THE ROYAL SOCIETY

Molecular phylogenetic evidence for the evolution of specialization in anemonefishes

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Anemonefishes (genera: *Amphiprion* and *Premnas*; family Pomacentridae) are a group of 28 species of coral reef fishes that are found in obligate symbiosis with large tropical sea anemones. A phylogenetic hypothesis based on morphological analyses of this group suggests that the ancestral anemonefish was a generalist with similar morphology to other pomacentrids, and that it gave rise to other anemonefish species that were more specialized for living with particular species of host anemones. To test this hypothesis we constructed a molecular phylogeny for the anemonefishes by sequencing 1140 base pairs of the cytochrome *b* gene and 522 base pairs of the 16S rRNA gene for six species of anemonefishes (representatives of all subgenera and species complexes) and two other pomacentrid species. Three methods of phylogenetic analysis all strongly supported the conclusion that anemonefishes are a monophyletic group. The molecular phylogeny differs from the tree based on morphological data in that the two species of specialized anemonefishes (*Premnas biaculeatus* and *Amphiprion ocellaris*) were assigned to a basal position within the clade, and the extreme host generalist (*Amphiprion clarkii*) to a more derived position. Thus, the initial anemonefish ancestors were probably host specialists and subsequent speciation events led to a combination of generalist and specialist groups. Further phylogenetic studies of additional anemonefish species are required to substantiate this hypothesis.

Keywords: anemonefish; phylogeny; cytochrome *b*; 16S rRNA; specialization; mutualism

1. INTRODUCTION

Many groups of organisms have undergone spectacular adaptive radiations. Classic adaptive radiation theory assumes that generalist ancestors gave rise to more-specialized descendent species that diversified into narrower ecological niches as the number of species increased (e.g. Mayr 1942). Some reviews of the theory suggest that the generalist to specialist progression may be a net evolutionary trend that can be found in virtually every taxon (Futuyma & Moreno 1988; Futuyma 1998), while others claim that specialization is often not a derived condition (Thompson 1994). Many recent studies have begun to test this theory using character reconstruction analyses, which involve mapping the characteristics of extant species onto well-corroborated phylogenetic trees. Phylogenies based on molecular data are considered to be most appropriate since the characters are independent of the phenotypic traits being examined. For example, molecular phylogenetic studies of the evolution of trophic types within the Middle American cichlid fishes have shown that generalized predators probably gave rise to a variety of specialized substratum sifting and piscivorous species (Roe *et al*. 1997). Whether the generalist to specialist progression is a common evolutionary trend in other adaptive radiations has yet to be determined, and studies of a variety of organismal groups are needed to provide a broader perspective on this issue.

The development of a specialist condition from a generalist ancestor is commonly used to explain the evolution of specialization of symbiotic organisms to their hosts (Futuyma & Moreno 1988). Much of the research in this area has been conducted on insects and their plant hosts (Bernays & Chapman 1994; Thompson 1994), but relatively few studies have determined whether this evolutionary trend is common in other types of symbioses. One of the most well-known symbioses in the marine environment is between small, colourful anemonefishes and large, tropical sea anemones (Fautin & Allen 1997). The ecology and behaviour of the organisms involved in this symbiosis have been studied extensively (review in Fautin 1991), but relatively little is known about their evolutionary history.

Anemonefishes are members of the family Pomacentridae, subfamily Amphiprioninae. Traditional taxonomic studies based on morphology (Allen 1972, 1980, 1991), have divided the anemonefishes into two genera: the monotypic genus *Premnas*, which includes *P. biaculeatus*; and the genus *Amphiprion*, which includes 27 species that are divided into four subgenera and two species complexes (table 1). Allen outlined the hypothetical relationships within the subfamily Amphiprioninae, and suggested that the members of the subgenus *Amphiprion* are generalists and 'perhaps represent a stage that is not far removed from the main branch of pomacentrid evolution' (Allen 1972, p. 51) (see figure 1). The members of this subgenus are similar to many other pomacentrids in that they are relatively deep-bodied and good swimmers. Also, these anemonefishes are less dependent on their host anemones for shelter than other species, and members of the *clarkii* complex are host generalists living with up to

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Received 28 October 1998 *Accepted* 17 November 1998

Table 1. *Host specificity patterns reported by Fautin & Allen (1997) for six species of anemonefishes* (Premnas (P) *and* Amphiprion (A)) *and ten species of host anemones*

(The subgenera of the anemonefishes and species complexes are after Allen (1991). Legend to abbreviations and symbols used in the table are given below.)

fish species[a]	anemone species[a]										
	CA	EQ	MD	HM	HC	HA	HU	SH	SG	SM	total number of species
genus: *Premnas*											
P. biaculeatus		+									1
genus: *Amphiprion*											
subgenus: *Actinicola*											
A. ocellaris				+					+	+	3
subgenus: *Paramphiprion*											
A. polymnus			+		+			+			3
subgenus: *Phalerebus*											
A. sandaracinos					+					+	2
subgenus: *Amphiprion* *ephippium*-complex											
A. frenatus		+									1
clarkii complex											
A. clarkii	+	+	+	+	+	+	+	+	+	+	10

[a]CA, *Cryptodendrum adhaesivum*; EQ, *Entacmaea quadricolor*; MD, *Macrodactyla doreensis*; HM, *Heteractis magnifica*; HC, *Heteractis crispa*; HA, *Heteractis aurora*; HU, *Heteractis malu*; SH, *Stichodactyla haddoni*; SG, *Stichodactyla gigantea*; SM, *Stichodactyla mertensii*. CA is the family Thalassianthidae. EQ and MD are in the family Actiniidae. The rest of the anemone species are in the family Stichodactylidae. Plus sign indicates that fish and anemone species have been observed together in the field.

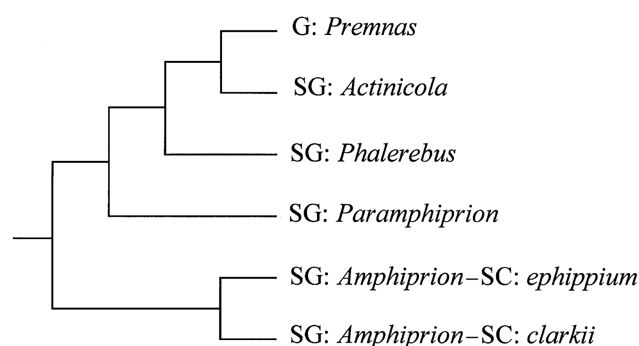

Figure 1. Evolutionary relationships among the subfamily Amphiprioninae as proposed by Allen (1972, p. 52) based on morphological characters. There are two genera, *Premnas* (one species) and *Amphiprion* (27 species), and the genus *Amphiprion* is divided into four subgenera (Allen 1991, p. 34). The subgenus *Amphiprion* is considered to be the most generalized group of anemonefishes, especially those members of the *clarkii* complex, and their body structure is most similar to other pomacentrid groups. G, genus; SG, subgenus; SC, species complex.

ten different host anemone species (table 1). Thus, members of the *clarkii* complex are considered to be the most ancestral group of anemonefishes because they are most similar to other pomacentrids in morphology and behaviour. Once the ancestral anemonefish became adapted to living with anemones, over evolutionary time the fishes are thought to have radiated into a variety of different niches by becoming more specialized for living with particular host species. These more specialized fish species are members of three other subgenera (table 1). They are relatively slender-bodied forms, poor swimmers and are very dependent on their hosts for protection, typically living with only one or a few species of hosts.

Traditional morphological analyses do not always provide enough informative characters to produce robust phylogenies (Hillis 1987, 1995). Recent advances in molecular analyses of DNA sequences allow comparisons of a large number of neutral or nearly neutral characters that can be used in phylogenetic analyses. The objective of the present study was to construct a molecular phylogeny for the major groups of anemonefishes using mitochondrial DNA (mtDNA) sequence data, and to compare the results with those of a phylogenetic hypothesis based on morphological characters (Allen 1972). The molecular phylogeny was then used to test the hypothesis that the ancestral condition within the anemonefishes was that of a generalist, and the derived species became specialized to live with particular species of host anemones.

2. MATERIALS AND METHODS

Mitochondrial DNA sequences were determined for *Premnas biaculeatus* and representative *Amphiprion* species (typically the most common species in each group) from each of the subgenera and the two species complexes of the *Amphiprion* subgenus (table 1). The host specificity patterns of the fishes range from extreme host generalists (*A. clarkii*) to extreme host specialists (*Premnas biaculeatus* and *A. frenatus*). For cytochrome *b* analysis, comparative sequences for two outgroup species were obtained from two other members of the Pomacentridae family, *Dascyllus melanurus* and *Chrysiptera cyanea*, that represent the pomacentrid subfamilies Chrominae and Pomacentrinae, respectively. For 16S rDNA,

we used sequence from a 'near' outgroup (*C. cyanea*) and a more distant one (*Lycodichthys dearborni*, order Perciformes, family Zoarcidae; GenBank Accession Number Z32730); reasons for not using *Dascyllus* for 16S analyses are outlined below. Pomacentrid specimens were collected from the field (The Philippines, Papua New Guinea and Palau; specific localities are available from authors), or purchased from aquarium fish suppliers. Fish were anaesthetized in a 5% solution of MS 222 and then preserved in 70% ethanol. Total genomic DNA was extracted from liver and muscle tissue and then purified using a GenomicPrep^{TM} DNA Isolation Kit (Pharmacia).

(a) *Cytochrome* b *sequences*

The polymerase chain reaction (PCR) was used to amplify the complete cytochrome *b* (cyt *b*) gene (1140 bp) for each species. Double-stranded amplifications were performed in 25-μl volumes using the methods of Palumbi (1996) in a Perkin Elmer GeneAmp PCR System 2400. The primers used were Gludg-L (Palumbi *et al*. 1991), and H15915 (Irwin *et al*. 1991). The PCR products were purified using a QIAquick PCR purification kit (QIAGEN) and then cycle sequenced using the above primers as well as L15299 (Lydeard & Roe 1997) and a custom-made primer, L15007 (5′-TACCTCCACATCGGACGAGG-3′). Most of the samples were cycle sequenced using an ABI PRISM dye terminator cycle sequencing ready reaction kit, and then sequenced on an ABI 373 automatic sequencer. Some samples were cycle sequenced using a SequiTherm Excel^{TM} Long-Read^{TM} DNA sequencing kit (Epicentre), and then sequenced on a Li-Cor 4200 automatic sequencer.

(b) *16S sequences*

An approximately 600 bp fragment was amplified using 16Sbr-H and 16Sar-L (Kocher *et al*. 1989; Palumbi *et al*. 1991, respectively), with the latter primer biotinylated at the 5′-end. Double-stranded amplifications were performed in 50-μl volumes using the methods of Palumbi (1996). Amplified products were cleaned and single-stranded template isolated using super-magnetic polystyrene beads (Dynabeads M-280 Streptavidin; Dynal) according to the manufacturer's instructions. Single-stranded 16S products were sequenced with 16S br-H and an additional primer of our design (16S an-H; 5′-GCGCTGTTATCCCTGGGGTAACTC-3′) using a ThermoSequenase cycle sequencing kit (Amersham Pharmacia Biotech) following the manufacturer's protocol. Samples were subjected to PAGE for short (2 h) and long (4.5 h) runs and visualized by autoradiography. Sequences were scored by hand.

(c) *Data analyses*

Cytochrome *b* sequences were aligned by eye in the program MacClade 3.05 (Maddison & Maddison 1995). The 16S sequences were aligned using GeneWorks (IntelliGenetics, Inc.) with further checking by eye. Because of difficulty obtaining clean 16S sequence for *Dascyllus melanurus*, it was excluded from all subsequent analyses. For the alignment of the 16S sequences, gaps and neighbouring sites for which homology across taxa was ambiguous were excluded.

DNA sequence variation and substitution patterns were examined using MEGA (Kumar *et al*. 1993). Maximum parsimony (MP) phylogenetic analyses were performed using PAUP v. 3.1 (Swofford 1993). An exhaustive search was performed, and bootstrap measures of stability were established using the heuristic search option over 500 bootstrap replications (Felsenstein 1985). Based on empirical average transition to transversion ratios (hereafter ts/tv) among the anemonefish species for cyt *b*, separate analyses were conducted with transversions weighted 2, 4 and 8 times over transitions at the third codon position.

Maximum likelihood (ML) analyses were performed using the DNAML option in PHYLIP (v. 3.5, Felsenstein 1993) with all characters unweighted and the transition to transversion ratio defined as weighted 2, 4 and 8 in separate analyses. Neighbour-joining (NJ) analyses (Saitou & Nei 1987) were performed with MEGA using three different methods to estimate evolutionary distances that accounted for multiple substitutions: Jukes–Cantor (Jukes & Cantor 1969), Kimura's two-parameter model (Kimura 1980), and Tamura–Nei's method (Tamura 1992). For both ML and NJ analyses, support for nodes within generated tree topologies was determined using bootstrap analyses as encoded in the respective programs, with 500 replicates.

The shortest mtDNA trees, determined in separate analyses of the cyt *b* and 16S data sets, were compared to the morphology-based phylogeny proposed by Allen (1972) (figure 1). Specific branches were swapped in MacClade to determine the difference in tree length between the two topologies. The evolution of specialization in anemonefishes was investigated using the character reconstruction methods of MacClade (Maddison & Maddison 1995) and the single shortest tree identified in the previous phylogenetic analyses. The following characters were mapped on to the phylogeny: (i) body depth, (ii) caudal fin shape, (iii) dependence on host for protection and (iv) number of hosts.

3. RESULTS

(a) *Examination for paralogous sequences*

There was no evidence for amplification of paralogous copies of either the cyt *b* or 16S data that might confound our analyses, as has been observed in other studies (e.g. Hu & Thilly 1994; Friesen & Anderson 1997). First, base-pair substitutions within non-functional paralogues should occur randomly. However, changes within cyt *b* are strongly skewed towards third base-pair substitutions and biased toward transitions. Further, no inappropriate stop codons, nor insertions or deletions were detected for cyt *b*, as would be expected in a non-functional paralogous copy where such events should incur no selective penalty. Second, based on known functional constraints for the products of these two genes, the least variable regions of cyt *b* correspond to proposed redox centres of the encoded protein product, while more variable regions relate to transmembrane domains (Howell 1989). For 16S, variable regions are confined to the looped domains in the proposed secondary structure of the rRNA (Gutell 1994; Maidak *et al*. 1997). One would expect this variation to be randomly distributed across the sequence in any non-functional nuclear copies.

(b) *Analysis of cytochrome* b *data*

Approximately 1140 base pairs of the cyt *b* gene were sequenced for each fish species (GenBank accession numbers AF097925–AF097931). Analyses of the aligned DNA sequences yielded 363 variable sites and 184 potentially phylogenetically informative sites. Out of the 184 informative sites, 19 (10%) were at first, 4 (2%) were at second and 161 (88%) were at third codon positions. The first codon position had relatively equal proportions of

Table 2. *Sequence differences for cytochrome* b *gene from anemonefishes and two outgroup taxa in the family Pomacentridae*

(Mean pairwise difference (uncorrected percentage sequence divergence) between sequences is shown above the diagonal. The transition/transversion ratio for each species pair is shown below the diagonal.)

species	1	2	3	4	5	6	7	8
1 *Dascyllus melanurus*	—	19	19	19	20	19	19	18
2 *Chrysiptera cyanea*	1.43	—	17	18	18	17	16	17
3 *Premnas biaculeatus*	1.34	1.76	—	12	12	10	11	11
4 *Amphiprion ocellaris*	1.04	1.69	2.21	—	13	13	12	13
5 *Amphiprion polymnus*	1.49	1.64	4.04	2.61	—	5	5	7
6 *Amphiprion sandaracinos*	1.32	1.51	3.61	3.00	8.17	—	5	7
7 *Amphiprion frenatus*	1.39	1.46	3.29	2.55	7.00	6.29	—	8
8 *Amphiprion clarkii*	1.46	1.62	4.08	2.80	5.38	5.91	5.07	—

each nucleotide (A = 0.24, C = 0.27, G = 0.26, T = 0.23), but there was an overall anti-G bias in the proportion of each nucleotide at positions two (A = 0.20, C = 0.25, G = 0.13, T = 0.42) and three (A = 0.34, C = 0.41, G = 0.05, T = 0.20). These values are typical for fish cyt *b* sequences (Lydeard & Roe 1997). Chi-square tests for each codon position indicated that the nucleotide frequencies were homogenous across taxa ($p > 0.05$).

The pairwise percentage sequence divergence (uncorrected) and the ts/tv ratios among the taxa are shown in table 2. The percentage sequence divergence was >18% between the outgroup taxa (*Dascyllus* and *Chrysiptera*) and each anemonefish species. Among the anemonefishes, the divergence ranged between 4.5 and 15%, with *A. ocellaris* having the furthest average distance from the other species (mean = 13.8%). The ts/tv ratio among taxa ranged from 1.04 to 8.17 (mean = 3.04). Plots of uncorrected genetic distance versus number of ts and tv at each codon position (not shown) indicated that there was the potential for saturation of transitions at the third codon position. Thus, we chose to weight transversions 2, 4 and 8 times over transitions at the third codon position.

Most MP analyses resulted in the topology shown in figure 2*a*, with the only variation being the placement of *Premnas* and *A. ocellaris*. All analyses indicated very strong support (i.e. bootstrap values of 100%) for the anemonefishes being a monophyletic group and an early divergence between the *Premnas* and *A. ocellaris* lineages and the rest of the anemonefishes. An exhaustive search using MP resulted in a single shortest tree of 790 steps and a consistency index of 0.78 with tv weighted 2 × ts. This tree differed from figure 2*a* in that there was a sister relationship between *Premnas* and *A. ocellaris*. There was moderate bootstrap support (73%) for this sister relationship in the 50% majority rule consensus tree with tv weighted 2 × ts. However, when tv were weighted 4 × ts the *Premnas–A. ocellaris* clade collapsed into a polytomy with the rest of the anemonefishes. When tv were weighted 8 × ts, *Premnas* was indicated as being basal to the remaining ingroup species, although bootstrap support for the clade comprised the remaining taxa did not exceed 60% in any analysis. There was strong support for the *A. polymnus–A. frenatus–A. sandaracinos* clade in all analyses (>85%), but only weak to moderate support for the *A. polymnus–A. frenatus* clade under the variety of weighting schemes.

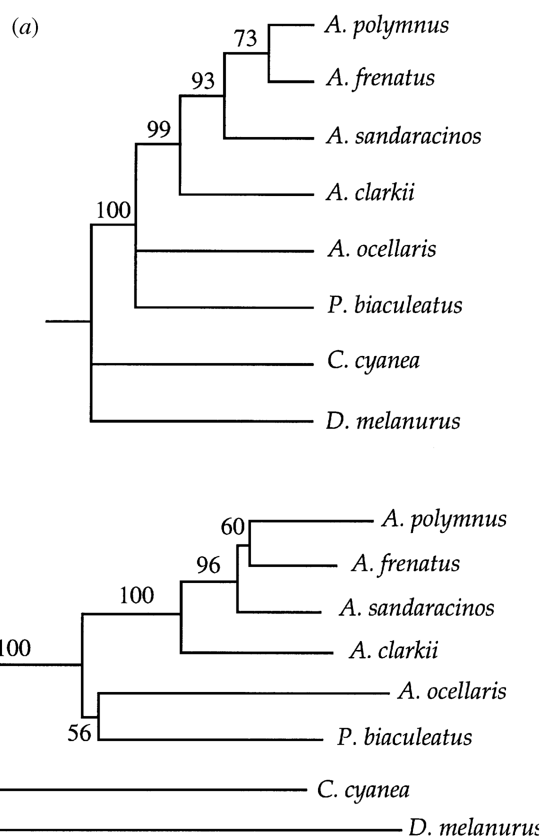

Figure 2. Cladograms resulting from maximum parsimony (MP) and neighbour-joining analyses (NJ) of cytochrome *b*. (*a*) Bootstrap 50%-majority-rule MP tree when tv were weighted 4 × ts. Numbers above branches indicate bootstrap values (Felsenstein 1985), when tv were weighted 4 × ts. (*b*) NJ tree based on a distance matrix that was corrected for multiple substitutions using the Kimura two-parameter model. The bar indicates 1% corrected sequence distance, and the branches are drawn according to the number of inferred substitutions. Numbers below branches indicate boostrap values.

All ML 50% majority-rule consensus trees (not shown) resulted in the same topology as in figure 2*a* except that the *A. polymnus–A. frenatus* clade collapsed into a polytomy with *A. sandaracinos*. Again, there was strong support for the anemonefishes being a monophyletic group, and an early divergence between the *Premnas* and *A. ocellaris* lineages and the rest of the

Table 3. *Sequence differences for 16S rRNA gene (505 aligned base pairs excluding gaps) from anemonefishes and two outgroup taxa, one from the family Pomacentridae and the other from the family Zoarcidae*

(Mean pairwise difference (uncorrected percentage sequence divergence) between sequences is shown above the diagonal. The transition/transversion ratio for each species pair is shown below the diagonal.)

species	1	2	3	4	5	6	7	8
1 *Lycodichthys dearborni*	—	12	13	12	14	14	14	13
2 *Chrysiptera cyanea*	1.00	—	7	7	8	8	8	7
3 *Premnas biaculeatus*	1.36	3.50	—	3	5	4	5	4
4 *Amphiprion ocellaris*	1.14	2.89	15.00	—	4	4	3	3
5 *Amphiprion polymnus*	1.15	2.15	3.80	2.50	—	2	2	2
6 *Amphiprion sandaracinos*	1.06	2.25	2.67	1.57	2.33	—	1	2
7 *Amphiprion frenatus*	1.19	2.50	5.00	2.40	7.00	2.00	—	1
8 *Amphiprion clarkii*	1.19	2.36	5.00	2.75	4.50	0.67	—	—

anemonefishes. ML tree as shown in figure 2*a*, except there was a sister relationship between *Premnas* and *A. ocellaris* when tv were weighted 2 and 4 × ts and *Premnas* was ancestral to all anemonefishes when tv were weighted 8 × ts.

All methods used to calculate evolutionary distances in three different NJ analyses resulted in the same topology (figure 2*b*), which was identical to the single shortest tree identified in the majority of the MP and ML exhaustive search analyses. The bootstrap support for each branch was similar to those for the MP analyses.

(c) *Analysis of 16S sequences*

Over 500 base pairs of the 16S rRNA gene were sequenced for each fish species (GenBank accession numbers AF114838–AF114844). Analyses of the aligned DNA sequences (excluding gaps) yielded 94 variable sites and 35 potentially phylogenetically informative sites. The average proportion of nucleotides was slightly skewed toward adenosine across all analysed taxa (A = 0.30, C = 0.24, G = 0.23, T = 0.23).

Pairwise percentage sequence divergence and the ts/tv ratios for the 16S rDNA gene are shown in table 3. The average percentage sequence divergence between *Lycodichthys* and members of the ingroup was 13.2% and between *Chrysiptera* and the anemonefish species 7.5%. Among the anemonefishes, the average divergence was 2.9% (range 1–5%), with *Premnas* having the largest average distance from the other ingroup species (mean = 4.1%). The ts/tv ratio among ingroup taxa ranged from 1.00 to 15.00 (mean = 3.04).

Tree topologies resulting from the various phylogenetic analyses of the 16S data set largely mirrored that of cyt *b*, with the anemonefish taxa comprising a well-supported clade in all (figure 3). MP exhaustive searches with every considered weighting scheme yielded a single most parsimonious tree (117 steps, CI = 0.89; see figure 3*a*). In contrast to cyt *b*, all MP (figure 3*a*), ML (not shown) and NJ (figure 3*b*) 16S rDNA trees consistently indicated *Premnas* as being basal to the remaining ingroup species, although bootstrap support for the clade comprised of the remaining taxa did not exceed 70% in any analysis (figure 3). The clade containing *A. clarkii*, *A. sandaracinos*, *A. frenatus* and *A. polymnus* was supported by bootstrap values in excess of 95% in all analyses, which is again coincident with analyses using cyt *b*.

(d) *Molecular versus morphology*

The most common tree identified in MP and ML analyses of the cytochrome *b* data was 605 steps (figure 2*a*), and when the data were constrained to the topology of the morphology-based tree of Allen (1972) it required 660 steps, an increase of 55 steps (9%). Similarly, constraining the 16S MP tree shown in figure 3*a* to Allen's topology resulted in an additional 14 steps, an increase from 117 to 131 (12%).

(e) *Character reconstruction*

The evolution of specialization in anemonefishes was investigated with MacClade (Maddison & Maddison 1995), using the most well-supported topology identified in the previous phylogenetic analyses (figure 3*a*). Allen (1972) suggested that the state of the following characters indicated whether a fish was specialized or generalized for living with anemones: (i) body depth, (ii) caudal fin shape, (iii) dependence on host for protection and (iv) number of hosts. Members of the *clarkii* complex were considered to be the most ancestral group of anemonefishes because they have the following morphological and behavioural characters: (i) deep-bodied, (ii) good swimmers with emarginate tails, (iii) least dependent on their host anemones for shelter and (iv) host generalists living with up to ten different anemone species. The more derived anemonefishes were considered to be: (i) slender-bodied, (ii) less efficient swimmers with truncate or rounded tails, (iii) more dependent on their host anemones for shelter and (iv) host specialists living with only a few different anemone species. Tracing the evolution of these characters within the Amphiprioninae (figure 4) indicates that the ancestral anemonefish was probably: (i) either deep- or slender-bodied, (ii) rounded tailed, (iii) highly dependent on its host for shelter from predators and (iv) living with few host anemone species. The reconstruction of the character 'body depth' had five equally parsimonious reconstructions of four steps, two with the ancestral anemonefish being slender-bodied and two as deep-bodied (figure 4*a*). There was only one most parsimonious reconstruction of all of the other characters (figure 4*b*–*d*).

4. DISCUSSION

This study provides the first molecular phylogenetic hypothesis for the anemonefishes, and the results have

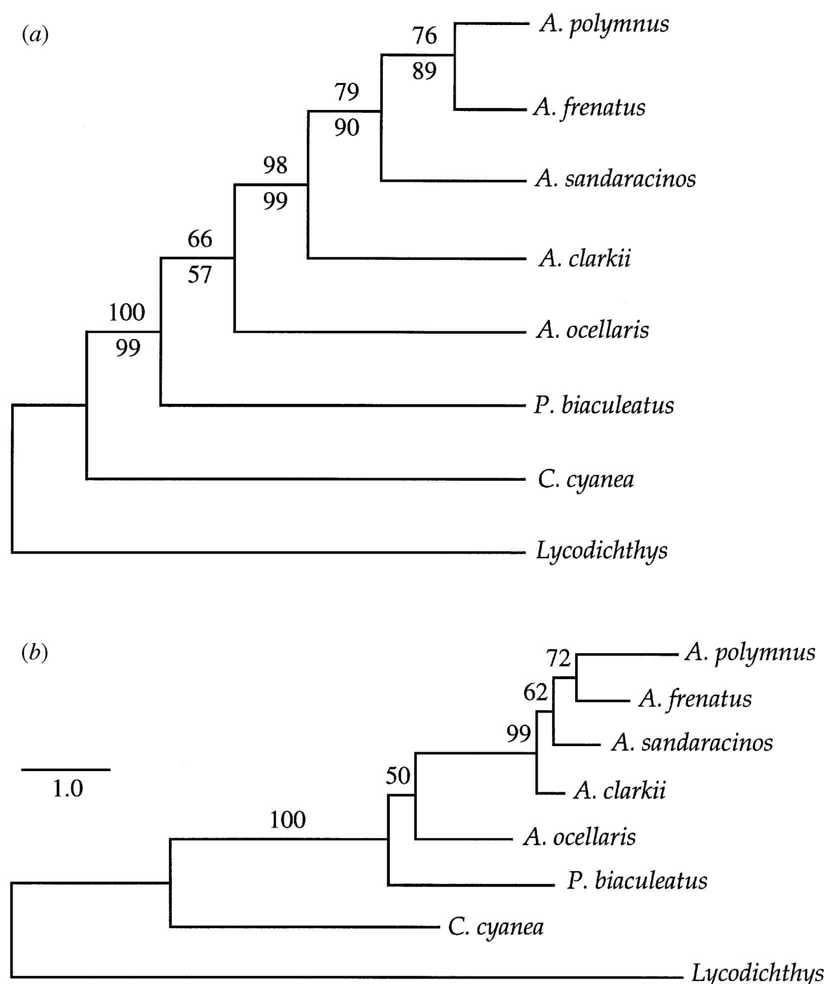

Figure 3. Cladograms resulting from maximum parsimony (MP) and neighbour-joining analyses (NJ) of the 16S rDNA data set. (*a*) Single most parsimonious tree resulting from an exhaustive search. Numbers indicate bootstrap values, above branch for unweighted, below branch for ts/tv = 8. (*b*) NJ tree based on a distance matrix that was corrected for multiple substitutions using the Kimura two-parameter model. The bar indicates 1% corrected sequence distance, and the branches are drawn according to the number of inferred substitutions. Numbers above branches indicate boostrap values.

important implications to our understanding of the taxonomy and evolutionary history of this group of coral reef fishes. The discussion will be limited to general trends that are well-supported by the molecular data, with the caveat that the results are based on a limited sample of only six out of the 28 recognized species of anemonefishes. Thus, the conclusions are based on the assumption that missing taxa do not significantly influence the inferred phylogenetic relationships. However, since each species was chosen to represent a particular genus, subgenus or species complex, each of which consists of only one species or a relatively homogeneous group of species, the data set is considered to be adequate for a discussion of the general phylogenetic trends detected.

(a) *Taxonomic implications*

There was very strong support for the monophyly of anemonefishes (figures 2 and 3). Thus, the subfamily Amphiprioninae is appropriate for this distinct clade within the family Pomacentridae. The separation of the subfamily into two genera (*Premnas* and *Amphiprion*) may not be warranted given that both *P. biaculeatus* and *A. ocellaris* were relatively far removed from the other anemonefishes. In some analyses of the cyt *b* data there was support for *P. biaculeatus* and *A. ocellaris* being sister taxa in a clade basal to the other anemonefishes, but this clade was not supported when tv were weighted 4 and 8 × ts, or in analyses of the 16S data. *Premnas* was identified as the most ancestral anemonefish species in the analyses of 16S and cyt *b* when tv were weighted 8 × ts. Since 16S evolves at a relatively slower rate than cytochrome *b* (Palumbi 1996), it may provide a more accurate phylogeny for these distantly related species.

Within the clade leading to the rest of the anemonefish species, *A. clarkii* and *A. frenatus* were not found to be sister taxa in the subgenus *Amphiprion*, as was expected from the phylogeny proposed by Allen (1972) (figure 1). There was relatively strong support for *A. frenatus* being a member of a clade along with *A. sandaracinos* and *A. polymnus*, and weak support for *A. frenatus* being the sister taxa of *A. polymnus*. Thus, the grouping of *A. clarkii* and *A. frenatus* in the subgenus *Amphiprion* was not supported and the validity of this subgenus awaits further analyses with more anemonefish species.

(b) *The evolution of anemonefish–anemone symbiotic relationships*

Contrary to the morphology-based phylogenetic hypothesis proposed by Allen (1972), with the basal anemonefish ancestor being a generalist (figure 1), the molecular data analysed in this study support the hypothesis that the ancestral anemonefish was specialized for living with certain species of anemones (figures 2–4). Evolution then proceeded towards generalization on the branch leading to *A. clarkii* (*clarkii* complex), with most

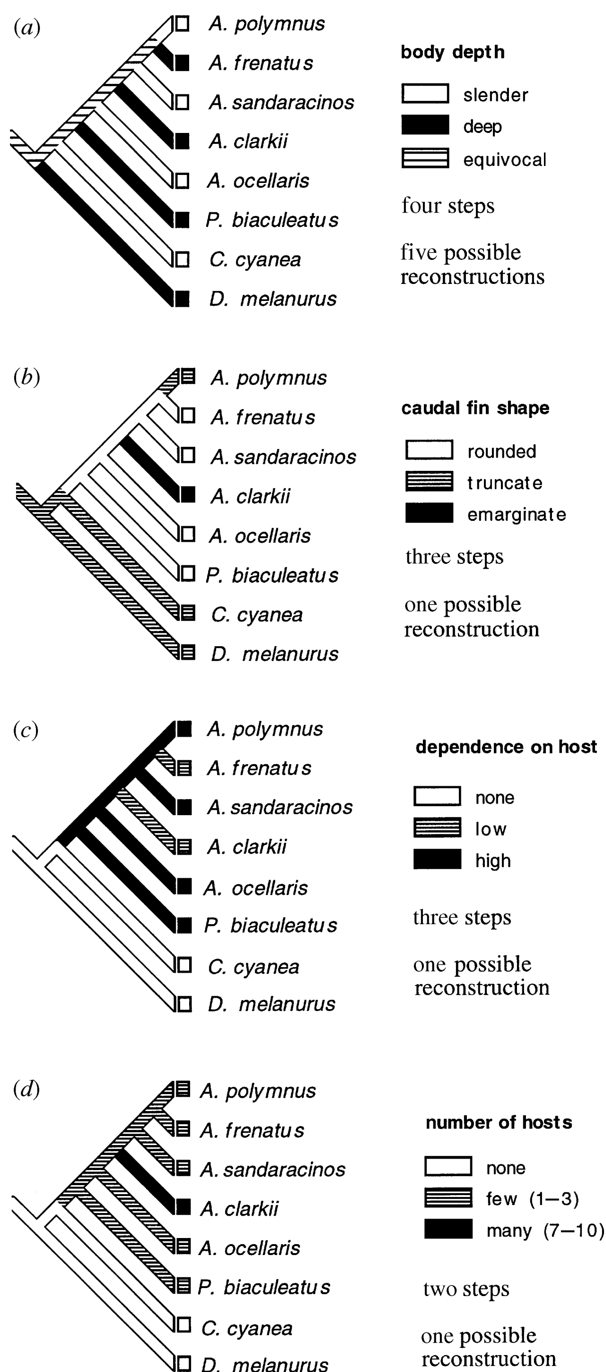

Figure 4. Reconstructions of the evolution of four characters using MacClade (Maddison & Maddison 1995) based on the topology given in figure 3*a*. (*a*) body depth, (*b*) caudal fin shape, (*c*) dependence on host for protection and (*d*) number of hosts.

other clades evolving towards specialization for living with other anemone species. However, this conclusion is given with the caution that there is uncertainty when estimating ancestral states, especially for distant ancestors. Whether the basal anemonefish ancestor was deep- or slender-bodied was equivocal in the character state analysis (figure 4*a*). Allen (1972) states that most other pomacentrids are deep-bodied, and that anemonefishes probably evolved from this body form. However, some pomacentrids, including one of the outgroup taxa, *C. cyanea*, are slender-bodied. This outgroup was also more similar genetically (for cyt *b*) to the anemonefishes than the other deep-bodied outgroup *Dascyllus melanurus* (table 2), which suggests that the ancestral anemonefish may have also had a slender-body form. Having a slender body may have allowed those anemonefishes to shelter in their host anemone's tentacles more effectively.

The parsimony reconstruction analysis of caudal fin shape suggests that the ancestral anemonefish had a rounded caudal fin (figure 4*b*). Fish with rounded caudal fins (low aspect ratio) are generally not as effective swimmers as those with truncate or emarginate fins which have a higher aspect ratio (Webb & Blake 1985). Anemonefishes with rounded caudal fins are probably adapted for quick, darting movements to capture plankton in the water column. A truncate or emarginate caudal fin, that allows for sustained, fast swimming, is not needed since they do not venture far away from the safety of their anemone's tentacles. Anemonefishes with truncate or emarginate caudal fins are better swimmers, and species such as *A. clarkii* are known to swim far away from their host anemone.

Anemonefishes that are better swimmers also are less dependent on their host anemones for shelter (figure 4*c*). *Amphiprion clarkii* is a very good swimmer and often shelters in coral crevices (instead of with its host), when attempts are made to capture it in the field. However, more-specialized anemonefishes such as *A. ocellaris* will always hide in the tentacles of their host when pursued. The ancestral anemonefish may have received a high level of protection from living with anemones, which allowed it to adapt its body and caudal fin shape for feeding instead of escaping predators. It is interesting to note that the most ancestral species of anemonefishes, *P. biaculeatus* and *A. ocellaris*, live with host anemones that offer excellent protection from predators. *Premnas biaculeatus* typically lives with large solitary individuals of *Entacmaea quadricolor* that attach their bases in deep crevices. The combination of a crevice and large anemone offer excellent protection for this fish species. Thus, the ancestral anemonefish may have been adapted for sheltering in crevices and then gained further protection by living with anemones in the same microhabitat. Once ancestral anemonefishes became adapted for living with anemones, they may have then been able to enter other anemone species that were large enough to provide shelter from predators. *Amphiprion ocellaris* is usually found with either *Heteractis magnifica* or with *Stichodactyla gigantea*, both of which are large and strongly stinging anemones (Elliott & Mariscal 1996; Fautin & Allen 1997). There was probably strong selection for the development of host specificity for those anemone species (Miyagawa 1989; Elliott *et al.* 1995).

Once the ancestral anemonefishes monopolized those anemone species that provided the best protection, subsequent speciation events in the anemonefishes may have resulted in more derived species of anemonefishes (e.g. the generalist *A. clarkii*) becoming adapted for living with a variety of other anemone species that offered less protection (but more protection than sheltering in a crevice on the reef), or living with anemones in habitats other than those occupied by the more ancestral species. It is interesting to note that *A. clarkii* is not chemically attracted to *E. quadricolor*, *H. magnifica* or *S. gigantea*, the hosts of *P. biaculeatus* and *A. ocellaris* (Elliott *et al.* 1995).

However, *A. clarkii* is chemically attracted to most other symbiotic anemone species.

If it is assumed that anemonefishes have coevolved with their host anemones, then the hosts of *P. biaculeatus* (*E. quadricolor*) or *A. ocellaris* (*H. magnifica*, *S. gigantea*, *S. mertensii*) may have been the initial hosts to the ancestral anemonefish. *Entacmaea quadricolor* is a member of the family Actiniidae, whereas the majority of the other symbiotic anemone species are in the family Stichodactylidae (table 1). Fautin (1991) suggests that symbiotic anemones may have evolved to reach such large sizes in response to the protection and nutrients (in nitrogenous wastes) provided by the symbiotic fishes. Phylogenetic studies of the host anemones are needed to determine whether their evolutionary history is related to that of the fishes.

(c) *Timing of diversification*

Phylogenetic analyses of both cyt *b* and 16S sequence data sets are concordant in placing of *P. biaculeatus* and *A. ocellaris* basally to the other ingroup taxa (figures 2 and 3). Based on this observation and assuming rates of change in cyt *b* of between 1 and 2.5% per million years (Myr) (Brown *et al*. 1982; Irwin *et al*. 1991; Martin *et al*. 1992), we may thus place the origin of anemonefishes somewhere between 5 and 13 Myr ago (table 2). Interestingly, this time-frame mirrors that suggested by McMillan & Palumbi (1995) for the origin of two Indo-West Pacific butterflyfish species groups (Chaetodontidae). Using the same calibration, other major diversification events within anemonefishes date to approximately the late Pliocene or the Pliocene–Pleistocene boundary.

5. CONCLUSION

The specificity of a symbiotic association refers to the number of host species that a symbiotic organism lives with in nature (Douglas 1994). Host specificity patterns range from extreme host specialists, in which a symbiont lives with only one host species, to extreme generalists that live with a variety of host species. Most explanations of host specificity have proposed that ancestral species are host generalists and then over evolutionary time become more specialized to live with particular host species (Futuyma & Moreno 1988). Allen (1972) provided a similar explanation for the evolution of host specificity in anemonefishes, and suggested that the first anemonefish species was probably a host generalist that evolved from a coral reef fish ancestor that was a habitat generalist. Then, over evolutionary time new anemonefish species evolved that became more specialized to live with particular species of host anemones. However, our analysis supports the hypothesis that the ancestral anemonefish lived with only one to a few species of host anemones (figure 4*d*). Host generalization is thus a derived trait that evolved in the clade that includes *A. clarkii*. The more-derived anemonefish species live with only one to a few species of hosts. Host specificity patterns are known to be a result of attraction behaviours of larval anemonefishes to chemical cues released by anemones (Miyagawa 1989; Elliott *et al*. 1995), and these behaviours are considered to be innate and have a strong genetic basis. Thus, host specialization was probably important in allowing the development of niche differentiation and the high level of adaptive radiation in this group of fishes.

This initial study into the phylogenetics of the anemonefishes has indicated some fundamental changes in our understanding of the evolution of this fascinating group of fishes. Future studies that include other anemonefish species and examine their ecological, behavioural and physiological traits with respect to their phylogeny will undoubtedly further illuminate trends in the evolution of this assemblage of symbiotic organisms.

We thank Peter Wimberger of the University of Puget Sound for technical assistance, laboratory hardware and reagents, and constructive suggestions for the manuscript. We also thank Pam Jensen of the Marine Molecular Biology Laboratory at the University of Washington for assistance in automatic sequencing. Denise Michaud provided insight and laboratory expertise at Queen's University. Pat and Lori Colin of the Coral Reef Research Foundation, Palau, are gratefully acknowledged for collecting most of the anemonefish tissue samples used in this study. The research was supported by grants from the University of Puget Sound Enrichment Grants programme and the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

Allen, G. R. 1972 *The anemonefishes: their classification and biology.* Neptune City: T. F. H. Publications, Inc.

Allen, G. R. 1980 *Anemonefishes of the world*. Mentor: Aquarium Systems.

Allen, G. R. 1991 *Damselfishes of the world*. Melle: MERGUS Publishers.

Bernays, E. A. & Chapman, R. F. 1994 *Host–plant selection by phytophagous insects*. New York: Chapman & Hall.

Brown, W. M., Pranger, E. M., Wanf, A. & Wilson, A.C. 1982 Mitochondrial DNA sequences of primates: tempo and mode of evolution. *J. Mol. Evol*. **18**, 225–239.

Douglas, A. E. 1994 *Symbiotic interactions*. New York: Oxford University Press.

Elliott, J. K. & Mariscal, R. N. 1996 Ontogenetic and interspecific variation in protection of anemonefishes from sea anemones. *J. Exp. Mar. Biol. Ecol*. **208**, 57–72.

Elliott, J. K., Elliott, J. M. & Mariscal, R. N. 1995 Host selection, location, and association behaviors of anemonefishes in field settlement experiments. *Mar. Biol*. **122**, 377–389.

Fautin, D. G. 1991 The anemonefish symbiosis: what is known and what is not. *Symbiosis* **10**, 23–46.

Fautin, D. G. & Allen, G.R. 1997 *Anemonefishes and their host sea anemones*, 2nd edn. Perth: Western Australian Museum.

Felsenstein, J. 1985 Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Felsenstein, J. 1993 *PHYLIP* (*Phylogeny Inference Package*) v. 3.5c. Seattle: Department of Genetics, University of Washington.

Friesen, V. L. & Anderson, D. J. 1997 Phylogeny and evolution of the Sulidae (Aves: Pelecaniformes): a test of alternative modes of speciation. *Mol. Phyl. Evol*. **7**, 252–260.

Futuyma, D. J. 1998 *Evolutionary biology*, 3rd edn. Sunderland: Sinauer.

Futuyma, D. J. & Moreno, G. 1988 The evolution of ecological specialization. *A. Rev. Ecol. Syst*. **19**, 207–233.

Gutell R. R. 1994 Collection of small subunit (16S- and 16S-like) ribosomal RNA structures. *Nucl. Acids Res*. **22**, 3502–3507.

Hillis, D. M. 1987 Molecular versus morphological approaches to systematics. *A. Rev. Ecol. Syst*. **18**, 23–42.

Hillis, D. M. 1995 Approaches for assessing phylogenetic accuracy. *Syst. Biol*. **44**, 3–16.

Howell, N. 1989 Evolutionary conservation of protein regions in the protein-motive cytochrome *b* and their possible roles in redox catalysis. *J. Mol. Evol.* **29**, 157–169.

Hu, G. & Thilly, W. G. 1994 Evolutionary trail of the mitochondrial genome as based on human 16S rDNA pseudogenes. *Gene* **147**, 197–204

Irwin, D. W., Kocher, T. D. & Wilson, A. C. 1991 Evolution of the cytochrome *b* gene in mammals. *J. Mol. Evol.* **32**, 128–144.

Jukes, T. H. & Cantor, C. R. 1969 Evolution of protein molecules. In *Mammalian protein metabolism* (ed. H. N. Munroe), pp. 21–132. New York: Academic Press.

Kimura, M. 1980 A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**, 11–120.

Kumar, S., Tamura, K. & Nei, M. 1993 *MEGA: molecular evolutionary genetics analysis*, v. 1.01. University Park: Pennsylvania State University.

Lydeard, C. & Roe, K. J. 1997 The phylogenetic utility of the mitochondrial cytochrome *b* gene for inferring intrarelationships of actinopterygian fishes. In *Molecular systematics of fishes* (ed. C. A. Stepien & T. Kocher), pp. 285–303. San Diego: Academic Press.

McMillan, W. O. & Palumbi, S. R. 1995 Concordant evolutionary patterns among Indo-West Pacific butterflyfishes. *Proc. R. Soc. Lond.* B **260**, 229–236.

Maddison, W. P. & Maddison, D. R. 1995 *MacClade, analysis of phylogeny and character evolution*, v. 3.05. Sunderland, MA: Sinauer Associates, Inc.

Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J. & Woese, C. R. 1997 The RDP (ribosomal database project). *Nucl. Acids Res.* **25**, 109–111.

Martin, A. P., Naylor, G. J. P. & Palumbi, S. R. 1992 Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. *Nature* **357**, 153–155.

Mayr, E. 1942. *Systematics and the origin of species*. New York: Columbia University Press.

Miyagawa, K. 1989 Experimental analysis of the symbiosis between anemonefish and sea anemones. *Ethology* **80**, 19–46.

Palumbi, S. R. 1996 Nucleic acids. II. The polymerase chain reaction. In *Molecular systematics*, 2nd edn (ed. D. M. Hillis, C. Moritz, C. & B. K. Mable), pp. 205–247. Sunderland, MA: Sinauer Associates, Inc.

Palumbi, S. R., Martin, A., Romano, S., McMillan, W. O., Stice, L. & Grabowski, G. 1991 *The simple fool's guide to PCR*. Honolulu: University of Hawaii Press.

Roe, K. J., Conkel, D. & Lydeard, C. 1997 Molecular systematics of middle American cichlid fishes and the evolution of trophic-types in 'Cichlasoma (amphilophus)' and 'C. (thorichthys)'. *Mol. Phyl. Evol.* **7**, 366–376.

Saitou, N. & Nei, M. 1987 The neighbor-joining method: a new method for constructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.

Swofford, D. L. 1993 *PAUP: phylogenetic analysis using parsimony.* v. 3.0. Sunderland, MA: Sinauer Associates, Inc.

Tamura, K. 1992 Estimation of the number of nucleotides when there are strong transition–transversion and G+C-content biases. *Mol. Biol. Evol.* **9,** 678–687.

Thompson, J. N. 1994 *The coevolutionary process*. University of Chicago Press.

Webb, P. W. & Blake, R. W. 1985 Swimming. In *Functional vertebrate morphology* (ed. M. Hildebrand, D. M. Bramble, K. F. Liem & D. B. Wake), pp. 110–128. Cambridge, MA: Belknap Press.

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