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Molecular phylogeny of 48 species of damselfishes (Perciformes: Pomacentridae) using 12S mtDNA sequences

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Abstract

Phylogenetic relationships within the family Pomacentridae (Teleostei: Perciformes) were inferred by analyzing a portion of the 12S mitochondrial ribosomal DNA gene. Thirty-four pomacentrid species were sequenced for this study and the resulting data were combined with previously published pomacentrid sequence data to form a combined matrix of 53 pomacentrids representing 48 different species in 18 genera. Four outgroup species were also drawn from published data; these taxa were taken from the other three putative families of the suborder Labroidei, as well as a single representative of the family Moronidae. The data set contained 1053 data columns after alignment according to ribosomal secondary structure and the removal of all ambiguously aligned positions. The resulting strict consensus tree topology generally agreed with the previous molecular hypothesis, and recovers a monophyletic Pomacentridae and subfamily Amphiprioninae. The two other subfamilies included, Chrominae and Pomacentrinae, were found to be polyphyletic. A monophyletic group consisting of the Amphiprioninae, *Pomacentrus*, *Acanthochromis*, *Amblyglyphidodon*, *Neoglyphidodon*, *Chrysiptera*, *Neopomacentrus*, and *Teixeirichthys* was found. This group was recovered as the sister group to a clade consisting of a paraphyletic *Chromis* and a monophyletic *Dascyllus*. A sister-group relationship between the genus *Pomacentrus* and the subfamily Amphiprioninae was observed.
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1. Introduction

Damselfishes (Teleostei: Pomacentridae) are a diverse and widespread family of primarily marine fishes found throughout the tropical oceans, forming a major component of coral reef communities (Allen, 1975). Although the bulk of damselfish diversity is concentrated in tropical regions, some species are found in temperate waters and others are known from freshwater and brackish environments (Allen, 1989). Pomacentrids are usually small in size, with reef species often no more than 100 mm in length, though several temperate species can get much larger, exceeding 250 mm in standard length (Allen, 1991). Because most species are associated with reefs and tidal zones, damselfishes are usually found near shore and in shallow water (within 20–25 m of the surface); however, several species do occur at depths greater than 100 m (Allen, 1975).

The Pomacentridae is currently recognized as a member of the perciform suborder Labroidei (Nelson, 1994), along with the families Cichlidae, Embiotocidae, and Labridae. However, there is some debate over the monophyly of this suborder. Based on morphological characters, these four families form a monophyletic group (Kaufman and Liem, 1982; Stiassny and Jensen, 1987). However, the monophyly of this suborder has been questioned (Johnson, 1993; Rosen and Patterson, 1990), with a recent molecular study suggesting that the suborder is not monophyletic (Streelman and Karl, 1997).

Currently, there are approximately 340 recognized pomacentrid species divided into 29 genera. The family is composed of four subfamilies: Amphiprioninae, Chrominae, Lepidozyginae, and Pomacentrinae (Allen, 1975, 1991). The members of the Amphiprioninae are unique because all of the species have an obligate

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symbiotic relationship with sea anemones. The approximately 28 species of this subfamily are united by a number of morphological (Allen, 1972, 1975; Fitzpatrick, 1992; Tang, unpublished) and molecular (Elliott et al., 1999; Tang, 2001) characters. The majority of pomacentrid diversity is concentrated in two of the subfamilies: the Chrominae, with over 80 species, and the Pomacentrinae, with more than 200 species. The Chrominae has been recognized as a natural group on the basis of some morphological characters (Allen, 1975); however, recent molecular analyses have cast some doubt on the monophyly of this subfamily (Tang, 2001). The subfamily Pomacentrinae is not considered a natural assemblage (Allen, 1975), a hypothesis corroborated by DNA data (Tang, 2001). Species placed in this subfamily are characterized simply as possessing an orbiculate to elongate body shape (Allen, 1975). Finally, *Lepidozygus tapeinosoma* is the sole representative of the monotypic Lepidozyginae. Prior to Allen (1975), who placed *Lepidozygus* in its own subfamily, *L. tapeinosoma* was considered a member of the Chrominae (Norman, 1957).

The damselfishes have long posed a challenge to systematists because of their diversity and intraspecific variation (e.g., species can exhibit enormous variation in coloration, both among adults and between juvenile and adult stages). The classification of the Pomacentridae that is in use today was established by Allen (1975, 1991), but, aside from the classification itself, no hypothesis of the relationships within the family was presented. Shao (1986) used morphometric measurements to examine generic relationships within the family. Using morphological characters, Fitzpatrick (1992) found characters supporting the monophyly of the family, but the generic and subfamilial relationships were almost completely unresolved in her analysis. Based on that work and others (Kaufman and Liem, 1982; Lauder and Liem, 1983; Stiassny, 1981), the monophyly of the Pomacentridae is supported by five synapomorphies: (1) a strong sheet of connective tissue originating from the dorsal border of the medial face of the dentary that merges with a cylindrical ligament and inserts onto the ceratohyal (anterior ceratohyal) bone (Stiassny, 1981); (2) a pair of nipple-like processes on the ventral surface of the lower pharyngeal jaw that act as insertion sites for the *pharyngohyoideus* muscle; (3) a pharyngo-cleithral articulation between the cleithra and the muscular processes of the lower pharyngeal jaw; (4) a prominent *obliquus posterior* muscle which is separated from the fourth *levator externus* muscle by a distinct aponeurosis (Kaufman and Liem, 1982; Lauder and Liem, 1983); and (5) the presence of two anal spines (Fitzpatrick, 1992; i.e., first anal pterygiophore with two supernumerary spines and a serially associated soft ray).

Tang (2001) used sequence data from the 12S and 16S mitochondrial ribosomal genes to investigate relationships within the family, producing a well-resolved phylogenetic hypothesis of relationships within the Pomacentridae. The data found strong support for the monophyly of both the family Pomacentridae and the subfamily Amphiprioninae. Within the Amphiprioninae, *Premnas* was found nested within the genus *Amphiprion*, as the sister group to an *Amphiprion ocellaris* + *A. percula* clade, thereby rendering *Amphiprion* paraphyletic. The Chrominae were found to be polyphyletic, because one putative member (*Mecaenichthys*) appeared in a more basal position in the tree. Although the subfamily Chrominae was not found to be a monophyletic subfamily, there was strong support for the monophyly of a *Chromis* + *Dascyllus* clade. Within this clade, *Chromis* was found to be paraphyletic relative to *Dascyllus*. Tang's (2001) analysis also recovered a novel clade, one composed of the Amphiprioninae, *Pomacentrus*, *Neoglyphidodon*, and *Amblyglyphidodon*. This large clade was the sister group of the *Chromis* + *Dascyllus* clade. The Pomacentrinae were recovered as a broadly polyphyletic assemblage; putative members of this subfamily were scattered throughout the tree. There was strong support for the monophyly of four genera as well: *Abudefduf*, *Dascyllus*, *Pomacentrus*, and *Stegastes*. A basal clade that included *Stegastes*, *Microspathodon*, *Hypsypops*, *Parma*, and *Plectroglyphidodon* was also recovered, although the support for this clade was weak.

The goal of this study is to use sequence data from a portion of the 12S gene to infer the phylogenetic relationships among representative species of the family Pomacentridae, and to compare these results with the phylogenetic framework that has been proposed previously (Tang, 2001). One of the possible avenues of future research suggested by Tang (2001) was the addition of more taxa to improve sampling and representation, which this study accomplishes. This analysis will resolve the phylogenetic position of four genera and 25 species that were not represented in that previous study, providing a more robust estimate of pomacentrid phylogeny.

2. Materials and methods

2.1. Material examined

Fishes used in this study were collected with a variety of methods, including the use of hand-nets while SCUBA diving, gillnets, and from aquarium specimens. Tissue samples were stored without preservation at −70 °C in an ultracold freezer. Thirty-four species of pomacentrids were sequenced for this study, representing 14 of the 29 recognized genera. These taxa represent three of the four subfamilies; tissue from the monotypic subfamily Lepidozyginae was not available. Four species (*Amphiprion frenatus*, *A. ocellaris*, *Dascyllus melanurus*, and *D. reticulatus*) originally sequenced for this

project were first published in a previous analysis (Tang, 2001). Sequence data from an additional 19 taxa were included from Tang (2001). Thus, data from a total of 53 pomacentrids, representing 48 different species and 18 genera, were compiled for the data matrix used in this study. Five species (*Abudefduf sexfasciatus*, *Amphiprion clarkii*, *Dascyllus aruanus*, *Plectroglyphidodon lacrymatus*, and *Premnas biaculeatus*) were represented by two different individuals in the data matrix. Following Eschmeyer et al. (1998), the name *Stegastes adustus* (Troschel) is used herein as the senior synonym for *S. dorsopunicans* (Poey). A complete list of ingroup representatives examined is given in Table 1.

Outgroup taxa were drawn from the Cichlidae, Embiotocidae, Labridae, and Moronidae. The first three families (Cichlidae, Embiotocidae, and Labridae) are outgroup candidates because they, along with the Pomacentridae, are considered members of the suborder Labroidei (Kaufman and Liem, 1982; Lauder and Liem, 1983; Stiassny and Jensen, 1987). However, considering the debate over the monophyly of the Labroidei (see Section 1), in addition to including a single species from each of those three putative labroid families as outgroups, one species from the family Moronidae was included as well to serve as a "generic" perciform outgroup; sequence data for this outgroup taxon were taken from Tang et al. (1999). A complete list of outgroup species examined is given in Table 1.

2.2. DNA amplification and sequencing

Only a portion of the 12S gene region was examined for this study. This project, which only entailed the sequencing of the 12S region, was completed independently of the work done by Tang (2001), so data from the 16S gene were not collected. Despite the absence of 16S data, this data matrix has almost 60% more sequenced base positions (54,574 bp, prior to alignment) than what was sequenced in the previous study (34,121 bp, prior to alignment; Tang, 2001) because of the increased number of taxa. For the 34 taxa sequenced in this study, genomic extractions were done from muscle tissue with standard phenol/chloroform extraction protocols, following Kocher et al. (1989). Target regions of the mtDNA were amplified using the polymerase chain reaction (PCR), following Saiki (1990). TaqDNA polymerase (HT Biotechnology) and the two 20-bp oligonucleotide primers listed in Table 2 were used to amplify an approximately 600-bp fragment of the 12S ribosomal gene, using the following thermal cycling profile: 94 °C denaturing for 60 s, 45 °C annealing for 70 s, and 72 °C extension for 2 min for 36 cycles (modified from Saiki, 1990). Each PCR reaction was preceded by a hot start at 94 °C for 2 min to improve the yield. The resulting amplified products were then reamplified to generate single-stranded DNA (ssDNA), following Sanger et al. (1977). The resulting ssDNA fragment was purified via agarose gel separation, electro-elution, and phenol/chloroform extraction, following Sambrook et al. (1989). The purified product was suspended in double-distilled water and prepared for manual sequencing, which was performed with an Amplicycle sequencing kit (Perkin–Elmer), [^{35}S]-dNTPs, and the primers indicated in Table 2. The sequencing reactions were carried out with the following thermal cycling profile: 95 °C for denaturing, 45 °C for annealing, and 72 °C for extension, 60 s for each step, for 30 cycles. Each sequencing reaction was electrophoresed on a 6% polyacrylamide gel and visualized by exposure to X-Omat X-ray film (Kodak) for 48–96 h. The sequences ranged from 378 bp (*Neopomacentrus azysron*) to 646 bp (*D. aruanus* and *P. biaculeatus*) in length, prior to alignment. All sequences were deposited in GenBank (Table 1). All DNA sequence data from Tang (2001) were collected via automated sequencing. See Tang (2001) for the DNA amplification and sequencing protocols used for the other 19 pomacentrids and the four outgroup species that were used in this analysis.

2.3. DNA alignment

All DNA sequences from the complementary light and heavy strands were spliced together to form a consensus light strand sequence. These 32 consensus sequences were first compiled into a data matrix using the Pileup option of the computer program GCG 8.01 (Genetic Computer Group, Wisconsin Sequence Analysis) and were subsequently aligned against a previously published data matrix (Tang, 2001). The published sequences were originally compiled using the computer program Sequence Navigator 1.01 (Applied Biosystems), where a preliminary alignment was performed using the CLUSTAL option included in that computer software. These data were then exported as a NEXUS file and the sequences were manually aligned in PAUP* 4.0b6 (Swofford, unpublished). The data were organized into stem and loop regions using published secondary structure models for the 12S mitochondrial ribosomal DNA gene (Van de Peer et al., 1994). See Tang (2001) for a more detailed treatment of sequence alignment procedures. All sequences generated for this study were incorporated into the existing data matrix used in that study. A PAUP/NEXUS file with the complete alignment is available from the corresponding author upon request; the file may also be downloaded at http://www.nhm.ku.edu/fishes/data.

2.4. Missing data

There was incomplete overlap in the two sets of sequence data. The sequences produced for this analysis begin approximately 300 bp from the 5′ end of the 12S

Table 1
List of species used in the study, following accepted classification (Allen, 1975, 1991), and GenBank accession numbers

Taxon	Catalog number (KU tissue number)	Accession no.
Order Perciformes		
Suborder Percoidel		
Family Moronidae		
Morone chrysops	KU 22901 (T823)	AF055589
Suborder Labroidei		
Family Cichlidae		
Crenicichla lepidota	KU 23532 (T605)	AF285917
Family Embiotocidae		
Embiotoca jacksoni	SIO uncataloged (T58)	AF285918
Family Labridae		
Halichoeres chrysus	KU 22975 (T29)	AF285919
Family Pomacentridae		
Subfamily Amphiprioninae		
Amphiprion clarkii	KU 22981 (T49)	AF285923
***A. clarkii* #2**	ASIZ uncataloged	AF081219
A. frenatus	ASIZ uncataloged	AF081220
A. ocellaris	ASIZ uncataloged	AF081221
A. percula	KU 27120 (T2928)	AF285924
A. perideraion	ASIZ uncataloged	AF081222
Premnas biaculeatus	KU 22965 (T20)	AF285936
***P. biaculeatus* #2**	ASIZ uncataloged	AF081234
Subfamily Chrominae		
Acanthochromis polyacanthus	ASIZ uncataloged	AF081217
Chromis analis	ASIZ uncataloged	AF081223
Chro. cyanea	USNM 343867 (T77)	AF285925
Chro. fumea	ASIZ uncataloged	AF081224
Chro. iomelas	USNM 334168 (T704)	AF285926
Chro.viridis	ASIZ uncataloged	AF081225
Dascyllus aruanus	USNM 334282 (T768)	AF285927
***D. aruanus* #2**	ASIZ uncataloged	AF081228
D. melanurus	ASIZ uncataloged	AF081229
D. reticulatus	ASIZ uncataloged	AF081230
Mecaenichthys immaculatus	AMS 38734004 (T3093)	AF285929
Subfamily Pomacentrinae		
Abudefduf saxatilis	USNM 349037 (T194)	AF285920
A. sexfasciatus	USNM 334161 (T714)	AF285921
***A. sexfasciatus* #2**	ASIZ uncataloged	AF081216
A. sordidus	ASIZ uncataloged	AF436879
A. vaigiensis	ASIZ uncataloged	AF436880
Amblyglyphidodon aureus	USNM 336462 (T773)	AF285922
A. curacao	ASIZ uncataloged	AF081218
Chrysiptera leucopoma	ASIZ uncataloged	AF081226
C. rex	ASIZ uncataloged	AF081227
Hypsypops rubicundus	KU 27890 (T3488)	AF285928
Microspathodon chrysurus	USNM 329833 (T142)	AF285930
Neoglyphidodon melas	ASIZ uncataloged	AF081231
N. nigroris	ASIZ uncataloged	AF081232
N. polyacanthus	AMS 34851001 (T3094)	AF285931
Neopomacentrus azysron	ASIZ uncataloged	AF081233
Parma oligolepis	AMS 31253055 (T3096)	AF285932
Plectroglyphidodon dickii	ASIZ uncataloged	AF081240
P. lacrymatus	USNM 334304 (T661)	AF285933
***P. lacrymatus* #2**	ASIZ uncataloged	AF081242
P. leucozonus	ASIZ uncataloged	AF081241
Pomacentrus auriventrus	ASIZ uncataloged	AF081235
P. bankanensis	ASIZ uncataloged	AF081236
P. brachialis	USNM 334307 (T763)	AF285934
P. chrysurus	ASIZ uncataloged	AF081237
P. coelestis	ASIZ uncataloged	AF081238
P. moluccensis	ASIZ uncataloged	AF081239
P. vaiuli	USNM 334312 (T690)	AF285935
Stegastes adustus	USNM 327594 (T86)	AF285937
S. altus	ASIZ uncataloged	AF081243

Table 1 (*continued*)

Taxon	Catalog number (KU tissue number)	Accession No.
S. fasciolatus	ASIZ uncataloged	AF081244
S. lividus	ASIZ uncataloged	AF081245
S. obreptus	ASIZ uncataloged	AF081246
S. variabilis	USNM 327596 (T188)	AF285938
Teixeirichthys jordani	ASIZ uncataloged	AF081247

Note. Species in boldface text were sequenced for this study, all other materials examined are from Tang et al. (1999) and Tang (2001).

Abbreviations. AMS, Australian Museum; ASIZ, Academia Sinica, Institute of Zoology, ROC; KU, University of Kansas; SIO, Scripps Institute of Oceanography; USNM, Smithsonian Institution, United States National Museum.

Table 2
Sequencing and amplification primers used in the study

Primer	Sequence (5′–3′)
PB2 (L)	CAAGTTGACAGACAACGGCG
PV (H)	GCACGGATGTCTTCTCGGTG

gene and extend through to the 3′ end of the 12S gene, whereas most of the sequences from Tang (2001) include the 5′ end of the 12S gene, beginning approximately 50 bp from the 3′ end of the tRNA-Phe gene, but do not extend to the 3′ end, stopping approximately 100 bp short of the start of the tRNA-Val gene. Therefore, all the sequences from Tang (2001) were missing at least 100 bp from the 3′ end of the 12S gene and all the new sequences generated are missing at least 300 bp from the 5′ end of the gene. One species, *Neopomacentrus azysron*, is missing approximately 600 bp from the 5′ end of the gene, and only 378 bp were collected from the 3′ end because of an error during sequencing. Although all of the taxa have some characters with missing data, studies have shown that, given a choice, including such characters in an analysis is better than simply excluding all those characters because doing so generally increases phylogenetic accuracy, provided the missing data are not abundant (Wiens, 1998). Therefore, rather than deleting hundreds of base positions (aligned data columns) for which only some data were missing, those characters were coded as missing and included for the phylogenetic analysis.

2.5. Site saturation

Analyses were performed to identify potential site saturation (i.e., multiple mutations at a single site) in both the stem and loop regions. Possible site saturation was examined by plotting the number of substitutions between pairs of taxa against mean patristic distance and Tamura–Nei distance (Tamura and Nei, 1993); both the patristic distance and Tamura–Nei values were generated in PAUP* 4.0 (Swofford, unpublished). Mean patristic distances were calculated from the 100 best trees retained in an initial heuristic parsimony search. The 100 patristic distances for each pairwise comparison were parsed and the mean was calculated by the computer program PatSat (Benson, unpublished).

2.6. Phylogenetic analyses

Phylogenetic analyses were carried out using the heuristic search option of PAUP* 4.0b6 (Swofford, unpublished), with 1000 random addition sequence replicates and tree-bisection-reconnection set as options. Gaps were common in loop regions and were included in all analyses as a fifth character state. All characters were equally weighted. Phylogenetic trees were evaluated using summary values reported by PAUP (e.g., tree length, consistency index). Support for each internode (i.e., monophyletic group) was evaluated by calculating decay index (Bremer, 1988, 1994) and bootstrap values (Felsenstein, 1985). Decay indices were generated using TreeRot (Sorenson, 1996) and bootstrap values were calculated in PAUP, using 1000 bootstrap replications of a simple heuristic search. Three constrained analyses were conducted to examine possible alternate topologies of relationships. The constraint trees enforced: (1) a monophyletic Chrominae; (2) a monophyletic *Chromis*; and (3) a monophyletic *Stegastes*. Templeton's tests (Templeton, 1983) were then performed to compare each of these alternate tree topologies to the most-parsimonious topologies. The character state differences were generated by MacClade 4.0 (Maddison and Maddison, 2000). The statistical tests were conducted with the computer program Minitab 10.5 (Minitab).

3. Results

The final aligned data matrix contained 1058 aligned base positions. Five such data columns were excluded from the analyses because of ambiguous alignment (i.e., an inability to confidently make homology statements between different taxa). Of the remaining 1053 base positions, 383 were parsimony-informative. Plotting the number of differences between pairs of taxa against their Tamura–Nei distance and mean patristic distance revealed no discernable evidence of site saturation in the stem regions or among the transversion substitutions in the loop regions. Among the least conservative class of mutations, transitions in loop regions, there was no conclusive evidence for site saturation. Plotting the number of loop transitions against Tamura–Nei distance shows little or no deviation from a linear relation

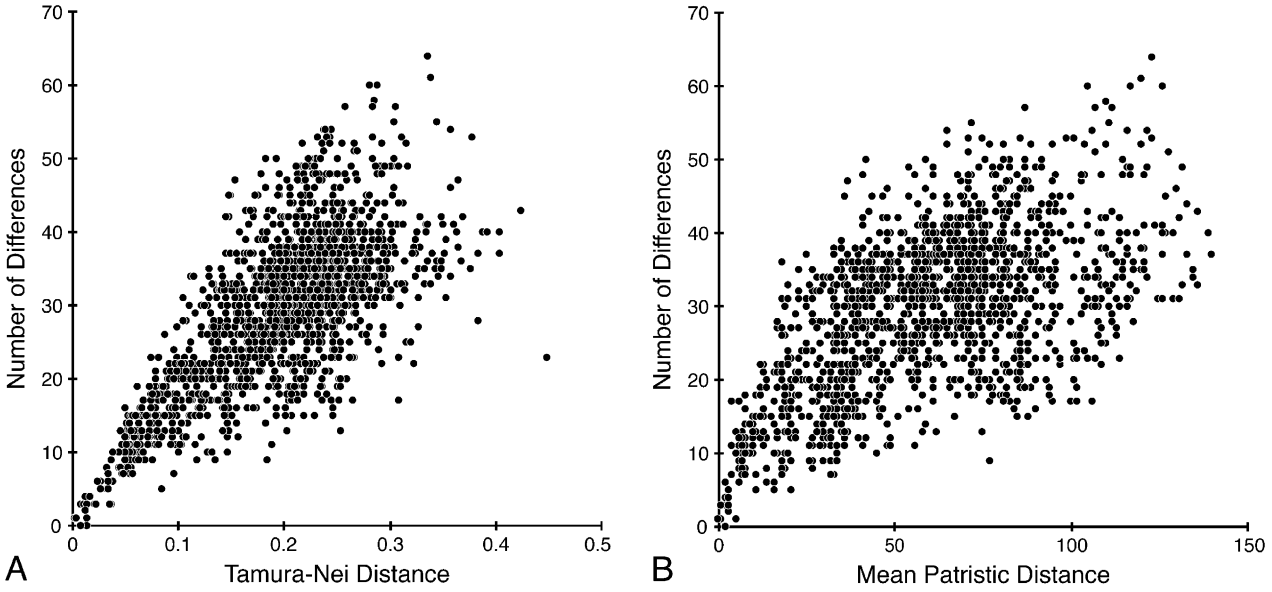

Fig. 1. Scatter-plot of the number of transition substitutions vs. the (A) Tamura–Nei distance and (B) mean patristic distance in pairwise comparisons in loop regions.

between the number of mutations and sequence divergence (Fig. 1A). The mean patristic distance plot displays an ambiguous result, there may or may not be a divergence from a linear relationship (Fig. 1B).

The phylogenetic analysis yielded 16 most-parsimonious trees each with a total length of 2124 steps (CI = 0.435; HI = 0.565; RI = 0.643; RC = 0.280). The strict consensus of the 16 trees is shown with decay index and bootstrap values at the appropriate internodes (Fig. 2). Bootstrap values below 50% are not shown. This strict consensus topology is well resolved, with only a few areas of uncertainty. The alternate topologies are entirely the result of instability within two clades, the genus *Abudefduf* and the subfamily Amphipioninae. The family itself, Pomacentridae, is recovered as a monophyletic group. Of the three subfamilies examined for this study, only the Amphiprioninae appears to be monophyletic. Within the Amphiprioninae, the position of *Premnas* is unstable relative to the species of *Amphiprion*: in four of the most-parsimonious trees *Premnas* is found to be the sister group to a monophyletic *Amphiprion*; in the other 12 trees, *Premnas* is recovered within the genus *Amphiprion*, as the sister group to an *A. ocellaris* + *A. percula* clade (subgenus *Actinicola*). Both the Chrominae and the Pomacentrinae are polyphyletic. Even though the subfamily Chrominae is not monophlyetic, there is support for a *Chromis* + *Dascyllus* clade (Fig. 2), but *Chromis* appears to be paraphyletic relative to a monophyletic *Dascyllus*. The other two chromine genera that were sequenced, *Acanthochromis* and *Mecaenichthys* (both monotypic genera), appear in very different parts of the tree. The subfamily Pomacentrinae is broadly polyphyletic, with putative members distributed throughout the tree. Among its genera, *Abudefduf*, *Amblyglyphidodon*, *Chrysiptera*, *Neoglyphidodon*, and *Pomacentrus* are monophyletic, with *Abudefduf* and *Chrysiptera* having the best branch support. *Pomacentrus* is recovered as the sister group to the Amphiprioninae and these taxa are part of a much larger clade which also includes *Acanthochromis*, *Amblyglyphidodon*, *Chrysiptera*, *Neoglyphidodon*, *Neopomacentrus*, and *Teixeirichthys*. The branch support for this large crown group is among the most robust in the tree, this group has the best support of any clade containing more than two genera. Within this group, *Acanthochromis*, a putative chromine, appears to be the sister group to an *Amblyglyphidodon* + *Neoglyphidodon* clade, and this clade of three genera is sister to the Amphiprioninae + *Pomacentrus* clade. *Chrysiptera* is the sister group of that clade, with *Neopomacentrus* as the next most basal member, and *Teixeirichthys* at the base of the large clade.

The constrained analysis enforcing a monophyletic *Stegastes* resulted in trees that were one step longer (TL = 2125) than the most-parsimonious resolution (TL = 2124). The results of the Templeton's test indicated that this alternate topology was not significantly different from the most-parsimonious one ($p = 0.40$). The constrained analysis enforcing a monophyletic Chrominae recovered trees that were 52 steps longer (TL = 2176) than the shortest trees, and comparison of the shortest trees with the constrained trees indicated that the alternate topologies were significantly different ($p < 0.0001$). Trees 10 steps longer (TL = 2134) resulted from the analysis enforcing a monophyletic *Chromis*, and the Templeton's test found that this topology was significantly different ($p = 0.02$).

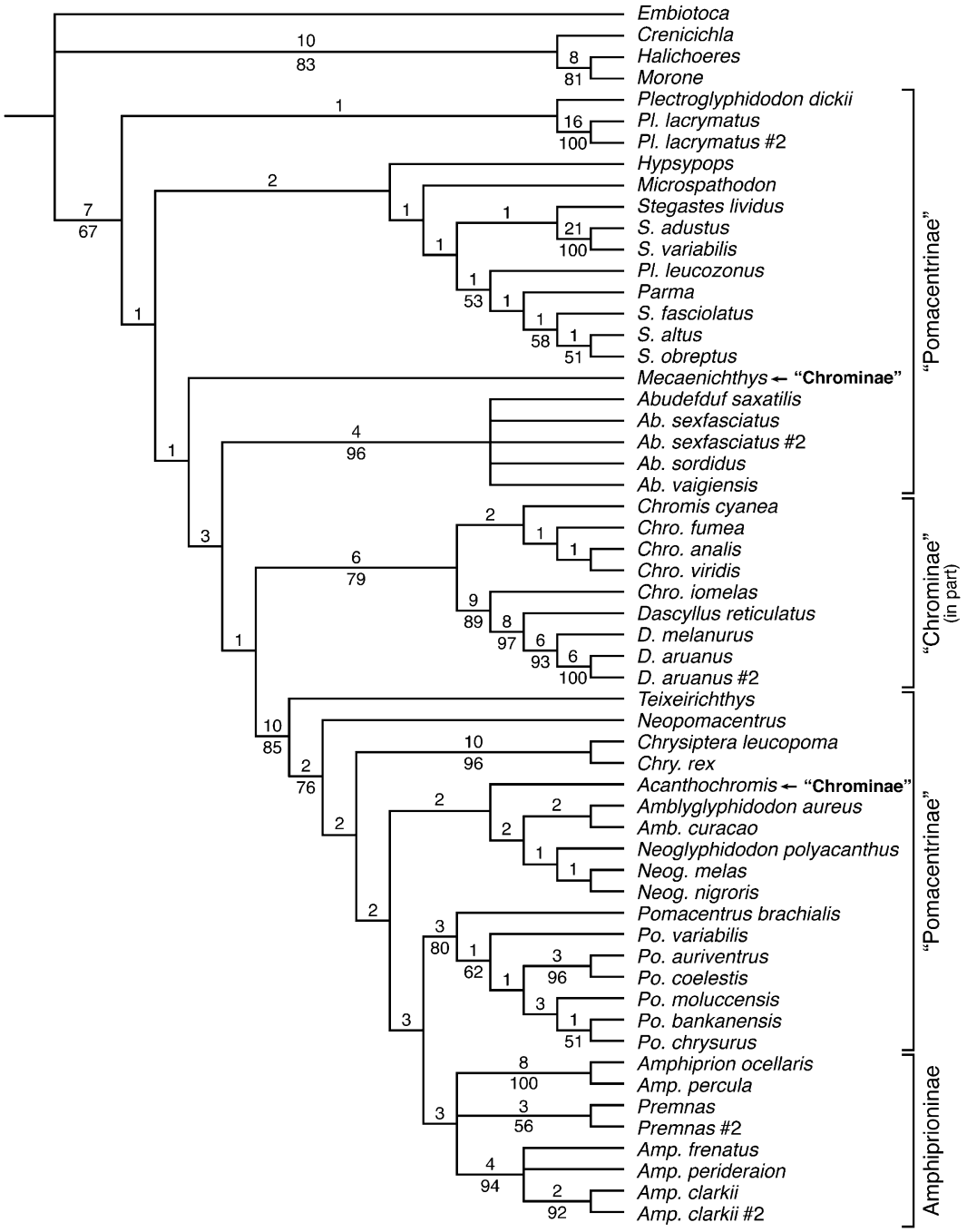

Fig. 2. Strict consensus of 16 most-parsimonious trees, total length = 2124 steps, CI = 0.435, HI = 0.565, RI = 0.643, RC = 0.280 (values are for each most-parsimonious tree). Decay index support (above) and bootstrap values for 1000 replicates (below) are indicated at each node, bootstrap values below 50% have been omitted.

4. Discussion

Sixteen most-parsimonious trees resulted from the phylogenetic analysis. The strict consensus of those most-parsimonious topologies finds a monophyletic family Pomacentridae (Fig. 2). Monophyly of the family is congruent with previous hypotheses, both molecular (Tang, 2001) and morphological (Fitzpatrick, 1992; Kaufman and Liem, 1982; Stiassny, 1981). Support for a monophyletic Pomacentridae was strong in the previous molecular analysis (Tang, 2001), and this tree topology is highly congruent with the relationships proposed in that study.

Of the three subfamilies examined herein, the subfamily Amphiprioninae (anemonefishes) is the only one that was found to be monophyletic. Support for this clade is relatively weak, which is surprising considering previous studies have found strong support for the

monophyly of this group: phylogenetic analyses using morphological data find that the group is united by a number of synapomorphies (Fitzpatrick, 1992; Tang, unpublished), and molecular studies have corroborated those findings (Elliott et al., 1999; Tang, 2001). Though they did not include many outgroup pomacentrids in their analysis, Elliott et al. (1999) found support for a monophyletic Amphiprioninae. Tang (2001) included additional genera and species of pomacentrids, and found very robust support for the monophyly of the subfamily.

Within the Amphiprioninae, the relationships are largely unresolved. In 12 of the 16 most-parsimonious trees, *Amphiprion* is not recovered as monophyletic because *Premnas* appears as the sister group to the subgenus *Actinicola* (*A. ocellaris* + *A. percula*). However, in four of the most-parsimonious resolutions, *Amphiprion* is monophyletic, with *Premnas* as its sister genus. These two conflicting results correspond with the two current hypotheses of anemonefish relationships. A monophyletic *Amphiprion* agrees with the preferred topology of Elliott et al. (1999, Fig. 3a), whereas a paraphyletic *Amphiprion*, with *Premnas* as the sister to an *A. ocellaris* + *A. percula* clade (subgenus *Actinicola*) agrees with the tree recovered by Tang (2001, Fig. 2). Such a sister-group relationship between the two species of *Actinicola* (the *percula* species complex of Allen, 1972) and *Premnas* has been proposed previously by Allen (1972, Fig. 12). Even though Elliott et al.'s (1999) preferred tree shows a monophyletic *Amphiprion*, some of their cytochrome *b* data supports an *A. ocellaris* + *Premnas* clade (Elliott et al., 1999, pp. 680–681). The data resolving this part of the tree are not conclusive, additional data are required to settle this issue.

These results resolve a monophyletic subgenus *Actinicola* (*A. ocellaris* + *A. percula*) within *Amphiprion*, with strong branch support, an outcome congruent with the classification proposed by Allen (1972) and the results of Tang (2001). Other relationships within the genus *Amphiprion* do not match those of previous studies. For example, the monophyly of the subgenus *Amphiprion* is not supported, which agrees with the results of Elliott et al. (1999); however, relationships among the species are not the same as those results. There is strong support for a clade composed of *A. clarkii*, *A. frenatus*, and *A. perideraion*, and two representatives of *A. clarkii* group together as well. Without more representatives, conclusions about *Amphiprion* relationships are tentative at best.

There is strong support for the monophyly of a large crown clade composed of the Amphiprioninae, *Pomacentrus*, *Acanthochromis*, *Amblyglyphidodon*, *Neoglyphidodon*, *Chrysiptera*, *Neopomacentrus*, and *Teixeirichthys* (Fig. 2). The relationships recovered in this clade suggest several novel relationships which are unlike anything that has been proposed in traditional classifications of the family (Allen, 1972, 1975). However, there is corroboration for such a clade from the previous molecular phylogeny of this group (Tang, 2001). That study did not have all the taxa examined for this analysis; however, all those that were included (*Amphiprion*, *Premnas*, *Pomacentrus*, *Amblyglyphidodon*, and *Neoglyphidodon*) did form a clade, with strong branch support (Tang, 2001, Fig. 2). The sister group of the subfamily Amphiprioninae appears to be *Pomacentrus*, a monophyletic genus. This relationship corroborates the results of Tang (2001), which found an Amphiprioninae + *Pomacentrus* clade.

The subfamily Chrominae is polyphyletic and there is good evidence for this. From the Templeton's test, a tree enforcing the monophyly of the subfamily based on these data would be not only much longer (52 steps) than the shortest tree, but also significantly different from that shortest tree ($p < 0.0001$). However, despite the polyphyly of the Chrominae, a substantial subset of the group does cluster together. There is strong support for a *Chromis* + *Dascyllus* clade, with some of the more robustly supported branches in the tree found within the genus *Dascyllus*. The topology of relationships found within *Dascyllus* is identical to the one found in Tang (2001), which is not surprising since that study included the same four species examined here; the only new addition is a second representative of *D. aruanus*. The nominate genus of the subfamily, *Chromis*, is paraphyletic relative to *Dascyllus*. This finding appears to be non-trivial as the branch uniting *C. iomelas* with the *Dascyllus* clade is well supported. The results of the Templeton's test provide additional evidence supporting this, as constraining a monophyletic *Chromis* would yield a tree significantly different from the most-parsimonious resolution. A paraphyletic *Chromis* is consistent with the results of Tang (2001), which had two *Chromis* representatives and found a similar result. Larval characteristics lend some support to this as well. Although larval characters have not been used before in a phylogenetic analysis, *Chromis* species display a great diversity among its larvae, a diversity of larval forms not seen in other pomacentrid groups (Kavanagh et al., 2000).

The other large clade found by this analysis is a basal one containing an assortment of different genera: *Hypsypops*, *Microspathodon*, *Parma*, *Plectroglyphidodon* (in part), and *Stegastes*. The presence of such a clade is corroborated by Tang (2001), which found a clade with a similar composition of genera, though with fewer representative species and a monophyletic *Stegastes*. The genus *Stegastes* appears polyphyletic in this analysis, but support in this part of the tree is weak; trees only one step away from the most-parsimonious trees recover a monophyletic *Stegastes*, making it hard to draw any conclusions about the status of *Stegastes* with confidence. Not surprisingly, the Templeton's test highlights

this problem, as constraining a monophyletic *Stegastes* results in trees that are not significantly different from the most-parsimonious ones. In general, support in this basal part of the tree is not robust. Aside from the two representatives of *Plectroglyphidodon lacrymatus* grouping together and a *Stegastes adustus* + *S. variabilis* clade, no branches have very strong support.

Overall, the relationships recovered in this analysis corroborate many of the findings of Tang (2001), though the two studies differ in several details. Both trees recover monophyletic Pomacentridae and Amphiprioninae, as well as monophyletic *Abudefduf*, *Dascyllus*, and *Pomacentrus*. Both studies find a clade which contains the Amphiprioninae, *Amblyglyphidodon*, *Neoglyphidodon*, and *Pomacentrus*, with *Pomacentrus* as the sister group of the Amphiprioninae. Both studies also agree on a sister-group relationship between this large clade and a *Chromis* + *Dascyllus* clade, and both find *Chromis* paraphyletic relative to *Dascyllus*.

4.1. Taxonomic implications

The results of this study support several relationships that do not agree with the traditional classification of the family Pomacentridae, though the trees yielded from this study largely corroborate the earlier results of Tang (2001). The most obvious difference between the relationships based on molecular data and the traditional hypothesis involves the status of the subfamilies within the Pomacentridae. Though only three of the four putative subfamilies were included in this study, it is clear that some of them do not accurately reflect monophyletic groups within the family. The one subfamily not examined, Lepidozyginae, is monotypic. Of the remaining three, only one, the Amphiprioninae, appears to be monophyletic. The other two are polyphyletic, with putative species of both subfamilies scattered in different parts of the tree. This situation presents some difficult taxonomic problems. The "Chrominae" likely will require a reduction in its constituent species. Currently, only the species of *Chromis* and *Dascyllus* form a monophyletic group; even the monophyly of *Chromis* itself is in doubt and the relationships of the other two chromine genera, *Altrichthys* and *Azurina*, which were not available for sequencing, are unknown. The far flung nature of the pomacentrine genera in the tree poses a larger problem. The nature of the tree topology does not appear to allow any easy division of the pomacentrine genera into convenient monophyletic subfamilies. Chief among these problems is that the nominate genus for the subfamily, *Pomacentrus*, is the sister group of the only monophyletic subfamily, the Amphiprioninae. Although this is far from a complete sampling of pomacentrid taxa, it seems unlikely that the addition of new taxa and data will drastically change the topology, rendering broadly polyphyletic groups like the "Pomacentrinae" monophyletic. Given this, eventually, when a more complete hypothesis of relationships within the family is available, a complete revision of this family and its component subfamilies will be necessary. More genera and species need to be examined, not only to establish the relationships within the family and subfamilies, but also to test the monophyly of speciose genera like *Amphiprion*, *Chromis*, *Chrysiptera*, *Pomacentrus*, and *Stegastes*.

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