CM: 2.5; CB to NB: 27.5; CM to NB: 2.5; CM to CB: 2.5. The estimated number of migrants per generation covered a range from 0.007 (corresponding to NB to CM and CB to CM) to 0.419 (corresponding to CB to NB).

Out of the 95 voucher specimens, 65 belong to the Gulf mitochondrial form and 30 to the Cape mitochondrial form (Table S3, Supporting Information). There are 36 males and 29 females of the Gulf form and 17 males and 13 females of the Cape form. The geographical distribution of *A. crockeri*

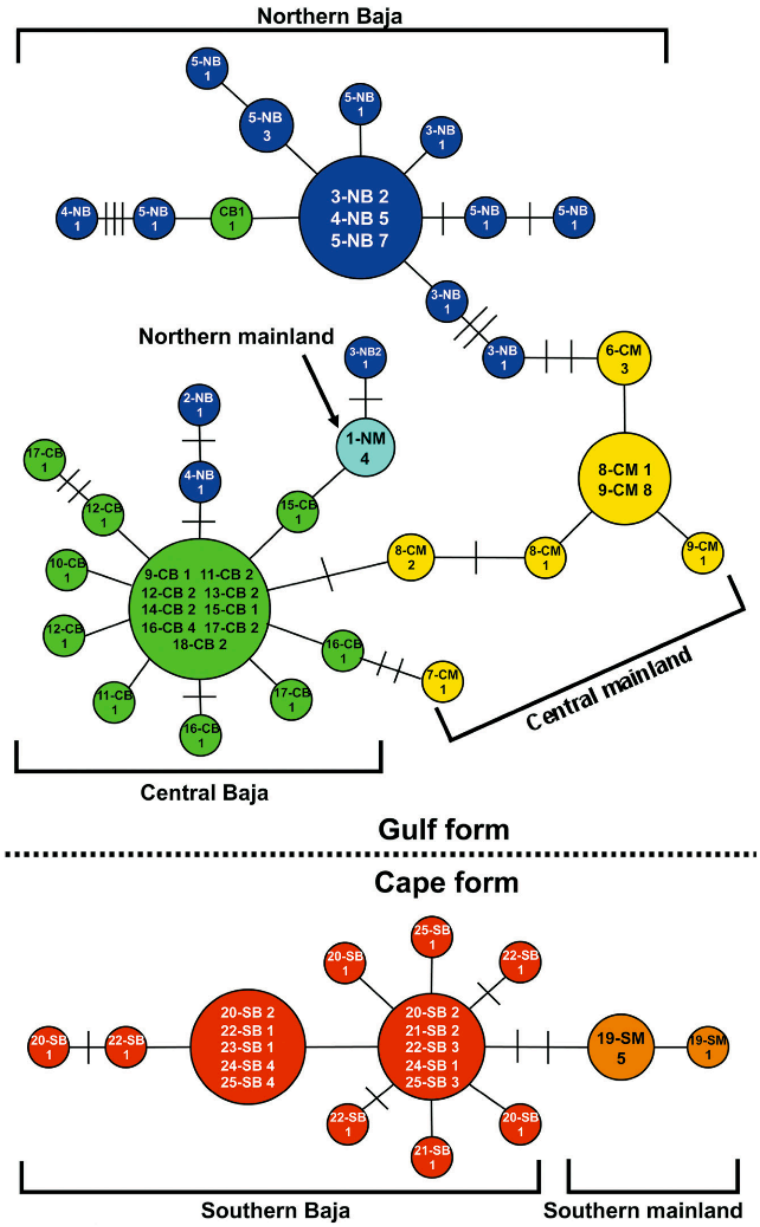

Fig. 3 Haplotype networks with estimated matriarchal phylogeny based on the tRNA-Pro/D-loop data set of 115 *Acanthemblemaria crockeri* derived under a criterion of statistical parsimony with probabilities above the 0.95 limit. Individual haplotypes are indicated by circles with sizes corresponding to the haplotype frequencies. Hatch marks on branches represent minimum numbers of additional substitutions separating haplotypes. The letters and numbers in each circle refer to the study sites where they were sampled, biogeographic regions and the haplotype frequencies (i.e. study site–biogeographic region–frequency).

male colour morphs is clear and abrupt (Fig. 4). The Gulf morph ranges from the most northern sites (sites 1 and 2) along both mainland and Baja coasts to Bahia San Carlos (site 9) and Tecolote (site 23). The Cape morph was present only at Cabo San Lucas (site 25). In contrast,the geographical distribution of female colour morphs is complex (Fig. 4). The Gulf morph ranges from the most northern site down to Punta Coyote (site 18) along the Baja coast and Bahia San Carlos (site 9) along the mainland coast. The Cape morph ranges from the most southern site, Cabo San Lucas, to Los Islotes along the Baja coast. In addition to these two morphs, two intermediate female morphs (I1 and I2) were present from Isla la Ventana (site 5) to Isla Espiritu Santo (site 21) along the Baja coast and Bahia San Carlos (site 9) along the mainland coast. In both sexes, the colour morphs are not congruent with the mitochondrial lineages.

(a)

	NB				CM			CB				
NM	1	3	4	5	6	8	9	10	11	12	16	17
1		0.8	0.8	0.9	1.4	1.3	1.7	0.7	0.6	0.6	0.6	0.8
3	**0.54**		0.7	0.6	1.2	1.2	1.5	0.9	1.0	1.0	1.0	1.1
4	**0.46**	–0.02		0.6	1.4	1.2	1.6	0.8	0.9	0.9	0.9	1.0
5	**0.67**	0.11	0.02		1.3	1.1	1.6	0.8	1.0	1.0	1.0	1.1
6	**0.99**	**0.59**	**0.58**	**0.74**		0.4	0.3	1.2	1.0	1.0	1.1	1.0
8	**0.83**	**0.51**	**0.48**	**0.65**	0.28		0.4	1.0	0.9	0.9	0.9	1.0
9	**0.98**	**0.81**	**0.79**	**0.84**	**0.83**	**0.59**		1.5	1.3	1.2	1.3	1.3
10	**0.53**	**0.21**	0.07	**0.39**	0.57	**0.42**	**0.85**		0.5	0.5	0.5	0.7
11	**0.88**	**0.52**	**0.40**	**0.65**	0.90	**0.62**	**0.94**	0.00		0.2	0.2	0.4
12	**0.80**	**0.53**	**0.43**	**0.65**	0.82	**0.61**	**0.91**	0.06	0.02		0.2	0.4
16	**0.76**	**0.57**	**0.46**	**0.65**	**0.82**	**0.64**	**0.91**	0.12	0.04	0.00		0.4
17	**0.62**	**0.43**	**0.35**	**0.59**	0.58	**0.48**	**0.83**	0.05	0.05	0.08	0.04	

(b)

	SM	SB				
	19	20	21	22	24	25
19		1.0	0.8	1.0	1.0	0.9
20	0.75		0.3	0.4	0.3	0.3
21	0.88	0.04		0.3	0.3	0.2
22	0.72	0.07	0.06		0.3	0.3
24	0.91	0.12	0.56	0.10		0.1
25	0.84	0.01	0.20	0.01	0.00	

Table 2 Pairwise Tamura–Nei divergence of tRNA-Pro/D-loop between study sites of (a) the northern Gulf species and (b) the southern Cape species. The number of base substitutions per site (%) from averaging over all sequence pairs is shown above the diagonal. The pairwise Φ_{ST} value and its significance level (bold if $P < 0.05$) is shown below the diagonal.

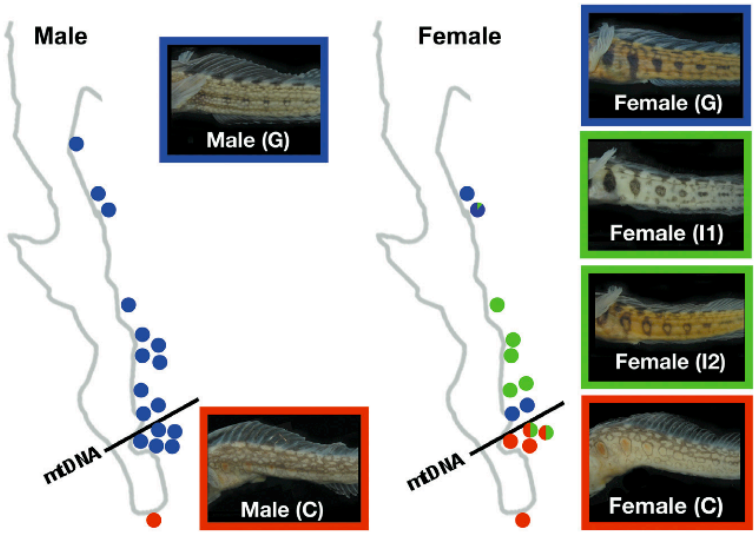

Fig. 4 Occurrences and relative frequencies of the northern Gulf colour morph (blue), southern Cape colour morph (red), and female intermediate morphs (green, I1 and I2 as described in text and Table S3) at each study site are indicated as pie charts based on Table S3. The 'mtDNA' line locates the boundary of the two mitochondrial species based on COI and tRNA-Pro/D-loop. The photos are the middle left side of the body of *A. crockeri*. G, Gulf morph; C, Cape morph; I1, intermediate 1; I2, intermediate 2.

Discussion

Cryptic speciation of Acanthemblemaria crockeri

The level of divergence between the Gulf form and the Cape form of *Acanthemblemaria crockeri* based on COI sequences is comparable to species level differences in other fishes. Two broad surveys of COI divergence levels reported average COI p-divergence value among 964 congeneric chordate species pairs and Kimura 2-parameter divergence value of 4259 comparisons among Australian fishes as 9.6% and 9.9%, respectively (Hebert *et al*. 2003a; Ward *et al*. 2005). An ongoing phylogenetic study of *Acanthemblemaria* reported a notably higher average species-pair COI p-distance of 20.15%, with a range from 11.77 to 23.39% (R. Eytan, unpublished). Although the divergence between the two forms of *A. crockeri* is not as high as its congeneric species pairs, the p-distance value of 9.1% and HKY + I divergence value of 11.9% approach the lower limit for *Acanthemblemaria* and are very close to the average species-level divergence of other fishes. Therefore, we conclude that the two monophyletic forms of *A. crockeri* are distinct cryptic species hereafter termed the Gulf species and the Cape species. This is the first demonstrated cryptic species pair for fishes found entirely within the Gulf of California.

The geographical boundary between the Gulf species and the Cape species is narrow, corresponding to a 30-km gap between Punta Coyote (site 18), Los Islotes (site 20) and southwest Bahia de La Paz (site 24) along the Baja coast (Fig. 1). Along the mainland coast, the two species are separated by a long stretch of mostly sandy coastline with few reefs. In both phylogenetic (based on COI) and haplotype network analyses (based on tRNA/D-loop) (Figs 2 and 3), the two species are exclusive in both geography and genetics and are separated with considerable nucleotide distance. Therefore, there is an abrupt genetic limit between the two nearly continuous (in Baja) but non-overlapping (parapatric) species.

What are the possible mechanisms underlying this speciation event? Riginos (2005) proposed that historical seaways across the Baja peninsula may have restricted the movement of nearshore fishes leading to genetic differentiation. Geographically, one of the hypothetical Baja transpeninsular seaways roughly corresponds to the speciation boundary identified in this study. This seaway across the Isthmus of La Paz isolated the cape region as an island from the rest of the peninsula during the Pliocene, about 3 to 4 Ma (Hausback 1984; Riddle *et al*. 2000; Murphy & Aguirre-Léon 2002). This seaway might have served as a dispersal barrier for *A. crockeri*, leading to reduced gene flow and ultimately to speciation. However, this hypothesized vicariant event is older than the estimated divergence time of these species (1.84 Ma). In addition, this vicariant event should have affected other lineages similarly (Knowlton *et al*. 1993), but available evidence confirms this only for terrestrial organisms, such as tree lizards and rodents (Aguirre-Léon *et al*. 1999; Riddle *et al*. 2000). One closely related chaenopsid, *Acanthemblemaria macrospilus*, that occurs in the central and southern Gulf, from Isla Danzante to Cabo San Lucas, shows little genetic variation in either COI or tRNA-Pro/D-loop ($P = 0.10547$, H-C. Lin, unpublished) across this range that encompasses the dividing line between the Gulf and Cape species of *A. crockeri*. These closely related species have similar habitat preferences, life history and feeding habits. Their significantly different genetic patterns suggest factors other than the physical environment may underlie the significant genetic structure seen in *A. crockeri* but not in *A. macrospilus*.

Colour morphs of the two species

Stephens (1963) first described a distinct colour pattern or Cape morph for individuals collected at a depth of 5 m off Cape San Lucas. Only the Cape morph is found at the extreme southern end of the Gulf, and only the Gulf morph is found at the extreme northern Gulf. Both colour morphs are found in a zone from Isla Espiritu Santo to Bahia Los Frailes (Stephens 1963). Lindquist (1980) re-examined the distributions of the two colour morphs based on fresh specimens and expanded the sympatric area along the mainland coast (Guaymas) and Baja coast (Isla del Carmen to Bahia Los Frailes). Additionally, he reported the distributions of colour morph frequency in males and females at the sympatric area individually with a gradual transition in frequency between Isla del Carmen and Bahia Los Frailes for males and Isla del Carmen to Tecolote for females. Similar to Stephens' and Lindquist's results, our data show a sympatric area of the two colour morphs and different distribution patterns in males and females (Table S3 and Fig. 4). In males, the Cape colour morph occurs exclusively at Cabo San Lucas (site 25) and no transitional area is found. Female specimens present a general pattern of a northern Gulf morph and a southern Cape morph with northern Los Islotes (site 20) as the boundary. However, the pattern of females is not as clear mainly because not all specimens can be assigned into either the Gulf morph (three to six dark-brown melanic bars on the anterior half of body) or the Cape morph (a series of ring-like spots along the midline with a second series on the ventral anterior half of body) based on Stephens (1963). These intermediate female colour morphs, i.e. with melanic spots (I1) or a single-row of ring-like spots (I2) along the mid-line of the body were collected from Isla la Ventana (site 5) southward to Isla Espiritu Santo (site 21; Table S3 and Fig. 4).

Our sampling of *A. crockeri* fully encompassed its distribution, thus providing a molecular perspective of the enigma of its colour variation. Instead of having an area of sympatry as suggested by Stephens's (1963), Lindquist's

(1980) and this study's analyses of colour morphs, we found an abrupt and distinct mtDNA separation. The Gulf species includes Gulf colour morphs of males and females, as well as intermediate colour morphs of females, while the Cape species includes both Cape and Gulf morphs of males, and Cape and intermediate morphs of females (Fig. 4). What is the relationship between the two colour morphs and the two mitochondrial species in *A. crockeri*? One possibility is that the colour morphs of *A. crockeri* first discussed by Stephens (1963) may represent 'residual geographic variation' that was present in the common ancestor that has been retained in descendant species (Wiley 1991) and does not play a direct role in the genetic isolation of these species. This phenomenon may apply to other cases where phenotypic and genetic geographic variations are incongruent. Other studies with similar findings in which mitochondrial lineages do not correspond to colour variation have included several reef fishes such as the three-spot damselfish (Bernardi *et al*. 2002), rockfish (Alesandrini & Giacomo 1999), hamlets (Ramon *et al*. 2003), *Pseudochromis* damselfish (Messmer *et al*. 2005), *Acanthochromis* damselfish (Herwerden & Doherty 2006) and pygmy angelfish (Bowen *et al*. 2006).

While quite distinctive based on mtDNA, little variation was found within three commonly used nuclear intron markers and a fourth had no parsimony informative variation. Thus, the nuclear markers we surveyed provide no additional insights on divergence in *A. crockeri*. Although mitochondrial DNA is maternally inherited, the genetic boundary does not correspond to female colour morphs or other noticeable phenotypic characters (Fig. 4). Also, there is no evidence of sex-biased dispersal in these or other blennies. Both males and females have a pelagic larval stage, occupy shelters and move very little as adults (Hastings 2002). Future work with fast-evolving nuclear markers (such as microsatellites) is needed to further test this species boundary and exclude the possibility of introgression.

Population subdivision within the two species

Because the *A. crockeri* lineage is abundant throughout the entire Gulf (Thomson *et al*. 2000; Thomson & Gilligan 2002), we were able to investigate biogeographic subregions within the Gulf of California. Four (NM, NB, CM and CB) and two (SM and SB) biogeographic populations within the Gulf and Cape species, respectively, were assigned based on prior knowledge (Walker 1960; Riginos & Nachman 2001) and supported by the presence of unique haplotypes (Fig. 3), the pairwise genetic divergence (Table 2), and significant Φ_{ST} values (Table 2). Recent debate has questioned the utility of F_{ST} related methods for estimating gene diversity and genetic differentiation in high-diversity systems (Jost 2008). In such systems, the F_{ST} methods tend to underestimate the divergence due to falsely assuming an additive relationship of 'between-population' and 'within-population' heterozygosity (Jost 2008). Although intraspecific variation within *A. crockeri* is high, the possible bias of underestimating the divergence does not affect our results; Φ_{ST} values were high and significant between populations and the parallel estimation of pairwise genetic distance showed complementary results (Table 2).

The level of population structure found within both the Gulf and Cape species of *A. crockeri* is surprising, given the restricted nature of the Gulf. Although the northern, central and southern Gulf divisions roughly correspond to those of Walker (1960) based on fish community composition, the further partitions between the mainland and Baja coasts are relatively new. This mainland-Baja differentiation has been reported in only one other Gulf fish species, the triplefin blenny *Axoclinus nigricaudus*, at the central and southern Gulf (Riginos & Nachman 2001). For small reef fishes, such as *A. crockeri* and *A. nigricaudus*, open-water masses like the central axis of the Gulf may serve as a dispersal barrier restricting gene flow, although a series of seasonal surface water gyres along the length of the Gulf (Pegau *et al*. 2002; Marinone 2003) might be expected to readily transport pelagic larvae across the Gulf. The deep population subdivisions in these species are likely related to the behaviour of their larvae that are able to remain close to reef substrates throughout development, thus decreasing the chance of being carried away from natal reefs by water currents (Brogan 1994).

Along the Baja coast, migration rates of five reef fishes, *Axoclinus nigricaudus* (Riginos & Nachman 2001), *Coralliozetus micropes* (Riginos 2005), *Malacoctenus hubbsi* (Riginos & Victor 2001), *Girella nigricans* (Terry *et al*. 2000) and *Parabrax maculatofasciatus* (Stepien *et al*. 2001), were estimated by Riginos (2005) using the software Migrate, permitting direct comparison of migration rates (M) in this study. Migration rates along the Baja coast of the *A. crockeri* Gulf species (NB→CB = 22.5 and CB→NB = 27.5, corresponding to 0.15 and 0.42 migrants per generation) are much smaller than any of the five species (191–1567). This limited gene flow between populations suggests a great differentiation potential of *A. crockeri*. *Axoclinus nigricaudus*, *C. micropes* and *P. maculatofasciatus*, along with the Gulf species of *A. crockeri*, have concordant genetic differentiation patterns (significant Φ_{ST} values and limited migrations) between the northern and central Gulf. This supports the hypothesis of a Plio–Pleistocene mid-peninsular seaway barrier to the movement of nearshore fishes (Riginos 2005).

The effective population sizes of Baja populations are all much larger than the mainland populations possibly because rocky reefs are ubiquitous and mostly continuous along the Baja coast but less extensive along the mainland coast especially south of Guaymas (Thomson *et al*. 2000). Based on the haplotype network (Fig. 3), Isla San Pedro

Martír (site 6), located south of Isla Tiburón (Walker's southern limit of the northern Gulf) seems to provide a transitional point between the northern and central Gulf and Baja and mainland shorelines. Geographically, this island is located in the centre of the Gulf and is about equidistant from NB and CM populations (Fig. 1). After examining the haplotype connection (Fig. 3), their genotype is closer to the CM population and intermediate between it and the NB population (Fig. 3). This evidence suggests that Isla San Pedro Martír may serve as a stepping stone for small reef-dwellers, such as *A. crockeri*, to disperse across the Gulf. This connection between subregions of the Gulf through an isolated island has not been reported before. An enhanced sampling survey and knowledge of oceanographic currents around this and neighbouring islands might provide further insights into the pattern and connection of population subdivisions within the Gulf of California.

Conclusions

Two exclusively monophyletic lineages were found in the Gulf of California endemic blennioid species, *Acanthemblemaria crockeri*, based on two mitochondrial markers, COI and tRNA-Pro/D-loop. These two parapatric species-level lineages were separated by a 30-km gap corresponding to a hypothesized former seaway across the Baja California peninsula north of La Paz. Surprisingly, the distributions of the northern Gulf and southern Cape mitochondrial species were not congruent with the formerly well-documented northern Gulf and southern Cape colour morphs in both males and females. In addition to the two mitochondrial species, subsequent diversification was found (NB, CB, NM and CM in the Gulf species; SB and SM in the Cape species) based on haplotype network analyses, Φ_{ST} values and pairwise genetic distances. Therefore, we find support for six biogeographic regions within the Gulf. Our data supported the NB/CB population division corresponding to the Plio–Pleistocene mid-peninsular seaway. The level of genetic divergence documented in *A. crockeri* is extraordinary for a marine fish with a pelagic larval stage within a semi-enclosed basin.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 GenBank accession numbers for each unique mitochondrial COI haplotype of *Acanthemblemaria crockeri* observed in this study

Table S2 GenBank accession numbers for each unique mitochondrial tRNA-Pro/D-loop haplotype of *Acanthemblemaria crockeri* observed in this study

Table S3 Morphological characters of 95 voucher specimens (20 out of the 115 specimens have no vouchers available) of *Acanthemblemaria crockeri* arranged from north to south

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Chapter 3, in full, is a reprint of the material as it appears in Molecular Ecology 2009. Lin, H.-C., Sanchez-Ortiz, C., Hastings, P. A. 2009. The dissertation author was the primary investigator and author of this paper.

CHAPTER 4

A new endemic species of Barnacle Blenny (Teleostei: Chaenopsidae: *Acanthemblemaria*) from the Gulf of California, Mexico.

Abstract

A new species of chaenopsid tube blenny, *Acanthemblemaria hastingsi* sp. nov., is described using molecular and morphological data. *A. hastingsi* sp. nov. is a member of the *hancocki* species group and closely related to *A. macrospilus* Brock (1940). Both genetic data (mitochondrial cytochrome C oxidase I and D-loop region, nuclear ribosomal protein S7 first intron) and coloration of these two species are distinct, and there is no geographic overlap in their ranges, as they occur exclusively on either side of the Sinaloan Gap. Vertebral, cephalic sensory pore, and fin ray counts, as well as color descriptions in life and in preservation, are included in the description.

Introduction

Acanthemblemaria is a genus of chaenopsid tube blennies found throughout the new world tropics in the Caribbean and the Tropical Eastern Pacific (TEP). The species of *Acanthemblemaria* are united by their combination of conspicuous frontal spines and two rows of palatine teeth (Stephens 1963; Stephens' 1970 description of a new species of *Emblemaria* with two rows of palatine teeth, however, implies that this combination might not be completely unique). This genus includes a monophyletic clade known as the *hancocki* species group after *A. hancocki* Meyers and Reid (1936). This group (also known as the barnacle blennies *sensu* Hastings and Robertson, 1998) was first recognized by Stephens (1963) and included three species from the TEP (*A. hancocki*, *A. balanorum* Brock 1940; and *A. macrospilus* Brock 1940). Since that publication, several new species in the *hancocki* group have been described based on morphological analyses, including: *A. castroi* Stephens and Hobson 1966, endemic to the Galapagos Islands; *A. rivasi* Smith-Vaniz and Placio 1974, from the southern Caribbean; *A. stephensi* Rosenblatt and McCosker 1988, endemic to Isla de Malpelo; *A. atrata* Hastings and Robertson 1998, endemic to Isla del Coco; and *A. mangognatha* Hastings and Robertson 1998, endemic to Islas Revillagigedo. A ninth species in this group, *A. hastingsi* sp. nov., endemic to the Gulf of California, Mexico (hereafter Gulf) is described herein.

Acanthemblemaria macrospilus, which formerly included all individuals of *A. hastingsi*, was first described as a subspecies of *A. hancocki* by Brock (1940). Stephens (1963) elevated it to species and reported that its range included the Gulf, the coast of southwestern Mexico, Isla del Coco, and Islas Revillagigedo. The Isla del Coco population was later described as *A. atrata*, and the Islas Revillagigedo population was

described as *A. mangognatha* (Hastings and Robertson, 1998). The remaining two populations are found in two distinct biogeographic regions, the Cortez and Mexican Provinces of the TEP that are separated by the Sinaloan Gap. This gap is an area of soft bottom that lacks appropriate habitat for many reef fishes and forms a barrier to dispersal for several species of blennies (Hastings, 2000). Based on coloration, Hastings and Robertson (1998) recognized two morphs of *A. macrospilus*, the northern "Cortez morph" and southern "Mexican morph" separated by the Sinaloan Gap. The type locality of *A. macrospilus* is Isla Maria Magdalena in the Tres Marias group off of mainland Mexico and represents the "Mexican morph."

In this paper, molecular (mitochondrial and nuclear DNA) and morphological data are evaluated in order to understand the relationship between the two color morphs. Molecular tools have recently been applied to distinguish between species in another *Acanthemblemaria* species pair where morphological evidence was ambiguous (Lin et al., 2009). Mitochondrial markers are especially informative because of their higher evolutionary rate compared to nuclear markers (reviewed by Avise, 2004), but the results can be biased because interpretation is based on a single evolutionary history. Therefore, we also analyze the pair with respect to a nuclear marker. Here, we provide molecular and morphological evidence that the two morphs are distinct species and describe the "Cortez morph" as *Acanthemblemaria hastingsi* sp. nov.

Materials and methods

Molecular analyses

We obtained samples of *A. macrospilus* from throughout its geographical distribution including the Gulf of California and southwestern Mexico (Fig. 1, Table 1). *A. hancocki* from Playa Cocos, Costa Rica was used as the outgroup taxon. Samples were collected by SCUBA or snorkeling, using the anesthetic quinaldine. Specimens were preserved in the field with 95-100% ethanol and subsequently archived in the Scripps Institution of Oceanography Marine Vertebrate Collection (SIO).

Total genomic DNA was extracted from muscle tissue with a Qiagen (Chatsworth, CA) QIAquick Tissue Kit following the manufacturer's instructions. The polymerase chain reaction (PCR) was used to amplify two mitochondrial and one nuclear DNA markers. The PCR was performed on a Mastercycler EP Gradient S (Eppendorf; Hamburg, Germany) with the following conditions: 94°C for one minute for initial denaturing, followed by 35 cycles of 94°C for 30 sec, 52-60°C for 45 seconds, and 72°C for 45 sec. Universal primers FishF2 and FishR2 (Ward et al., 2005), A and E (Lee et al., 1995) and S7RPEX1F and S7RPEX2R (Chow and Hazama, 1998) were used in amplifying cytochrome C oxidase I (COI), tRNA-Pro/D-loop (D-loop region), and ribosomal protein S7 first intron (S7-1), respectively. Resulting amplicons were purified with Exonuclease I (20U/μl, New England Biolabs) and Shrimp Alkaline Phosphatase (1U/μl, Roche) to remove excess single-stranded DNA and unincorporated dNTPs. Direct sequencing of PCR products was performed in both forward and reverse directions with the amplification primers (except an additional internal primer DloopF1 (Lin et al., 2009) for D-loop) using DYEnamicTM ET dye terminator chemistry on an automated MegaBACE^{TM} 500 DNA sequencer (Amersham Biosciences Corp., Piscataway, NJ). Sequences were assembled and edited with Sequencher 4.5 (Gene Codes Coporation,

Ann Arbor, MI), then aligned with CLUSTAL X (Thompson et al., 1997) using default settings and adjusted by eye in MacClade 4.07 (Maddison and Maddison, 1997).

To resolve the S7-1 sequences of two strands in each heterozygous individual, the program PHASE v2.1 implemented Bayesian statistical method was used to predict allelic phase (Stephens and Scheet, 2005; Stephens et al., 2001). PHASE reconstructs unknown haplotypes from population genotype data with the goal of assigning identical or similar known haplotypes from homozygous individuals (Stephens et al., 2001). The data matrix running in PHASE composes bi-allelic polymorphic sites of all the specimens. For consistency, five independent runs were executed using different seeds with default settings and the prediction of the run with the best "goodness of fit" was reported.

Genealogical relationships within *A. macrospilus* were constructed by Bayesian MCMC estimation with *A. hancocki* as the outgroup. Bayesian methods were implemented in MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) and run under the best-fit nucleotide substitution models selected by MrModeltest v2.2 (Nylander, 2004) under Akaike Information Criterion (AIC). HKY+G, HKY+I+G, and HKY+I were selected as the best-fit models for COI, D-loop, and S7-1, respectively. Two simulated independent runs were performed starting from different random trees for 10^6 generations. Each run comprised four chains (one cold and three heated) and was sampled every 100 generations. The mitochondrial combined dataset (COI+ D-loop) was executed with partitioned mixed-model where the optimal molecular evolution model was applied to each of the gene partitions and model parameter values were "unlinked"

among partitions (Ronquist and Huelsenbeck, 2003). The first 10% of the generations were discarded as burn-in while the convergence between the two runs was not reached.

Analyses of Molecular Variance (AMOVA) (Excoffier et al., 1992) based on Tamura-Nei distance method (TN93) (Tamura and Nei, 1993) were performed in Arlequin v3.11 (Excoffier and Schneider, 2005) to evaluate the genetic variance hierarchically. Statistical significance of these analyses was determined on the basis of the distribution of values obtained from 10,000 permutations. Pairwise Φ_{ST} value as an indication of population structure was also calculated in Arlequin. The mean evolutionary divergence of individuals collected from the Gulf and southwestern Mexico was estimated with TN93 method in MEGA4 (Tamura et al., 2007). The divergence time was estimated using the average mutational rate of COI as 6.87% TN93-distance per million years based on three geminate pairs of chaenopsids across the Isthmus of Panama, *Acanthemblemaria betinensis* and *A. exilispinus*, *A. castroi* and *A. rivasi* and *Ekemblemaria myersi* and *E. nigra*, that hypothetically diverged at the final closure of the transisthmian seaway 3.5 million years ago (Hastings, 2000).

Morphological analyses

Meristic counts and morphological measurements follow Hubbs and Lagler (1958) with the following exceptions: predorsal length is defined as the diagonal between the dorsal fin origin and the snout, and preanal length is the diagonal between the anal fin origin and the snout. Meristic data were taken from 40 cleared and stained specimens, and morphological measurements were taken from 43 additional specimens (total specimens examined = 83). Cephalic sensory pores were counted on the latter 43

individuals, following Smith-Vaniz and Palacio (1974) and Hastings (1990). Pores on lateral bones were counted only on the left side. Measurements were recorded to the nearest 0.1mm using digital calipers. In the description, an asterisk indicates data representative of the holotype. Institutional abbreviations follow Leviton et al. (1985).

Specimens from the Islas Revillagigedo species *A. mangognatha* and the now distinct southwestern Mexico species *A. macrospilus* were examined for coloration comparisons. Fin element counts for these species are from Stephens (1963).

Results

DNA sequence data

After trimming the sequences, 560bp for COI, 422bp of D-loop and 648bp (647bp in *A. hancocki*) of S7-1 were obtained for the 32 specimens of *A. macrospilus* (30 for D-loop) and 2 specimens of *A. hancocki*. All unique haplotypes were submitted to GenBank (Table 2). The alignments were unambiguous in all the markers. However, three indels (two insertions and one deletion) were found in S7-1 of *A. macrospilus* compared to *A. hancocki*. Phylogenetic relationships within *A. macrospilus* based on independent or concatenated mitochondrial markers indicated the presence of two distinct monophyletic lineages (Fig. 2). These two lineages geographically corresponded to individuals collected from the Gulf (Sites 1-9) and the southwestern Mexican coast (Sites 10-15) (Table 1).

Ten haplotypes were found in COI with haplotypes 1-6 collected from the Gulf and 7-10 from southwestern Mexico (Table 2). Seventeen haplotypes were found in D-loop region with haplotypes 1-7 collected from the Gulf and 8-17 from southwestern

Mexico. The mitochondrial genetic variation between the Gulf and southwestern Mexico populations can explain 99.13% and 93.36% of the total variation in COI and D-loop region, respectively. Seventy-one and 20 fixed mutations were found in COI and D-loop region, respectively, which clearly diagnose the Gulf and southwestern Mexican lineages. The mean TN93 divergences between the two lineages were estimated as 15.50% and 7.26% for COI and D-loop, respectively. A divergence time of 2.26 million years ago (Lin and Hastings, unpubl.) was further estimated using the COI mutational rate as 6.87% TN93-distance per million years.

Twenty-one genotypes were found in the nuclear S7-1 gene (Table 2) and no fixed mutations were found. The Φ_{ST} value between the two lineages was highly significant (0.83, $P<0.001$). Out of the 638bp, 38 were bi-allelic polymorphic loci and thus further analyzed with PHASE to reconstruct haplotypes. The best reconstruction included 19 haplotypes out of 32 individuals with 11 homozygous individuals (Table 3). Haplotype 3 and 6 were the most common in the Gulf and southwestern Mexico individually and only haplotype 5 was shared by individuals from both areas (Table 3b).

***Acanthemblemaria hastingsi* new species**

Cortez Barnacle Blenny

(Fig. 3)

Acanthemblemaria hancocki macrospilus (in part): Brock, 1940.

Acanthemblemaria macrospilus (in part): Stephens, 1963; Rosenblatt and McCosker, 1988; Hastings and Robertson, 1998; Hastings, 2000; Thomson, et al., 2000.

Acanthemblemaria macrospilus: Lindquist, 1985; Kotrschal and Lindquist, 1986; Lindquist and Kotrschal, 1987; Thomson and Gilligan, 2002.

Acanthemblemaria nsp.: Hastings, 2009.

Holotype.—SIO 65-272, 43.6 mm SL, male, Mexico, Gulf of California, Canal San Jose, 24°60' N, 110°46' W, 3 m depth, collected with Chemfish, 8-July-1965.

Paratypes.—SIO 65-272, 51, 22-46 mm SL, collected with the holotype; SIO 65-342, 69, 23-49 mm SL, Mexico, Gulf of California, Isla Santa Cruz, 10 m depth, collected with Chemfish, 22-July-1965; SIO 59-228-J, 121, 14.5-45 mm SL, Mexico, Gulf of California, Isla San Ignacio de Farallon, 5-10 m depth, collected with Rotenone, 1-April-1959; SIO 59-225-Q, 266, 20-54 mm SL, Mexico, Gulf of California, Punta Pescadero, 5 m depth, collected with Rotenone, 28-March-1959; UNSM 317625, 3, 32-40.2 mm SL, Mexico, Gulf of California, Isla San Pedro Nolasco, 6 m depth, 20-June-1990.

Diagnosis.—*Acanthemblemaria hastingsi* can be distinguished from its closest relatives (*A. macrospilus* and *A. mangognatha*) by a dark swath of melanophores on the dorsal fin in both males and females that highlights the bright orange coloration on that fin. The lower jaw is covered with scattered melanophores that reach the tip. Primary bright color on the head is also orange.

Description.—Variable meristic data are summarized in Table 4. All paratypes have 11 precaudal vertebrae; the holotype has 12. There was no variation in pectoral-fin rays (13), primary caudal-fin rays (13), anal-fin spines (2), pelvic-fin spines (1), and pelvic-fin rays (3). Dorsal and anal fin meristics for the closely related *A. macrospilus* and *A. mangognatha* are also presented in Table 4.

Individuals of *A. hastingsi* are long and slender (body depth in standard length nearly 6.5 times, Table 5) and have long heads (contributing to nearly 20% of standard length, Table 5). This body type probably reflects *A. hastingsi*'s ecology of colonizing vacant invertebrate tests as mature adults. Individuals have one pair of supraorbital cirri that are usually unbranched, but occasionally shallowly branched and rarely deeply branched, never more than once. Nasal cirri are located on anterior nostrils and are always branched, occasionally more than once. Posterior nostrils lack cirri. The single dorsal fin is notched –at the 24th spine on the holotype. The caudal fin is truncate. The upper jaw is large (about 1.8 times in the head, Table 5) and always extends beyond the level of the posterior edge of the orbit and nearly as far back as the dorsal fin origin. Several bones of the neurocranium are covered with spines (Fig. 4). The frontals have the most well developed spine field, forming a diamond-shaped patch, extending posteriorly from a point in the interorbital space (Fig. 4).

The following measurements were taken from the holotype and are reported in mm: standard length 43.6; head length 10.9; upper jaw length 6.0; orbital diameter 2.6; snout length 2.2; interorbital width 1.9; predorsal length 7.8; preanal length 19.0; caudal peduncle depth 3.4; body depth at anal fin origin 6.5. These measurements for the paratypes, are summarized in Table 5, sorted by sex.

Cephalic pore counts are as follows, with numbers of specimens (n=44) in parentheses. Mandibular: 4(43*), 5(1); common: 1(44*); preopercular: 5(43*), 6(1); posttemporal: 4(43*), 5(1); lateral supratemporal: 3(4), 4(33*), 5(6); median supratemporal: 1(1), 2(3), 3(39*), 4(1); anterior infraorbital: 3(43*), 4(1); posterior infraorbital: 4(5), 5(29*), 6(9), 7(1); supraorbital: 3(3), 4(36*), 5(5); frontal: 3(7), 4(9*),

5(13), 6(2), 8(1); commissural: 0(3), 1(37*), 2(3); anterofrontal: 1(3), 2(41*); and nasal: 1(44*).

Head and Body Coloration.—Both males and females exhibit a series of distinct saddles, from the nape to the posterior end of the dorsal fin, as a result of dense melanophore expression in these areas. Most often, individuals (including the holotype) have eight saddles, but individuals with seven (as a result of the combination of the two posterior-most saddles) or nine (as a result of an area of no dark coloration dividing the posterior saddle) were observed. In some males, dark head and anterior body coloration mask the distinction of the first or first two saddles. Individuals of both sexes also exhibit seven blotches along the lateral midline of the body, almost always more distinct in females than in males (which are generally darker; only five easily distinguishable in one especially dark male). The most anterior blotch is hidden by the adpressed pectoral fin, and the remaining blotches are roughly offset from the dorsal saddles. The most posterior blotch is also the most elongate, stretching to (but not onto) the caudal fin, and often contains regions of fewer melanophores, obscuring its overall shape. No other dark coloration is present on the bodies of either males or females. Females also have distinct dark blotches on the pectoral base and the cheek. These two marks are often masked by dark head coloration in males but are occasionally present. Melanophores are present to the tip of the lower jaw in both sexes (more so in males than in females). In life, the primary bright color on the head in *A. hastingsi* is orange, and faint blue spots are occasionally located on the cheeks and head. Finally, there is no unique noticeable difference in coloration of individuals fixed in formalin and those preserved in ethanol.

Fin Coloration.—Individuals of both sexes have a very dark, distinct swath of melanophores along the anterior, spinous dorsal fin. In males, this swath begins at the base of the first dorsal spine and extends posteriorly (Fig. 3). Some individuals have fewer melanophores at the base of the first or first two spines than in the rest of the swath, but they always have some. In females, the base of the first or first two dorsal-fin spines is usually free of melanophores, creating a triangular patch of no color and causing the swath to be more j-shaped. In life, the primary bright color on the dorsal fin is orange, which is windowed by the swath and by a fainter band of melanophores along the top edge of the fin. Scattered melanophores are present along the remainder of the spinous dorsal fin. The anal fin is characterized by one broad band of melanophores running the full length of the fin in both males and females. The caudal fin, pelvic fins, and pectoral fins have very few, randomly scattered melanophores but are otherwise colorless.

Sexual Dimorphism.—In addition to the differences in coloration discussed above, the sexes are distinguishable by the shape of the genital papilla, which is pointed and simple in males and broader and fimbriate in females (Böhlke, 1957). Males and females also differ slightly in body shape (Table 5). The orbital diameter consistently fits fewer times into the snout length of females than males ($P<0.0001$, unpaired t-test) implying that females either have larger eyes or shorter snouts than males. These data are corroborated by the fact that the orbital diameter fits fewer times in the head length in females than in males ($P<0.0001$). Finally, females seem to be more slender, with their body depth fitting in their standard length more times than in males ($P<0.001$). There are no noticeable differences in head pore pattern or count or in meristics between males and females.

Distribution.—*Acanthemblemaria hastingsi* is endemic to the Gulf of California and is known to occur from Mulegé to Cabo San Lucas along the Baja Peninsula and between Isla San Pedro Nolasco and Isla San Ignacio de Farallon along the Mexican continental mainland (Hastings and Robertson, 1998). Gulf species in this genus are known to exhibit depth partitioning, and *A. hastingsi* is typically found 0-13 m deep (Lindquist, 1985).

Etymology.—*Acanthemblemaria hastingsi* is named for Philip A. Hastings who has contributed to our knowledge of chaenopsid blennies for more than 25 years.

Remarks.—We provide molecular evidence from both mitochondrial and nuclear markers that the formerly recognized color morphs of *A. macrospilus* are distinct species. This observation of closely related species living across, and separated by, the Sinaloan Gap is not unique and occurs in at least three other pairs of chaenopsid tube blennies (Hastings, 2000). The speciation is identified by reciprocal monophyly of lineages (Fig. 2), unique haplotypes (Table 2), and abundant fixed mutations based on the two mitochondrial markers. An ongoing phylogenetic study of the Chaenopsidae has found notably higher than average congeneric species-pair COI divergence values with a range from 21.2% to 35.8% in TN93 distance (Lin and Hastings, unpubl.). Although the COI divergence value of 15.5% between *A. macrospilus* and *A. hastingsi* is lower than their congeneric species, it still suggests a species-level divergence about 2.25 million years ago. To avoid interpretation purely relying on single evolutionary history from mitochondrial genes, we also analyzed one nuclear intron gene, S7-1. As expected from the four-fold higher effective population sizes in nuclear genes than in mitochondrial genes, less variation was found in the sequences of S7-1. However, only one out of the

nineteen S7-1 haplotypes was shared by these two sister species (Table 3b) and there is evident variation between their genotypes (Φ_{ST}=0.83).

Rosenblatt and McCosker (1988) presented a morphological key to the Pacific species of *Acanthemblemaria* that were known at that time of publication. Using their publication, individuals of *A. hastingsi*, as well as individuals of the closely related and morphologically similar *A. macrospilus* and *A. mangognatha*, all key out to *A. macrospilus*. Head and dorsal-fin coloration constitutes the best character to discern individuals of these species.

Unlike in *A. hastingsi,* the melanophores on the lower jaws of individuals of *A. macrospilus* do not reach all the way to the distal end. Furthermore, the primary bright head color in *A. macrospilus* is red. The dorsal fin melanophore patterns help further distinguish individuals of *A. hastingsi* from *A. macrospilus* and *A. mangognatha*. Individuals of *A. macrospilus* almost never have melanophores reaching the base of the first dorsal fin and more typically have a dark, round spot or stretched out spot instead of a swath. Also, the primary bright color on the dorsal is red, and the windowing effect around that color is less distinct. Individuals of *A. mangognatha* have more broadly scattered, less dense melanophores throughout the anterior dorsal fin that do not form as distinct of a swath or spot and do not create a windowed patch of color.

Materials examined

Acanthemblemaria macrospilus: SIO H46-245-A, 4, Mexico, Guerrero, near Acapulco, 15-September-1946; UAZ 70-22-8, 5, Mexico, Oaxaca, Puerto Escondido, 8-June-1970; UAZ 71-61-2, 34, Mexico, Jalisco, Bahia Banderas, Puerto Vallarta, Los

Arcos Rocks, 26-July-1971; UAZ 77-41, 71, Mexico, Punta Santiago, near Manzanillo, 30-June-1977. *Acanthemblemaria mangognatha*: SIO 97-216, 12, Mexico, Islas Revillagigedos, Isla Socorro, 29-October-1990.

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Table 4.1 Study sites and sample sizes of *Acanthemblemaria macrospilus* for molecular analyses. Voucher specimens of all are cataloged in the Marine Vertebrate Collection, Scripps Institution of Oceanography.

Site	SIO collection #	Locality	Latitude	Longitude	Sample size
		Gulf of California			
1	07-113-1~2	Isla Danzante	25º42’	111º15’	2
2	07-114-1~2	Islotes Las Galeras	25º41’	111º03’	2
3	07-115-1~2	Isla Santa Cruz	25º17’	110º43’	2
4	07-116-1~2	Isla La Habana	25º08’	110º52’	2
5	07-119-1~2	Isla San Francisco	24º51’	110º35’	2
6	05-122-1~2	Puerto Escondido	24º26’	110º38’	2
7	03-79-1~2	La Paz: Isla Espiritu Santo	24º33’	110º23’	2
8	06-1-1~2	Bahia Los Frailes	23º23’	109º25’	2
9	02-22-1~3	San Jose del Cabo	23º00’	109º43’	3
					19
		Southwestern Mexico			
10	02-23-1~3	Mazatlán: Isla Pajaro	23º15’	106º30’	3
11	03-152-1~3	Isla Isabela	21º51’	105º55’	3
12	01-182-1~4	Puerto Vallarta	20º37’	105º15’	4
13	01-48-1	Bahia Maguey	15º47’	96º09’	1
14	01-49-1	Bahia Conejos	15º45’	96º05’	1
15	01-41-1	El Tigre, Huatulco	15º43’	96º10’	1
					13
Total					32

Table 4.2 Collecting sites and accession numbers for COI and D-loop haplotypes and S7-1 genotypes. Numbers within parentheses are frequencies.

COI	Collecting sites	Accession number
Haplotype1	1(2), 2, 4, 5(2), 6(2), 7, 8, 9	FJ884556
Haplotype2	2, 7, 9	FJ884553
Haplotype3	3	FJ884554
Haplotype4	3, 4	FJ884555
Haplotype5	8	FJ884552
Haplotype6	9	FJ884548
Haplotype7	10(3)	FJ884549
Haplotype8	11	FJ884550
Haplotype9	11(2), 12(4), 13, 15	FJ884551
Haplotype10	14	FJ884547
A. hancocki1	Playa Cocos, Costa Rica	FJ884557
A. hancocki2	Playa Cocos, Costa Rica	FJ884558

D-loop	Collecting sites	Accession number
Haplotype1	1	FJ884573
Haplotype2	3	FJ884574
Haplotype3	1, 2(2), 3, 4(2), 5(2), 6(2), 7, 9	FJ884575
Haplotype4	7	FJ884570
Haplotype5	8	FJ884571
Haplotype6	8	FJ884572
Haplotype7	9(2)	FJ884564
Haplotype8	10	FJ884565
Haplotype9	10(2)	FJ884566
Haplotype10	11	FJ884567
Haplotype11	11	FJ884568
Haplotype12	11	FJ884569
Haplotype13	12	FJ884559
Haplotype14	12	FJ884560
Haplotype15	12	FJ884561
Haplotype16	14	FJ884563
Haplotype17	15	FJ884562
A. hancocki1	Playa Cocos, Costa Rica	FJ884576
A. hancocki2	Playa Cocos, Costa Rica	FJ884577

S7-1	Collecting sites	Accession number
Genotype1	1	FJ915175
Genotype2	2	FJ915176
Genotype3	3	FJ915177
Genotype4	1, 2, 4, 6, 7, 9	FJ915178
Genotype5	4	FJ915179
Genotype6	3, 5(2), 9	FJ915180
Genotype7	6	FJ915172
Genotype8	7	FJ915171
Genotype9	8	FJ915173
Genotype10	8	FJ915174
Genotype11	10	FJ915166
Genotype12	10	FJ915167
Genotype13	11	FJ915168
Genotype14	11	FJ915169
Genotype15	11	FJ915170
Genotype16	12	FJ915160
Genotype17	12	FJ915161
Genotype18	12	FJ915162
Genotype19	12	FJ915163
Genotype20	13	FJ915164
Genotype21	14	FJ915165
A. hancocki1	Playa Cocos, Costa Rica	FJ915181
A. hancocki2	Playa Cocos, Costa Rica	FJ915182

Table 4.3 (a) Nineteen S7-1 haplotypes predicted from PHASE and (b) the haplotype phase of each specimen collected from the Gulf of California and southwestern Mexico.

(a)

	Haplotype
1	TTGTCTAGACCCAGAGCCGGTCCTACGATTAGTTCCCT
2	TTGTCTAGACCCAGAGCCGGTCCTACGTTTAGTTCCCT
3	TTGCGTAGACCCAGAGCCGGCCCTACGATTTGTCTCCT
4	TTGCGTAGACCCAGAGCCGGTCCTACGTTTTGTCTCCT
5	TTGCGTAGACCCAGAGCCGCTCCTACGTTTTGTCTCCT
6	TTGCCTAGACCCAGAGCCGCTCCTATGTTTTGTTCCCT
7	TTGCCTAGACCCAGAGCCGCTCCTATGTTTTATTCCCT
8	TTGCCTAGACCCAGAGTCACTCCTATGTTTTGTTCTCA
9	TTGCCTAGACCCTGAGCCGGTCCCACGTTTTGTCTCCT
10	TTGCCTAGACCCTGAGCCGCTCCTCCGTTTTGTTCCCT
11	TTGCCTAGACCCTGTGCCGCTCCTACGTTTTGTTCCCT
12	TTGCCTACACCCAGAGCCGCTCCTATGTTCTGTTCCCT
13	TTGCCTACACCCAGAGTCGCTCCTATGTTTTGTTCCCT
14	TTGCCTACACCCAGAGTCGCTCCTATGTCTTGTTCCCT
15	TTGCCTACACACAGAGTCGCTCCTATGTTTTGTTCCCT
16	TTACCTGGACCCAAATCCGGTTCTATATTTTGTTCCGT
17	TCGCCTAGACCTTGAGCTGGTCATACGTTTTGTTCCCT
18	ATGCCTAGTCCCAGAGCCGCTCCTATGTTTTATTCCCT
19	ATACCAAGATCCAGATCCGCTCCTACGTTTTGCTCCCT

(b)

Gulf of California		Southwestern Mexico	
07-113-1	(3,4)	02-23-1	(6,6)
07-113-2	(3,3)	02-23-2	(5,12)
07-114-1	(3,3)	02-23-3	(6,6)
07-114-2	(5,5)	03-152-1	(7,9)
07-115-1	(3,5)	03-152-2	(5,15)
07-115-2	(2,5)	03-152-3	(9,16)
07-116-1	(3,3)	01-182-1	(7,18)
07-116-2	(2,3)	01-182-2	(6,8)
07-119-1	(3,5)	01-182-3	(6,14)
07-119-2	(3,5)	01-182-4	(6,19)
05-122-1	(2,3)	01-48-1	(13,17)
05-122-2	(3,3)	01-49-1	(6,10)
03-79-1	(3,3)	01-41-1	(6,6)
03-79-2	(4,11)		
06-1-1	(1,3)		
06-1-2	(2,2)		
02-22-1	(3,3)		
02-22-2	(2,3)		
02-22-3	(3,5)		

Table 4.4 Vertebral and fin elements of 40 cleared and stained paratypes of *A. hastingsi*.

Site	Vertebrae						Dorsal									Anal			
	Caudal			Total			Spines			Soft Rays			Total			Soft Rays			
	31	32*	33	42	43	44*	23	24*	25	12	13*	14	35	36	37*	38	23	24	25	26*
SIO 65-272 (n=10)	0	7	3	0	7	3	0	4	6	2	6	2	0	0	4	6	0	0	3	7
SIO 59-229-J (n=10)	3	6	1	3	6	1	1	5	4	2	7	1	1	0	5	4	0	0	6	4
SIO 59-225-Q (n=10)	0	7	3	0	7	3	0	7	3	0	6	4	0	0	3	7	0	0	5	5
SIO 65-342 (n=10)	1	8	1	1	8	1	0	5	5	3	7	0	0	0	8	2	0	1	8	1
A. macrospilus							3	25	2	3	26	1	0	5	23	2	1	11	16	2
A. mangognatha							1	8	0	1	7	1	0	2	6	1	0	0	8	1

* marks the category that includes the type specimen. *A. macrospilus* and *A. mangognatha* data from Stephens (1963) Table 2.

Table 4.5 Size ratios sorted by sex.

	head length in standard length	jaw length in head length	orbit diameter in head length^{+}	body depth in standard length^{+}	orbit diameter in snout length^{+}	predorsal length in preanal length
Males (n=22)	3.85 (0.15)	1.81 (0.06)	4.35 (0.39)	6.28 (0.34)	1.01 (0.11)	2.46 (0.15)
Females (n=21)	3.85 (0.18)	1.81 (0.08)	3.87 (0.24)	6.63 (0.3)	0.86 (0.08)	2.50 (0.11)
Total Mean (n=43)	3.85 (0.16)	1.81 (0.07)	4.12 (0.4)	6.45 (.036)	0.94 (0.12)	2.48 (0.13)

Male standard length range = 33.7-51.0 mm. Female standard length range = 29.8-40.0 mm. $^{+}$ indicates a ratio where the values for males and females are significantly different according to an unpaired t-test.

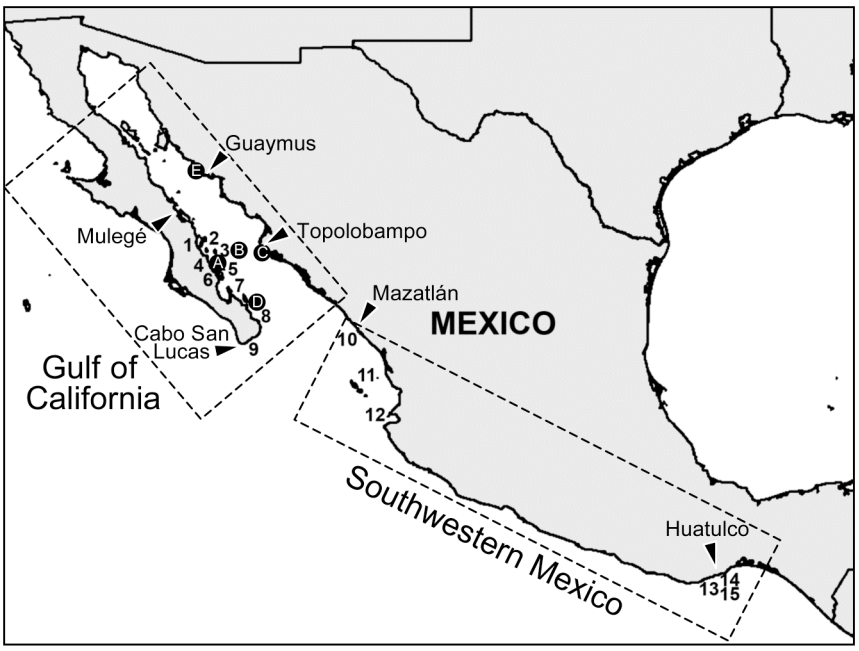

Fig. 4.1 Collecting sites for molecular (1-15) and morphological data (A-E) in the Tropical Eastern Pacific. The site labels, latitude and longitude information are as listed in Table 1.

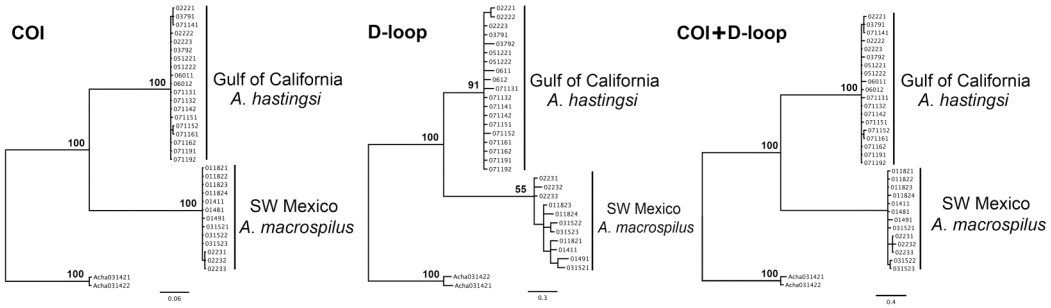

Fig. 4.2 Bayesian Inference phylogeny based on 560bp COI, 422bp D-loop and the combined data with *A. hancocki* as the outgroup. Numbers above the nodes are posterior probabilities above 50.

Fig. 4.3 (A) *Acanthemblemaria hastingsi*, sp. nov., holotype (SIO 65-272), 43.6 mm SL. (B) *Acanthemblemaria hastingsi*, sp. nov., life colors.

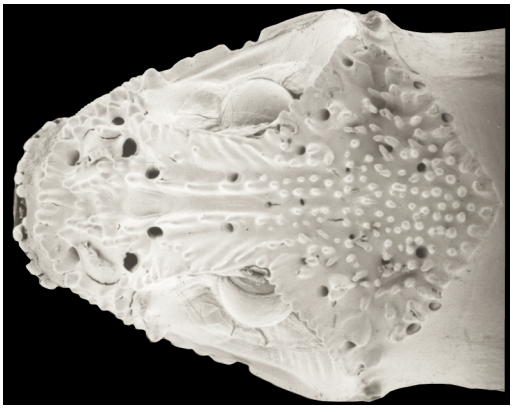

Fig. 4.4 Scanning electron microscope image of *Acanthemblemaria hastingsi* head spination (SIO 65-318) (from Rosenblatt and McCosker, 1988, Fig. 2c).

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